ANALYSIS OF BRCA1 AND BRCA2 GENES IN TURKISH BREAST CANCER PATIENTS

A THESIS SUBMITTED TO THE DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS AND THE INSTITUTE OF ENGINEERING AND SCIENCE OF BILKENT UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

BY

HİLÂL ÖZDAĞ

June 2000

ANALYSIS OF *BRCA1* AND *BRCA2* GENES IN TURKISH BREAST CANCER PATIENTS

A THESIS SUBMITTED TO THE DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS AND THE INSTITUTE OF ENGINEERING AND SCIENCE OF BILKENT UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

BY

HİLÂL ÖZDAĞ

June 2000

Bu çalışma TTGV 172 no'lu proje

ve

TÜBİTAK Bütünleştirilmiş Doktora Programı

ile desteklenmiştir.

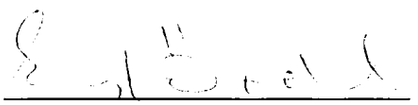
İlim ilim bilmektir
İlim kendin bilmektir
Sen kendini bilmezsin
Ya nice okumaktır

Okumaktan mana ne
Kişi hakkı bilmektir
Çün okudun bilmezsin
Ha bir kuru emektir

Yunus Emre

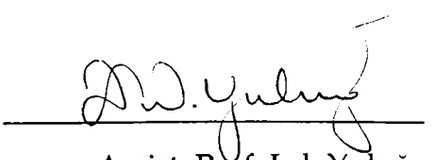
Sevgili Anneannem
Fahriye Erk'e

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Philosophy.



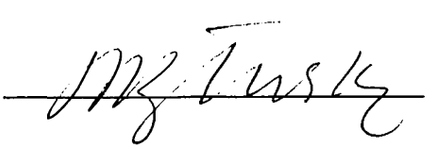
Assoc. Prof. Tayfun Özçelik

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Philosophy.



Assist. Prof. Işık Yuluğ

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Philosophy.



Prof. Dr. Mehmet Öztürk

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Philosophy.

Prof. Dr. Ay Öğüş

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Philosophy.

Prof. Dr. Ayşe Ayhan

Approved for the Institute of Engineering and Science

Prof. Dr. Mehmet Baray

ABSTRACT

ANALYSIS OF *BRCA1* AND *BRCA2* GENES IN TURKISH BREAST CANCER PATIENTS

Hilâl Özdağ

Ph.D. in Molecular Biology and Genetics

Supervisor: Assoc.Prof. Tayfun Özçelik

June 2000, 165 Pages

Breast cancer is the most frequent cancer type and the second cause of death among women. It is estimated that 10 to 15% of breast cancer cases are hereditary. The majority of hereditary breast cancers can be attributed to germ-line mutations in *BR*east *CA*ncer susceptibility genes *BRCA1* and *BRCA2*.

In this study, germ-line *BRCA1* and/or *BRCA2* gene mutations were screened in 50 Turkish breast and/or ovarian cancer patients divided into four groups of hereditary, familial, early onset, and male cancer by heteroduplex analysis and DNA sequencing. Two *BRCA2* mutations, one novel (6880insG) and one previously reported (3034delAAAC), were found in the hereditary group. A novel *BRCA1* (1200insA) mutation was found in the early onset group. All three mutations cause premature-termination codons. In addition, five *BRCA1* sequence variants have been identified in 23 patients. K654E (2080 A—>G), D693N (2196 G—>A), P871L (2731 C—>T), and K1183R (3667 A—>G) result in a change of amino acids. 1013 T—>C and 2201 C—>T are silent mutations. One patient in the early onset group was compound heterozygote for K654E and D693N. These results indicate that *BRCA1* and *BRCA2* genes are involved in some but not all hereditary breast cancers in the Turkish population.

ÖZET

TÜRK MEME KANSERİ HASTALARINDA *BRCA1* VE *BRCA2* GENLERİNİN İNCELENMESİ

Hilâl Özdağ
Moleküler Biyoloji ve Genetik Doktorası
Tez Yöneticisi: Doç. Dr. Tayfun Özçelik
Haziran 2000, 165 Sayfa

Meme kanseri kadınlar arasında en sık görülen kanser tipidir ve ölüm nedenleri arasında ikinci sırada yer alır. Meme kanseri olgularının %10 ile 15'inin kalıtımsal olduğu tahmin edilmektedir. Kalıtımsal meme kanserlerinin büyük bir kısmı *BRCA1* ve *BRCA2* Meme Kanseri yatkınlık genlerindeki eşey hücre mutasyonlarına atfedilebilir.

Bu çalışmada kalıtımsal, ailesel, erken yaş ve erkek meme kanseri gruplarına ayrılmış 50 Türk meme ve/veya over kanser olgusunda *BRCA1* ve /veya *BRCA2* esey hücre mutasyonları heteroduplekş analizi ve DNA dizilemesi ile taranmıştır. Kalıtımsal meme kanseri grubunda biri yeni (6880insG) biri önceden bildirilmiş (3034delAAAC) iki *BRCA2* mutasyonu saptanmıştır. Erken yaş grubunda yeni bir BRCA1 mutasyonu (1200 insA bulunmuştur. Her üç mutasyonun da proteinde erken sonlanma kodonu oluşumuna neden olduğu tespit edilmiştir. Ayrıca 23 hastada beş adet BRCA1 dizi varyantı tanımlanmıştır. Bunlardan K654E (2080 A→G), D693N (2196 G→A), P871L (2731 C→T) ve K1183R (3667 A→G) tranzisyonları aminoasit değişimine yol açmaktadır. 1013 T→C ve 2201 C→T ise sessiz mutasyonlardır. Erken yaş grubundaki bir hastanın K654E ve D693N için "compound"heterozigot olduğu tespit edilmiştir. Bütün bu sonuçlar *BRCA1* ve *BRCA2* genlerinin Türk popülasyonundaki kalıtımsal meme kanserlerinin bir kısmında rol aldığını ortaya koymaktadır.

TEŞEKKÜR

Yalnızca yazan kişi, jüri üyeleri ve belki konu ile ilgilenen birkaç araştırıcı dışında kimsenin okumadığı her tezin ardında uzun ve yorucu bir hikaye vardır. Bu uzun ve yorucu hikayenin başlamasında etkili olanlar, ilerlemesi ve sonlanmasında desteği olanlar olay örgüsünün önemli birer parçasıdırlar.

Bu hikayenin başlaması iki kişi ile yakından ilgili. Hayatım boyunca beni olayları sorgulamaya ve incelemeye iten ve böylece akılcılık çizgisini bilim yolunda bulmamda en önemli etken olan Sevgili Anne ve Babam'a verdikleri tüm desteğin yanısıra özellikle bu nedenle teşekkür borçluyum.

Bu yolculuğa çıkmaya karar vermemi sağladığı için, üniversiteye başladığım ilk yıl, ilk gün, ilk saat karşıma çıkan ve öğrencilerine bilim felsefesini öğretmeye bütün bir hayatını adamış olan değerli hocam Prof. Dr. Ali Demirsoy'a ve aslında paslanmış çarkların bile kişisel gayret ile çevrilebileceğini göstererek yolun henüz başındaki genç bir araştırıcıya belki de en önemli teşviği vermiş olan değerli hocalarım Prof. Dr. Reyhan Öner ve Prof. Dr. Cihan Öner'e,

Bu uzun yolun başından itibaren, her çıkmaz yol levhasının belirişinde yanımda bulduğum ve bilimsel perspektifi ile yolumu aydınlatan değerli hocam Prof. Dr. Mehmet Öztürk'e,

Değerli tecrübelerini benimle paylaşan ve dostlukları ile bu yılları zenginleştiren Doç. Dr. Uğur Yavuzer ve Dr. Cengiz Yakıcıer'e; özellikle hakkı teslim etmekteki titizliği için destek ve yardımlarını esirgemeyen Doç. Dr. Işık Yuluğ'a,

Beraber gülüp ağlamanın, aynı evi, mutluluğu, hüznü ve dostluğu paylaşmanın güzelliğini yaşadığım Tuba Dinçer'e,

Uzun muhabbetlerini, her zor anda omuzunu, dertlerimizi ve mutluluklarımızı paylaştığı için inatçı, KÜÇÜK kardeşim Tolga Çağatay'a; sonsuz yardımseverliği, dosdoğru dostluğu için Cemaliye Akyerliye'ye; yardımları ve arkadaşlığı için Ayça Sayı'ya;

dostluğu için Arzu Atalay'a; sakin ve soğukkanlı tavrıyla her an yardıma hazır olan Hani Al'Otabi'ye,

İnanılmaz keyifli, derin ve felsefi geyikleri, uzun yaz ve kış gecelerindeki lezzetli sofralar için sevgili kuzucuklarım, Aslı Öztan (The Aquarius), Gülayşe İnce (Gülbahar Teyze), Bleda Bilican (Mösyö-Ahmet Can), N. Tolga Emre (Temre)'ye, (Toprak ananız sizleri seviyor)

On senelik kesintisiz dostluğun her anını doyasıya destek ve sevgileri ile paylaştığım Sibel Songür ve Yasemin Güler'e; uzun geceler ve telefon sohbetlerinde dostluğu için sevgili kuzenim Aylin Özgen'e,

Eşsiz dostluğu, güveni ve arkası kesilmez desteği ile hep yanımda bulduğum Lütfiye Mesci'ye; hayatı ayrıntılarda aramanın keyfi, sınırsız güven ve desteği ile yanımda olduğu için Dr. Gürol Tunçman'a,

Farklı bilim dallarında aynı uzun yolu katetmiş olan Doç. Dr. Ümit Özdağ, Dr. Hakan Özdağ ve Av. Savaş Özdağ (müstakbel Dr)'a, destek ve sevgileri ve herbiri nev'i şahsına münhasır ağabeylerim oldukları için,

en içten teşekkürlerimle.....

TABLE OF CONTENTS

SIGNATURE PAGE ____ i

ABSTRACT ____ ii

ÖZET ____ iii

TESEKKÜR ____ iv

TABLE OF CONTENTS ____ vii

LIST OF TABLES ____ xiii

LIST OF FIGURES ____ xiv

ABBREVIATIONS ____ xvii

1. INTRODUCTION ____ *1*

1.1. Cancer ____ **3**

1.2. Oncogenes ____ **5**

1. 3. Tumor suppressors "Gatekeepers and Caretakers" ____ **7**

1. 4. Breast cancer ____ **10**

1. 4. 1. Etiology of breast cancer ____ 13

1. 4. 2 Genetic factors in Breast Cancer ____ 17

1.4.2.1. Somatic Mutations in Breast Cancer ____ 17

1.4.2.2. Germline Mutations in Breast Cancer ____ 19

1.4.2.2.1. Li-Fraumeni Syndrome (*p53*) ____ 19

1.4.2.2.2. Lynch Type II Syndrome (*MLH1*) ____ 19

1.4.2.2.3. Ataxia Teleangiectasia (*ATM*) ____ 20

1.4.2.2.4. Cowden Disease (*PTEN*) ____ 20

1.4.2.2.5. Genetic Polymorphisms ____ 21

1.5. Hereditary Breast Cancer Genes ____ **23**

1.5.1 Breast Cancer susceptibility gene 1 (*BRCA1*) ____ 24

1.5.1.1 Identification of *BRCA1* ____ 24

1.5.1.2. The Function of BRCA1 ____ 26

1.5.1.3. Mutations in *BRCA1* ____ 30

1.5.2. Breast Cancer susceptibility gene 2 (*BRCA2*) ____ 36

1.5.2.1. Identification of *BRCA2* ____ 36

1.5.2.2. The Function of *BRCA2* ____ 39

1.5.2.3. Mutations in *BRCA2* ____ 40

1.5.3. Penetrance and Histoprognosis of *BRCA1* and *BRCA2* genes ____ 46

1.5.4. Breast Cancer Susceptibility Genes and Risk Assesment 47
1.5.5. Homozygous germ-line *BRCA1* mutations 51

1.6. Mutation Screening 52
1.6.1. Protein Truncation Test 53
1.6.2. Denaturing Gradient Gel Electrophoresis (DGGE) 55
1.6.3. Denaturing High Performance Liquid Chromatography (DHPLC) 56
1.6.4. Single Strand Confirmation Polymorphism (SSCP) 57
1.6.5. Heteroduplex Analysis (HA) 58
1.6.6. Direct Sequencing 59

2. MATERIALS AND METHODS 62

2.1. Materials 62
2.1.1. Families 62
2.1.1.1. Hereditary Breast Cancer Families 62
2.1.1.2. Familial.Breast Cancer Families 64
2.1.2. Oligonucleotides 65
2.1.3. Chemicals and Reagents 69
2.1.4. PCR Materials 70
2.1.5. Sequencing Materials 71
2.1.6. Standard Solutions and Buffers 72

2.2. Methods 76
2.2.1.DNA isolation from peripheral blood 76
2.2.2. Agarose Gel Electrophoresis 77
2.2.3. Polymerase Chain Reaction 78
2.2.3.1. PCR for Heteroduplex analysis 78
2.2.3.2. PCR for DNA Sequencing 79
2.2.4. Protein Truncation Test 81

2.2.5. Cloning 82
2.2.5.1.Ligation and Transformation 82
2.2.5.2.Low scale DNA Isolation 83
2.2.5.3. Restriction Endonuclease Digestion 84
2.2.6. DNA Sequencing 84
2.2.6.1. Cycle Sequencing 84
2.2.7. Polyacrylamide Gel Electrophoresis (PAGE) 85
2.2.7.1 PAGE for Heteroduplex Analysis 85
2.2.7.2. PAGE for PTT 86
2.2.7.2. Denaturing PAGE for DNA Sequencing 86

2.2.8. Capillary Electrophoresis 87

3. RESULTS 88

3.1. DNA Isolation 88

3.2. Polymerase chain reaction 89

3.3. Protein Truncation Test 91

3.4. Heteroduplex analysis 92
3.3.1. *BRCA1* exon 2 92
3.3.2. *BRCA1* exon 5 93
3.3.3. *BRCA1* exon 11A 94
3.3.4. *BRCA1* exon 11B 95
3.3.5. *BRCA1* exon 11C 96
3.3.6. *BRCA1* exon 11D 97
3.3.7. *BRCA1* exon 11 E 98

3.3.8. *BRCA1* exon 11F 99
3.3.8. *BRCA1* exon 11G 100
3.3.9. *BRCA1* exon 11H 101
3.3.10. *BRCA1* exon 11I 102
3.3.11. *BRCA1* exon 11J 103
3.3.12. *BRCA1* exon 11K 104
3.3.13. *BRCA1* exon 13 105
3.3.14. *BRCA1* exon 20 106
3.3.15. *BRCA1* exon 24 107
3.3.16. *BRCA2* exon 11a 108
3.3.17. *BRCA2* exon 11b 109
3.3.18. *BRCA2* exon 11a-b 110
3.3.19. *BRCA2* exon 11c 111
3.3.20. *BRCA2* exon 11d 112
3.3.21. *BRCA2* exon 11e 113
3.3.21. *BRCA2* exon 11f 114
3.3.22. *BRCA2* exon 11g 115
3.3.23. *BRCA2* exon 11g-26 116

3.4. Cloning 120
3.4.1. Mini prep DNA Isolation 120
3.4.2. Restriction Endonuclease Analysis 121

3.4. DNA Sequence Analysis 123
3.4.1. Hereditary Breast and/or Ovarian Cancer 123
3.4.2. Familial Breast Cancer 126
3.4.3. Early Onset Breast Cancer 130
3.4.4. Male Breast Cancer 134

4. DISCUSSION 135

4.1. Mutation Screening 138

4.2.Hereditary Breast Cancer 139

4.3. Familial Breast Cancer 142

4.4. Early Onset Breast Cancer 142

4.5. Male Breast Cancer 145

4.6. Conclusion 145

REFERENCES 149

LIST OF TABLES

Table 1. Leading cause of death and their mortality rates in WHO member states ____ 4

Table 2. TNM Staging_____ 12

Table 4. BRCA1 Primer sequences, expected fragments' sizes and optimal [$MgCl_2$] _ 66

Table 5. BRCA2 Primer sequences, expected fragments' sizes and optimal [$MgCl_2$] _ 67

Table 6. Heteroduplex analysis results of BRCA1 and BRCA2 genes in Turkish breast cancer patients_____117

Table 7. Mutations and polymorphisms identified in Turkish breast and breast-ovarian cancer patients. _____ 137

Table 8. Phenotypes and mutations identified in Turkish Hereditary, and Familial breast and breast-ovarian cancer groups. _____ 140

Table 9. BRCA1 and BRCA2 mutation frequencies in patient groups_____147

LIST OF FIGURES

Figure 1. Caretaker-Gatekeeper Model (from Kinzler and Vogelstein, 1997). 9
Figure 2. Main anatomic structures of breast. (from NCI database) 10
Figure 3. Chromosomal location and exonic organisation of BRCA1 25
Figure 4. Mutation type ratio in BRCA1 (adapted from Bic) 32
Figure 6. BRCA1 mutation frequency in different populations in families with three or more cases of female breast and/or ovarian cancer (Adapted from Szabo and King,1997) 34
Figure 7. BRCA1 mutation frequency in US, Hungarian and Icelandic families with male and female breast cancer. 34
Figure 8. BRCA1 mutation frequency in Italian, Israeli and Japanese population in breast and/or ovarian cancer patients not selected for family history. 35
Figure 9. Chromosomal location* and exonic organization of BRCA2**. 37
Figure 10. Mutation type ratio of BRCA2 41
Figure 11. Frequency of mutations according to type in BRCA2 exon 11. 41
Figure 12. BRCA2 mutation frequency in English, Canadian, Finnish, French, Hungarian, Icelandic, Swedish, Danish, and US populations in families with three or more cases of female breast and/or ovarian cancer. 42
Figure 13. BRCA2 mutation frequency in US, Hungarian and Icelandic families with male and female breast cancer cases. 44
Figure 16. Schematic representation of protein truncation test. 54
Figure 17. Comparison of SSCP, DGGE and HA in mutation detection. 59
Figure18. Pedigrees of hereditary breast cancer families. 63
Figure 19. Pedigrees of familial breast cancer families. 65
Figure 19. Analysis of extracted genomic DNA samples. 89
Figure 20. Analysis of PCR products. 90
Figure 21. PTT of BRCA1 exon 11. 91
Figure 22. Heteroduplex analysis of BRCA1 exon 2. 92

Figure 23. Heteroduplex analysis of BRCA1 exon 5. 93
Figure 24. Heteroduplex analysis of BRCA1 exon 11A. 94
Figure 25. Heteroduplex analysis of BRCA1 exon 11B. 95
Figure 26. Heteroduplex analysis of BRCA1 exon 11C. 96
Figure 27. Heteroduplex analysis of BRCA1 exon 11D. 97
Figure 28. Heteroduplex analysis of BRCA1 exon 11F. 98
Figure 29. Heteroduplex analysis of BRCA1 exon 11F 99
Figure 30. Heteroduplex analysis of BRCA1 exon 11G. 100
Figure 31. Heteroduplex analysis of BRCA1 exon 11H. 101
Figure 32. Heteroduplex analysis of BRCA1 exon 11I. 102
Figure 33. Heteroduplex analysis of BRCA1 exon 11J. 103
Figure 34: Heteroduplex analysis of BRCA1 exon 11K. 104
Figure 35. Heteroduplex analysis of BRCA1 exon 13. 105
Figure 36. Heteroduplex analysis BRCA1 exon 20. 106
Figure 37. Heteroduplex analysis of BRCA1 exon 24. 107
Figure 38. Heteroduplex analysis of BRCA2 exon 11a. 108
Figure 39. Heteroduplex analysis of BRCA2 exon 11b. 109
Figure 40. Heteroduplex analysis of BRCA2 exon 11a-b. 110
Figure 41. Heteroduplex analysis of BRCA2 exon 11c 111
Figure 42. Heteroduplex analysis of BRCA2 exon 11d 112
Figure 43. Heteroduplex analysis of BRCA2 exon 11e 113
Figure 44. Heteroduplex analysis of BRCA2 exon 11f. 114
Figure 45. Heteroduplex analysis of BRCA2 exon 11g. 115
Figure 46. Heteroduplex analysis of BRCA2 exon 11g-26. 116
Figure 47. Agarose gel electrophoresis of extracted mini prep DNA samples of 97/641 BRCA1 exon 11B. 121
Figure 48. Restriction endonuclease analysis of 97/641 mini prep DNA samples. 122
Figure 57. Secondary structure prediction of K654E substitution. 144

ABBREVIATIONS

APS	ammonium persulfate
BIC	Breast cancer Information Core
bisacrylamide	N,N, methylene bis-acrylamide
bp	base pair
BRCA1	BReast CAncer susceptibility gene 1
BRCA2	BReast CAncer susceptibility gene 2
cDNA	complementary DNA
cm	centimeter
dCTP	cytosine deoxyribonucleoside triphosphate
del	deletion
ddH2O	deionized water
ddNTP	dideoxynucleotide triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
g	gram
HA	heteroduplex analysis
ins	insertion
IPTG	isopropyl -β–D thiogalactopyranoside
kb	kilobase
lt	liter
M	Molar
MCi	MiliCurie
min	minute
ml	mililiter
mM	milimolar
NCI	National Cancer Institute
ng	nanogram

nt	nucleotide
P^{32}	phosphore 32
PCR	polymerase chain reaction
pmol	picomol
PTT	protein truncation test
RNA	dibonucleic acid
rpm	revolution per minute
S^{35}	sulphure 35
SDS	sodium dodecyl sulphate
sec	second
SSCP	single strand conformation polymorphism
TBE	Tris, Boric acid, EDTA
TEMED	N,N,N,N-tetramethyl-1,2 diaminoethane
U	unit
uv	ultraviolet
V	volt
WHO	World Health Organization
Xgal	5 Bromo-4 chloro-3 indolyl, β-D-galactopyranoside
µCi	microCurie
µl	microliter

1. INTRODUCTION

Cancer is one of the leading causes of death in human populations[1]. There have been many studies that aim to elucidate the mechanism of tumorigenesis, and many attempts to cure cancer in the 20th century. These efforts accumulate a huge amount of data about the nature and mechanism of tumorigenesis. Although the accumulated data is just the tip of the iceberg, it is now known that cancer appears as a result of the disruption of the genomic integrity. This disruption may arise in chromosomal or gene levels. In both ways there is either a gain (oncogene) or a loss (tumor supressor) of function of a particular set of genes that control the growth and the life time of a cell (Haber and Harlow, 1997). Thus the researches dealing with tumorigenesis are focused on either oncogenes or tumor supressor genes at several different scopes, all aiming to find out the underlying mechanisms of a cell's life cycle.

Disruption of genomic integrity may occur in several different ways. It may develop sporadically, because of epidemiologic factors, lifestyle, or it may be hereditary. In either case, genomic integrity is disrupted. Recent experimental data has shown that some individuals are more susceptible to cancer because of their genomic heritage. Actually, more than a century ago, Paul Broca described four generations of breast

[1] (WHO Report 1998)

cancer in his wife's family which underlined, probably for the first time, contribution of the heredity factor in tumorigenesis (Lynch *et al.* 1994).

Breast cancer is the most frequent cancer type and the second cause of death among women. Like many other cancer types, breast cancer may arise sporadically or hereditary factors may contribute to its development. It is estimated that 10 to 15% of breast cancer cases are hereditary (Feunteun 1999). In 1994 and 1995, as a result of an international collaboration of many research groups, two loci have been found to be linked to hereditary breast cancer (Miki *et al.*1994, Wooster *et al.* 1995). The subsequently identified genes were named as *BR*east *CA*ncer susceptibility genes *BRCA1* and *BRCA2* are now considered to be responsible for approximately 60% of hereditary breast cancer cases based on mutation analysis studies in many different populations (Szabo and King 1997). Functional studies, although not complete, show that these two genes are tumor suppressors that have roles in the maintenance of genomic integrity as caretakers (Vogelstein and Kinzler 1998).

Characterization of the mutations of a particular gene renders important information regarding the function of that gene. Besides, documentation of the mutation spectrum of a gene is one of the most reliable reflection of a population's dynamics. The *BRCA1* and *BRCA2* mutation status of most Western populations has been studied during the last five years. However, there is not enough data on these two structurally gigantic genes' status in Eastern populations. Breast cancer is among the most common malignancies in Turkish women (Karaoguz and Içli, 1993). The frequency and the types of germ-line mutations involved in Turkish breast/ovarian cancers are not well known. In order to

determine the contributions of *BRCA1* and *BRCA2* mutations to the development of breast/ovarian cancer in the Turkish population, we screened pre-selected regions of these genes in four groups of patients composed of hereditary and familial cancer. As well as early onset and male breast cancer.

1.1. Cancer

Cancer is a common term used for all malignant tumors, and 2.3 million individuals die (Table 1) from cancer each year according to the 1998 estimates of WHO (The World Health Report, 1999). Although the ancient origins of this term is somewhat uncertain, it probably derives from the Latin for crab, "cancer", presumably because a cancer "adheres to any part that it seizes upon in an obstinate manner like the crab". Neoplasia means "new growth". The term tumor was originally applied to the swelling caused by inflammation. Neoplasms also may induce swellings, and by long precedent the non-neoplastic usage of tumor has passed into limbo; thus, the term is now equated with neoplasm. Oncology (greek "oncos"= tumor) is the study of tumors or neoplasms (Robbins S. *et al*, 1984). Since the beginning of the century, researchers have studied cancer to understand the mechanisms of tumorigenesis. The original concept of negative regulators of tumor development can be traced to the earliest descriptions of cancer as a genetic disease. The earliest known reference is to the German cell biologist Theodor Boveri, who proposed in 1902 that cancer might arise from the effects of both positive and negative regulators (Haber D. and Harlow E., 1997). It is known for many years that cancer arise as the result of genetic disruption which leads the cell to be immortalized and to proliferate in an uncontrolled fashion.

Table 1. Leading cause of death and their mortality rates in WHO member states

Mortality in WHO Member States			
	rank	*% of total*	*(000)*
Both sexes			
Ischaemic heart disease	1	13.7	7 375
Cerebrovascular disease	2	9.5	5 106
Acute lower respiratory infections	3	6.4	3 452
HIV/AIDS	4	4.2	2 285
Chronic obstructive pulmonary disease	5	4.2	2 249
Diarrhoeal diseases	6	4.1	2 219
Perinatal conditions	7	4.0	2 155
Tuberculosis	8	2.8	1 498
Cancer of trachea/bronchus/lung	9	2.3	1 244
Road traffic accidents	10	2.2	1 171
Males			
Ischaemic heart disease	1	12.8	3 659
Cerebrovascular disease	2	8.2	2 340
Acutelowerrespiratory infections	3	6.1	1 753
Chronic obstructive pulmonary disease	4	4.3	1 240
HIV/AIDS	5	4.1	1 164
Diarrhoeal diseases	6	4.0	1 149
Perinatal conditions	7	3.9	1 121
Cancer of trachea/bronchus/lung	8	3.2	911
Tuberculosis	9	3.1	893
Road traffic accidents	10	3.0	855
Females			
Ischaemic heart disease	1	14.6	3 717
Cerebrovascular disease	2	10.9	2 766
Acutelowerrespiratory infections	3	6.7	1 699
HIV/AIDS	4	4.4	1 121
Diarrhoeal diseases	5	4.2	1 070
Perinatal conditions	6	4.1	1 034
Chronic obstructive pulmonary disease	7	4.0	1 010
Tuberculosis	8	2.4	605
Malaria	9	2.1	538
Measles	10	1.7	432

Genetic disruption leading to tumorigenesis cause either a negative regulation – loss of function of tumor supressor genes, or a positive regulation – gain of function of oncogenes, in a cell's life cycle. Tumorigenesis is a multistep process. Thus the genetic disruption is an accumulation of many genetic alterations. Some individuals are genetically susceptible to cancer, although most of the cases arise sporadically. This susceptibility comes from what is termed as "the first hit". Knudson postulated that individuals with an autosomal dominant cancer susceptibility inherited a germ-line mutation in one of the alleles of a particular gene and that subsequent genetic alterations were required for tumor formation. Over the years Knudson's hypothesis was refined to include a second hit at the same locus to inactivate the remaining normal allele. The past decade has seen numerous molecular confirmations of Knudson's hypothesis (ex. *APC*, *RB*) and concrete demonstration of the multiple genetic events required for tumorigenesis (ex. colon cancer) (Vogelstein and Kinzler, 1997).

1.2. Oncogenes

For many years it has been realised that damage to the DNA of a cell (mutation) is associated with changes that lead to cancer. It was initially believed that the genetic mutations responsible for cancer caused a deletion of essential regulatory genes restraining cell growth. Evidence for this has also come from cell fusion studies, which implied that malignant cells have lost functions that are dominantly expressed in normal cells, and are capable of suppressing malignancy. However, the discovery of retroviruses radically changed these ideas concerning the alterations that occur to genes in cancer; it led to the discovery of oncogenes and gave a new perspective of cancer as being caused

by genes that actively promote uncontrolled growth. The vast majority of non-viral oncogenes appear to be altered forms of cellular genes that encode proteins that participate in the pathway of cellular proliferation. The normal, intact cellular genes are known as proto-oncogenes. Cells receive signals to proliferate via growth factors that bind to receptors located on the outside surface of the cell. The signal is then transmitted into the cell, across the cytoplasm to the nucleus. There, the signal is converted into a growth response by means of transcriptional activation of the genes for proliferation. As a general rule, oncogenes are mutated forms of the cellular proto-oncogenes which encode the components of this signalling pathway. Direct comparisons of the DNA sequences of normal proto-oncogenes with their mutated oncogene derivatives have revealed the mechanisms by which proto-oncogenes become activated. Activation can occur either by changes to the coding sequence of the proto-oncogene so that a mutated protein –an oncoprotein- is produced with aberrant biochemical properties; or it can occur by mutations that deregulate the levels, and/or timing, of expression of the structurally unaltered proto-oncogene (Cooper GM, 1995).

The result of proto-oncogene activation is the gain of function in genes signalling proliferation to occur in cells which should not normally be proliferating. This inappropriate cell division will disrupt the normally ordered differentiation program of the cell. These activation processes can occur in a number of ways, which do not necessarily involve alteration of the structure of the proto-oncogene, but rather its forced expression at times in the development of the cell when proliferative genes should be silent and differentiative genes should be expressed (Burck KB *et al*, 1988).

Similarly, just as the overexpression of a normal gene product at the inappropriate time can lead to partial cell transformation, so the alteration of the coding sequence of a proto-oncogene can change the function of the protein product so that it relays its proliferative signals inappropriately. Such activation events can occur by deletions of portions of the coding sequence, by fusion of the sequence to other protein domains that alter the activity of the signalling domain, or by point mutations to the sequence at critical bases such that the amino acid sequence of the protein product is critically altered. The ras class of proto-oncogenes can become oncogenic by a change as small as a single base being changed in the sequence of the gene (Vile R., 1992)

1. 3. Tumor suppressors "Gatekeepers and Caretakers"

The molecular concepts of tumor suppressor genes stem from three lines of experimental evidence. The first is explained by Knudson's model predicting the development of the childhood tumor retinoblastoma requires two rate-limiting genetic hits, subsequently shown to represent mutations of both alleles of a tumor suppressor gene. The hypothesis that children with inherited susceptibility to retinoblastoma harbour a germline hit, while sporadic cases have two somatic mutations, was confirmed with the cloning of the *RB* gene. A second essential observation was the fact that these genetic hits consisted of loss-of –function mutations. Loss of heterozygosity showed how loss of genetic material was the mechanism by which recessive mutations became manifest. A third experimental avenue was explored by Harris and co-workers in 1969, who found they could suppress the malignant phenotype by fusion of cancer cells with non-transformed cells.

Many of the known tumor suppressor genes, including *RB*, *p53*, *WT1*, *APC* and *p16*, fulfill these basic early predictions-loss-of-function mutations, inactivation in both familial and sporadic tumors, and rescue of the tumor phenotype by the wild-type allele. For example, the classical concept that re-introduction of tumor suppressors into malignant cells inhibits their proliferation, is challenged by the new class of genes implicated in genomic integrity and DNA repair, whose loss leads to genetic damage that cannot be remedied simply by restoration of a wild-type allele.

The functions of known tumor suppressor genes are in a wide range of different cellular pathways. The current family of tumor suppressors includes DNA-binding transcription factors (*p53* and *WT1*), genes that may indirectly modulate transcriptional regulation (*RB*, *APC*, possibly *BRCA1*), an inhibitor of kinases required for cell-cycle progression (*p16*), a cell structural component (*NF2*), a novel phosphatase (*PTEN MMAC1*), a potential mediator of mRNA processing (*VHL*), and genes implicated in signaling pathways including those of ras (*NF1*, *TGF*.) Tumor suppressor genes involved in DNA repair and genomic stability (*MSH2*, *MLH1*, *BRCA1* and *BRCA2*) have also been described as caretakers.

Actually, recently Vogelstein and Kinzler divided most tumor suppressors broadly into two groups, named as "gatekeepers" and "caretakers" (Figure 1). Gatekeepers are genes that directly regulate tumor development by inhibiting its growth or by promoting its death. The functions of these genes are rate limiting for tumor growth, and as a result both the maternal and paternal copies of these genes must be inactivated for a tumor to

develop. The identity of gatekeepers varies with each tissue, such that inactivation of a given gene leads to specific forms of cancer predisposition. Because gatekeeping genes are rate limiting for tumor initiation, they tend to be frequently mutated in sporadic cancers through somatic mutation as well as in the germline of predisposed individuals.

In contrast, inactivation of caretakers does not directly promote growth of tumors. Rather, inactivation of caretakers leads to a genetic instability that only indirectly promotes growth by causing an increased mutation rate. Because numerous mutations are required for the full development of a cancer, inactivation of caretakers, with the consequent increase in genetic instability, can greatly accelerate the development of cancer (Vogelstein B. 1998).

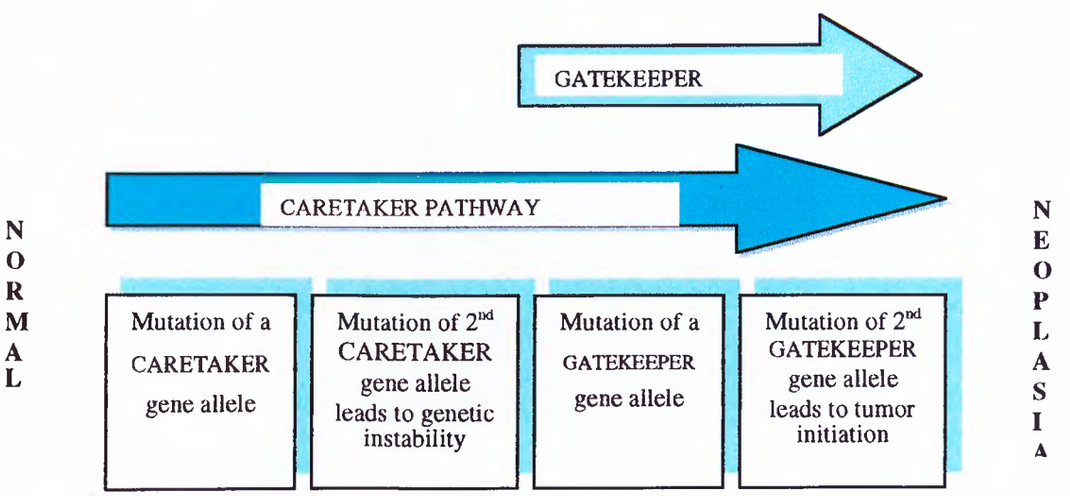


Figure 1. Caretaker-Gatekeeper Model (from Kinzler and Vogelstein, 1997).

1. 4. Breast cancer

Neoplasms constitute the most important lesions of the female breast. A great variety of tumors may occur in the female breast, made up as it is of a covering integument, adult fat, mesenchymal connective tissue, and epithelial structures (Robbins *et al*, 1984). Breast cancer is the second leading cause of death among women of all ages according to the National Cancer Institute statistics. It becomes the first leading cause of death among women with age between 15-54.

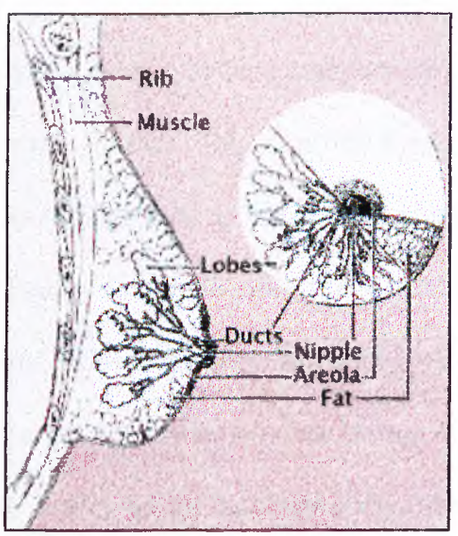


Figure 2. Main anatomic structures of breast. (from NCI database)

Each breast has 15 to 20 overlapping sections called lobes. Within each lobe are many smaller lobules, which end in dozens of tiny bulbs that can produce milk. The lobes,

lobules, and buibs are all linked by thin tubes called ducts. These ducts lead to the nipple in the center of a dark area of skin called the areola. Fat fills the spaces around the lobules and ducts. There are no muscles in the breast, but muscles lie under each breast and cover the ribs. Each breast also contains blood vessels and vessels that carry colorless fluid called lymph. The lymph vessels lead to small bean-shaped organs called lymph nodes. Clusters of lymph nodes are found near the breast in the axilla (under the arm), above the collarbone, and in the chest. Lymph nodes are also found in many other parts of the body.

Breast carcinoma arises from the epithelium of the mammary gland, which includes the milk-producing lobules and the ducts that carry milk to the nipple. Malignant transformation of the stromal, vascular, or fatty components of the breast is not included in this definition and is extremely rare. The transition from normal to malignant breast epithelium has not been as well studied as the parallel changes in colonic epithelium; however, there is increasing evidence that the breast epithelium undergoes a transformation from normal to hyperplastic, followed by the appearance of atypia in association with the hyperplasia, ultimately becoming malignant. Malignant cells continue to evolve from noninvasive carcinoma, typified by in situ to invasive carcinoma, and ultimately to cells with metastatic potential (Kinzler and Vogelstein, 1997). Breast cancer is generally classified as invasive or non-invasive. Invasive cancer originates in the lobules and/or milk ducts, while while non-invasive or in situ cancers are confined to the lining of the lobules or ducts. The most important criteria for assessing future risk of invasive disease is to determine whether cancer cells have invaded the lymph nodes. Breast cancer is commonly categorized into three grades

according to growth rate. Well-differentiated tumors are termed as grade I; moderately differentiated tumors as grade II; and poorly differentiated tumors are termed as grade III. The most common type of breast cancer begins in the lining of the ducts and is called ductal carcinoma. Another type, called lobular carcinoma, arises in the lobules. When breast cancer spreads outside the breast, cancer cells are often found in the lymph nodes under the arm (axillary lymph nodes). If the cancer has reached these nodes, it may mean that cancer cells have spread to other parts of the body--other lymph nodes and other organs, such as the bones, liver, or lungs via the lymphatic system or the bloodstream.

The treatment and prognosis of a women with breast cancer are strongly influenced by the stage at the time of diagnosis. Multiple staging systems have been proposed, but the most commonly used system is the one adopted by both the American Joint Committee (AJC) and the International Union against Cancer (UICC). This staging system is a detailed TNM (tumor, nodes, metastasis) but can be summarized as in Table 2.

Table 2. TNM Staging

Stage 0	Carcinoma in situ
Stage I	Tumor 2 cm, axillary nodes not involved
Stage II	Tumor between 2 and 5 cm and/or involved but mobile axillary lymph nodes
Stage III	Tumor larger than 5 cm and/or fixed axillary lymph nodes; includes inflammatory breast cancer
Stage IV	Distant metastases beyond ipsilateral axillary lymph nodes

1. 4. 1. Etiology of breast cancer

There are several determinants of breast cancer occurrence such as reproductive history, diet and alcohol consumption, endogenous hormones and estrogen receptor expression, and exogenous hormones. There are many case-control studies analyzing the effect of reproductive factors on breast cancer. The possibility of a protective effect for mothers of twins versus mothers of single births (odds ratio 0.7) received support from a large nested case-control study conducted in Sweden (Murphy *et al*, 1997). This association may be correlated with either to the influence of hormonal characteristics of pregnancy or to genetic differences between women who give birth to twins and those who do not. Women appear to be at higher risk of developing breast cancer during or shortly after pregnancy, and the prognosis for such pregnancy associated breast cancers was observed to be poorer than for tumors unrelated to pregnancy (Bonnier,. *et al*, 1997). The possible influence of lactation on the risk of breast cancer is also under investigation. Some recent studies suggest that there is a protective association between breast feeding and breast cancer among both premenopausal and postmenopausal women (Enger *et al*, 1997).

Numerous studies have reported on the relationship between drinking alcohol and breast cancer, and the results have tended to indicate that alcohol consumption is associated with a slightly increased risk of breast cancer (Schatzkin, *et al* 1994)

Hormone replacement therapy is used in cardiovascular disease, in osteoporosis, and in the relief of menopausal symptoms. An analysis of studies on this topic showed that current hormone replacement therapy users were approximately 20% more likely to develop breast cancer than those who never used this therapy. The risk decreases in a nonuser 5 year after cessation of use (Coll. Group on Horm. Fact. In Breast Ca, 1997). These results suggest that estrogen acts late in the sequence of breast carcinogenesis and its tumor-promoting effects may be reversible (Alberg *et al*, 1998).

Table 3. Mortality Rates in five leading cancer among men and women according to age of onset

All Ages		Under 15		15-34		35-54		55-74		75+	
Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Lung Bronchus	Lung Bronchus	Leukemia	Leukemia	Leukemia	Breast	Lung Bronchus	Breast	Lung Bronchus	Lung Bronchus	Lung	Lung
51	37	43	41	31	32	53	46	59	44	40	33
Prostate	Breast	Brain CNS	Brain CNS	NHL	Leukemia	Colon Rectum	Lung Bronchus	Colon Rectum	Breast	Prostate	Colon Rectum
19	27	30	33	25	24	16	27	15	25	31	25
Colon Rectum	Colon Rectum	Endocrine	Endocrine	Brain CNS	Cervix	NHL	Colon Rectum	Prostate	Colon Rectum	Colon Rectum	Breast
16	19	13	13	23	18	12	10	13	14	16	23
Pancreas	Pancreas	NHL	Bone Joints	Soft Tissue	Brain CNS	Brain CNS	Ovary	Pancreas	Ovary	Pancreas	Pancreas
7	9	55	38	11	15	10	9	7	9	7	11
NHL	Ovary	Soft Tissue	Kidney	Hodgkin's	NHL	Pancreas	Cervix	NHL	Pancreas	Leukemia	NHL
7	8	7	35	10	11	9	8	6	8	6	8

(According to National Cancer Institute 1998 Statistical Data)

Evidence concerning reproductive history and exogenous estrogen use suggest that estrogen is involved in the etiology of breast cancer (Alberg *et al* 1998). Several studies, including a multicountry study for the World Health Organization, suggest that oral contraceptives (used by 61 million women worldwide) are associated with a relative risk of 1/3 to 1/5 for breast cancer that will be diagnosed before the age of 40-45 (Hulka and Stark, 1995).

That hormones influence risk and prognosis of breast cancer has been known for decades. Breast cancer is rare in men, suggesting an influence of sex-steroid hormones. Women who have had bilateral oophorectomy early in life are at markedly reduced risk of subsequently developing breast cancer; the earlier oophorectomy is done, the greater the reduction. Early age at menarche (11 years or less) and late age at natural menopause (55 or older) are associated with increased risk (Hulka and Stark, 1995). Antiestrogens are employed clinically because of their ability to inhibit the growth of breast cancer cells. In addition, disease progression inevitably occurs, indicating a transition to hormone resistance even in the presence of the estrogen receptor (Eisen and Weber, 1998). The responsiveness of breast tissue to estrogen exposure would be expected to lead to differences in susceptibility to breast cancer. This is supported by the results of a case-control study which showed that the normal breast tissue of women with breast cancer was three times more likely than the breast tissue of the control patients to have estrogen receptor overexpression (Khan, *et al*, 1997).

1.4.2 Genetic factors in Breast Cancer

1.4.2.1. Somatic Mutations in Breast Cancer

The study of sporadic breast cancers is important in order to understand the pathogenesis of breast cancer. Sporadic breast cancer which constitutes approximately 90% of all breast cancer cases, have fundamental molecular genetic differences. These somatic alterations are in growth factors and receptors, intracellular signalling molecules, regulators of cell cycle, adhesion molecules and proteases. In addition, LOH of many loci, such as chromosomes 17p, 17q, 16q, 13q, 11p, 1p, 3p and 18q (Cleton–Jansen, 1994; Gudmundsson, 1993; Cropp,1993; Mathew, 1994; Dorion-Bonnet, 1995).

Members of the epidermal growth factor receptor (EGFR) family are frequently altered in sporadic breast cancer. These proto-oncogenes, EGFR, erbB-2 or HER-2, and erbB4, become oncogenic through gene amplification or overexpression resulting in the aberrations in signal transduction pathways and thus deregulation of cellular proliferation (Bacus, 1994).

Disruption of the cell cycle checkpoints leads to uncontrolled growth and cancer. The tumor suppressor protein p53 which is known also as genome guardian, plays a central role in regulating progression through cell cycle. p53 Alterations in breast cancer are shown by analyzing the coding region for mutations or using antibody demonstrating aberrant localization or altered levels of the protein. p53 mutations have been detected in

15 to 45% of human breast cancer specimens in several studies (Deng *et al*, 1994; Andersen *et al*, 1993; Saitoh *et al*, 1994)

Like p53, RB regulates cell cycle progression. Dephosphorylated RB inhibits cellular proliferation by halting cell cycle progression in G1. Structural rearrangements and inactivation of RB is detected in breast cancer (T'ang *et al*, 1988; Lee *et al*, 1988). In addition it is shown that estradiol decreases expression of RB (Gottardis *et al*, 1995). Another growth inhibitory protein TGF-β which is shown to be hormonally regulated is found to be decreased in breast cancer (Jeng *et al*, 1993). Cyclins which play important roles in different cell cycle checkpoints are studied in breast cancer. Cyclin D1 which regulates the G1-S transition, is shown to be overexpressed in breast cancer (Musgrove *et al*, 1991).

The programmed cell death, apoptosis, is an important physiological defense mechanism of cells to avoid cancer. The proto-oncogene, bcl-2, which functions to suppress apoptosis is found to be overexpressed in 30-45% of breast cancer cases (Johnston *et al*, 1994). Another proto-oncogene, c-myc, is also found to be overexpressed in breast cancer (Pavelic *et al*, 1992).

1.4.2.2. Germline Mutations in Breast Cancer

The search for reliable genetic markers for the susceptibility to develop breast cancer has proven to be difficult, since the disease itself is not due to a single gene mutation and even the predisposition can be based on different gene defects. There are several tumor suppressor genes associated with several syndromes that are thought to be involved in genetic predisposition to breast cancer.

1.4.2.2.1. Li-Fraumeni Syndrome (*p53*)

This rare dominantly inherited cancer syndrome is characterized by a predisposition to sarcomas, breast cancer, brain tumors, leukemia and adrenocortical carcinoma in children and young adults. Approximately 50% of the women with the Li-Fraumeni syndrome may develop breast cancer. Germ-line mutations found in the *p53* tumor suppressor gene explains this disorder (Malkin *et al*, 1990). However, it has been shown that less then 1% of the women who develop breast cancer at a very young age carry a germ-line mutation in the *p53* tumor suppressor gene (Borresen *et al*, 1992)

1.4.2.2.2. Lynch Type II Syndrome (*MLH1*)

The Lynch type II syndome is associated with a dominantly inherited susceptibility to a variety of tumors including breast, colon, uterine and ovarian cancers and melanomas. The risk of developing breast cancer is increased in individuals from families with the

Lynch type II syndrome, and the relative risk for breast cancer in first-degree relatives of index cases identified because of colon cancer has been estimated to be about 5% (Krainer, 1994).

1.4.2.2.3. Ataxia Teleangiectasia (*ATM*)

It has been suggested that heterozygotes for the gene for ataxia teleangiectasia (*AT*) may represent a large proportion of those at high risk for breast cancer, since the relative risk for breast cancer in *AT* heterozygotes is said to be between 7% and 15%, and the heterozygote frequency is estimated to be 1.4%. However, a linkage to the *AT* locus in familial breast cancer could not be found (Wooster, *et al*, 1993).

1.4.2.2.4. Cowden Disease (*PTEN*)

Cowden disease (CD) is an autosomal dominant syndrome characterized by the development of hamartomas in multiple organ systems, and an increased risk of developing breast and thyroid cancers. The CD locus was mapped to chromosome 10q22-23 in 1996 (Nelen, *et al*, 1997). Two groups independently identified a tumor suppressor gene (*PTEN*) at 10q23 using loss of heterozygosity analysis in glioblostoma multiforme (Steck *et al*, 1997; Li *et al*, 1997). Mutations in this gene were found in primary gliomas and carcinomas of the breast and kidney as well as in related cell lines. Subsequently, mutations in *PTEN* have been identified in multiple CD kindreds identifying *PTEN* as the gene responsible for CD when mutated. So far no germ-line

mutations in breast cancer families have been identified. These data provide strong evidence that *PTEN* does not account for hereditary breast cancer susceptibility outside of families that are affected by CD. Finally, somatic *PTEN* mutations in primary breast cancer specimens have been identified only very rarely (Rhei *et al*, 1997, Teng, *et al*, 1997).

1.4.2.2.5. Genetic Polymorphisms

Environmental contaminants have potential to influence breast cancer risk. The most well-defined environmental risk factors are radiation exposure and alcohol ingestion. Diet is clearly related to the increased incidence of breast cancer in developed countries, but its precise role is not yet established. Epidemiologic and ecologic investigations must take into account the very complex etiology of breast cancer, and the knowledge that tumorigenesis can arise from different mechanisms. Genetic polymorphisms exist in genes that govern capacity to metabolize environmental contaminants. Higher risk may occur among persons whose enzymes either are more active in the production of procarcinogens or fail to detoxify carcinogenic intermediates formed from chemicals in the environment (Wolff and Weston, 1997).

Catechol-O-methyltransferase (*COMT*) participates in the metabolism of potentially harmful estrogen metabolites. In a nested case-control study, the low activity *COMT* allele was not strongly associated with breast cancer overall, but was associated with increased risk among postmenopausal women, particularly those with higher body mass index. A second study also showed no association between the low activity *COMT* allele

and breast cancer overall, but it was associated with decreased risk among postmenopausal women, and increased risk among premenopausal women in the study (Thompson *et al*, 1998).

The results of a nested case-control study showed a strong association between putative high risk genotypes for three of the glutathione S-transferases (*GSTs*), which act to detoxify carcinogens. Women with all three hypothesized high risk genotypes were over three times more likely to develop breast cancer than women with all three putative low risk genotypes (Charrier *et al*, 1999). Other studies, however, did not observe significant associations between breast cancer and the *GSTM1* positive or *GSTM1* null genotypes (Ambrosone *et al*, 1999).

The *CYP17* gene may influence the risk of breast cancer by influencing estradiol production. In a case-control study, little association was present between *CYP17* polymorphisms and breast cancer overall but the presence of an A2 allele was associated with a 2.5-fold excess risk among women with advanced stage disease (Haiman *et al*, 1999).

Slow and rapid acetylator individuals exist in most human populations, and the mutations responsible for the slow-acetylator genotype have been determined. Slow acetylators, is at higher risk of developing breast cancer, and fast acetylators, have an increased risk of developing colon cancer (Rodrigo *et al*, 1999). Previous findings suggested that cigarette smoking was a risk factor for postmenopausal breast cancer

among women with the N-acetyltransferase 2 slow acetylation genotype (Zheng *et al*, 1999).

Besides all of these genetic factors that are supposed to be involved in genetic predisposition to breast cancer, it has been found that two genes are acting a major role in familial breast cancer.

1.5. Hereditary Breast Cancer Genes

Most genetic changes arise spontaneously (sporadic mutations) and are not inherited from either parent. Familial and hereditary are terms that refer to increased cancer risk within families; however, they are not synonymous. Familial cancer is defined as the simple clustering of disease within families. Hereditary cancer is a more specific term, referring to a subtype of familial disease with a pattern of distribution consistent with Mendelian inheritance of a susceptibility gene. Families in which the predisposition for malignant disorders appears to be restricted to breast cancer are known as "breast specific" families. There are also families with an increased risk for ovarian cancer along with breast cancer. Furthermore, there is evidence that these families show a higher incidence of prostate, colon and pancreas cancer (Krainer, 1994). Women are not all at equal risk for contracting breast cancer. The most important factor other than age that determines who will and who will not manifest breast cancer is the family history of breast and related cancers. Descriptive studies of pedigrees of breast cancer-prone families and epidemiologic studies of breast cancer in the general population are the keys to identifying differences in cancer susceptibility. Since Paul Broca's report, where

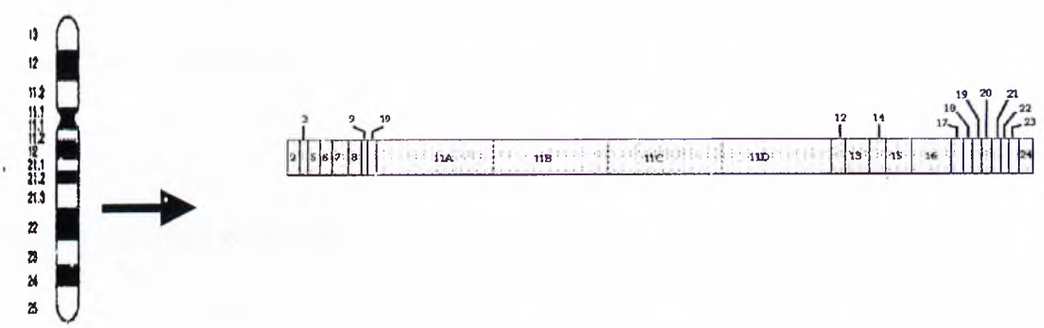
he described four generations of breast cancer in his wife's family, an enormous number of breast cancer-prone families have been reported. Usually the pattern of breast cancer occurrences in these families is most consistent with an autosomal dominant mode of inheritance (Lynch, *et al,* 1994).

1.5.1 Breast Cancer susceptibility gene 1 (*BRCA1*)

1.5.1.1 Identification of *BRCA1*

Description of families with multiple cases of breast cancer has led the researchers to hunt for a breast cancer susceptibility genes. Linkage analysis allows the localization of human disease genes in families with high incidence of the disease. This analysis relies on the identification of one or more markers in the genome that segregate with the disease. In 1990, Hall *et al.*, made a genetic analysis on 23 extended families with 146 cases of breast cancer. These families share the epidemiological features that are characteristic of familial, versus sporadic, breast cancer: younger age at diagnosis, frequent bilateral disease, and more frequent occurrence of disease among men. The result of this study showed that familial breast cancer is linked to 17q21 (Hall, *et al,* 1990). Several confirmations of this result were subsequently published. After the first confirmation by Narod and his coworkers in 1991 (Narod, *et al.* 1991) this breast-ovarian cancer locus has been formally labelled "*BRCA1*" (Solomon and Ledbetter, 1991). Then a third confirming report came from Breast Cancer Linkage Consortium for the locus on 17q21 (Easton, *et al,* 1993).

After the localization of *BRCA1* to 17q21, great efforts were made to identify the gene, its transcript, and protein. Miki and his coworkers developed a detailed map of transcripts for the 600 kb region of 17q21 between D17S1321 and D17S1325 (Figure 3). Sixty-five candidate expressed sequences within this region were identified. Three expressed sequences eventually were merged into a single transcription unit whose characteristics strongly suggest that it is *BRCA1*. Conceptual translation of the cDNA revealed a single, long open reading frame encoding a protein of 1863 amino acids. Homology searches revealed that the protein contains a zinc finger domain in its amino terminus. Probing of genomic DNA samples from different species with *BRCA1* sequences revealed strong hybridizations in tissues from humans, mice, rats, rabbits, sheep, and pigs. In the same study of Miki and co-workers an 11 bp deletion, a 1 bp insertion, a nonsense, a missense and a regulatory mutation that were segregating with breast cancer in families linked to 17q21 were identified (Miki Y. *et al*, 1994).



Chromosome 17

Figure 3. Chromosomal location and exonic organisation of *BRCA1*
*adapted from NCBI database
**adapted from Breast Cancer Information Core database

BRCA1 appeared to encode a tumor suppressor protein. In order for *BRCA1* to be a tumor suppressor gene it must bear the most important characteristic of a tumour suppressor. That is *BRCA1* protein should be present in normal breast and ovarian tissue and should be altered, reduced or absent in breast and ovarian tumors to fulfill the loss-of-function characteristic of a tumor suppressor gene. After the localization of *BRCA1* to 17q21 many research groups studied whether *BRCA1* has this characteristic or not, by loss of heterozygosity studies (Smith, *et al*, 1992; Lindblom, *et al*, 1993; Cornelis, *et al*, 1993; Neuhausen, *et al*, 1994). Just six months before the identification of *BRCA1* it was shown that transfer of an intact human chromosome 17 led a breast cancer cell line (MDA-MB-231) to loose its capacity to induce tumors in nude mice. This result obtained from one clone (MDA-231/H17) which bears the long arm of chromosome17, indicated that at least one gene mapping to this region could be a tumor suppressor, confirming the *BRCA1* identification and LOH studies (Negrini, *et al*, 1994). Another study by Rao and co-workers also provided evidence for the tumor suppressive effect of *BRCA1*. In this report, it is stated that antisense RNA to BRCA1 transforms mouse fibroblasts (Rao, *et al*, 1996).

1.5.1.2. The Function of BRCA1

After the identification of *BRCA1* and definition of a zinc finger motif in its amino terminus in 1994, it is thought that the gene product is a tumor suppressor that may be a transcription factor (Miki, *et al*, 1994). Until 1996 there was no definite evidence about

the function of BRCA1 protein. That year two successive reports stated that the carboxy-terminal of the protein (nt 1528-1863) bears a transcriptional activation function (Chapman and Verma, 1996; Monteiro, *et al*, 1996).

A very exciting finding appeared in 1996 by Jensen *et al*, stating that BRCA1 is actually a protein that is secreted, and exhibited properties of a granin. This result was relied on homology search and immunological studies. This was a very important finding because BRCA1 would be the first secreted tumor suppressor protein (Jensen, *et al*, 1996). But four months later a report by Wilson *et al* discussed the specificity of the antibody used in this paper, and found out that the detected protein was actually EGFR but not BRCA1 (Wilson *et al*, 1996).

Gudas and co-workers found that *BRCA1* mRNA and protein levels are regulated by the steroid hormones and progesterone (Gudas, *et al*, 1995). Later, another group has suggested that *BRCA1* expression is not directly responsive to estrogen but it is induced as a result of the mitogenic activity of estrogen in estrogen receptor positive cells (Marks, *et al*, 1997).

Experimental inhibition of BRCA1 expression with antisense oligonucleotides produced accelerated growth of normal and malignant mammary cells, but had no effect on non-mammary epithelial cells (Thompson, *et al*, 1995). It was also shown that retroviral transfer of the wild-type *BRCA1* gene inhibits in vitro growth of all breast and ovarian cancer cell lines tested (Holt, *et al*, 1996). An evidence on how *BRCA1* mediate growth regulation and tumor suppression came from the report stating that *BRCA1*

transactivated expression of cyclin-dependent kinase inhibitor *p21* in a *p53*-independent manner, and that *BRCA1* inhibits cell-cycle progression into the S-phase (Somasundaram *et al*, 1997). There is also a report on the induction of apoptosis by *BRCA1* (Shao *et al*, 1996).

Little is known about the role of *BRCA1* in human development. But there are several studies on the role of *BRCA1* in mice development. These results suggest that *BRCA1* is crucial in the embriyonic development and that *BRCA1* homozygous mutants were not able to survive (Liu *et al*, 1996; Lane *et al*, 1995, Gowen *et al*, 1996). It is also found that *BRCA1* is expressed in rapidly proliferating cell types undergoing differentiation. In the mammary gland, *BRCA1* expression is induced during puberty, pregnancy, and following treatment of ovariectomized animals with estrogen and progesterone (Marquis *et al*, 1995). The role of *BRCA1* in human development remains unclear. There is one report on a human *BRCA1* gene knockout. The reported case was a women who had breast cancer diagnosed at age 32 (Boyd *et al*, 1995). This data implies that the role of *BRCA1* in human development is not as crucial as in mice development or *BRCA1* mutations' penetrance are rather wide.

It was in 1994 just before the publication of *BRCA1* identification in a review Vogelstein and Kinzler stated some predictions on the possible functions of BRCA1 protein. There they suggested that *BRCA1* gene product was part of a repair or replication complex that only indirectly affects the growth of breast cancer cells in a similar way to the mismatch repair genes (Kinzler and Vogelstein, 1994). Their predictions were actually confirmed by several different studies. First report has come from Scully and his coworkers noting

that BRCA1 is associated with Rad51, a protein that functions in DNA double stranded break repair, in mitotic and meiotic cells (Scully *et al*, 1997). In a following report, Scully and his coworkers stated that BRCA1 is a component of the RNA polymerase II holoenzyme, linking BRCA1 with the transcription process (Scully *et al*, 1997). The finding of BRCT module (BRCA1 C-terminus) has emphasized the potential importance of the BRCA1 C-terminal region for BRCA1-mediated breast cancer suppression, as this domain showed similarities with the C-terminal regions of a p53-binding protein (53BP1), the yeast RAD9 protein involved in DNA repair.

Another evidence confirming and supporting the function of BRCA1 in DNA repair and transcription process came from Gowen and co-workers stating that mouse embryonic stem cells deficient in BRCA1 are defective in the ability to carry out transcription-coupled repair of oxidative DNA damage, and are hypersensitive to ionizing radiation and hydrogen peroxide (Gowen *et al*, 1997). A more precise evidence that links BRCA1 to transcription process noted that BRCA1 protein is linked to the RNA polymerase II holoenzyme complex via RNA helicase A (Anderson *et al*, 1998). The most recent evidence that supports the role of BRCA1 protein in DNA repair shows that BRCA1 interacts in vitro and in vivo with hRad50, which forms a complex with hMre11 and p95/nibrin. Upon irradiation, BRCA1 was detected in discrete foci in the nucleus, which colocalize with hRad50. Formation of irradiation-induced foci positive for BRCA1, hRad50, hMre11, or p95 dramatically reduced in HCC/1937 breast cancer cells carrying a homozygous mutation in *BRCA1* but was restored by transfection of wild-type BRCA1 (Zhong *et al*, 1999).

The important function of *BRCA1* defined in DNA repair puts it in a crucial role as a caretaker gene. This finding lead to the studies that search its relation with the genome guardian, *p53*. It was shown that BRCA1 physically associates with p53 and stimulates its transcriptional activity. BRCA1 and p53 cooperatively induce apoptosis of cancer cells indicating that they may coordinately regulate gene expression in their role as tumor suppressors (Zhang *et al*, 1998; Ouchi *et al*, 1998). It was also found that breast tumors with mutant alleles of *BRCA1* show high frequency of *p53* mutations (Glebov, 1994). This finding is attributed to its role in DNA repair implying a mutator phenotype to *BRCA1* associated breast tumors. Although this finding is supported by a study demonstrating that disruption of *BRCA1* causes genetic instability and triggers further alterations, including the inactivation of p53, that lead to tumour formation (Xu *et al*, 1999). Another study shows that the correlation between *BRCA1* and *p53* is not because of the attributed role to *BRCA1* as a mutator, but to multiple mechanisms (Crook *et al*, 1998). Another finding suggested that *BRCA1* and *BRCA2* respond to DNA damaging agents, regulated by a p53-sensitive component (Andres *et al*, 1998).

It is found that in sporadic breast cancer cells 5' aberrant methylation of *BRCA1* CpG island leads to decrease in *BRCA1* mRNA suggesting a mechanism of *BRCA1* repression in sporadic breast cancer (Rice *et al*, 1998).

1.5.1.3. Mutations in *BRCA1*

Demonstration of germ-line mutations that disrupt the function of BRCA1 protein was crucial to establish primary involvement of this gene in hereditary breast cancer. The

first publication identifying *BRCA1* on 17q21 spanning to 100 kb region on the genomic DNA, encoding a protein of 1863 amino acids, reported also the first mutations that were actually disrupting the function of *BRCA1*, thus segregating with hereditary breast cancer (Miki *et al*, 1994). This initial report was followed by many studies on *BRCA1* mutation status of several populations and patient groups. To date 697 distinct mutations, polymorphisms, and variants have been reported 2887 times.

The mutation data collected in Breast Information Core shows several different pictures. Frameshift and nonsense mutations that result in truncated gene product covers 53% of all the identified mutations in *BRCA1* (Figure 4.). The second and third important points are related to the status of exon 11. Mutations found in exon 11 constitutes 53% of the total number of recorded mutations in *BRCA1*. Besides 68% of the mutations found in exon 11 result in premature stop codon formation. The number of frameshift and nonsense mutations found in exon 11 are nearly twice that of the remaining exons (Figure 5.)

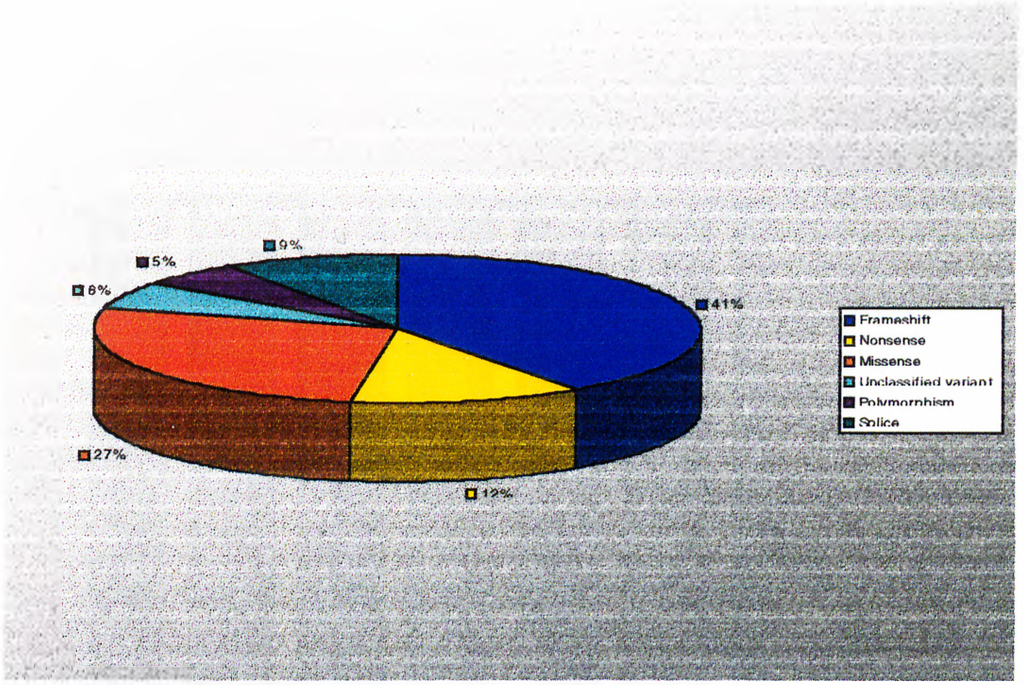


Figure 4. Mutation type ratio in *BRCA1* (adapted from Bic)

Most of the mutations are reported more than once. If the data is reanalyzed based on the total number of entries of a mutation, exon 11 re-emerges as the leading region of mutations in *BRCA1*.

Besides these frameshift, nonsense, missense mutations, unclassified variants and polymorphisms caused by small changes on DNA sequence, several reports of rearrangements mediated by Alu repeats, causing big deletions and duplications have appeared (Swensen *et al*, 1997; Puget *et al*, 1997; Puget *et al*, 1999a; Puget *et al*, 1999b, Mazoyer and BRCA1 exon 13 dup. Scr. Gr., 2000).

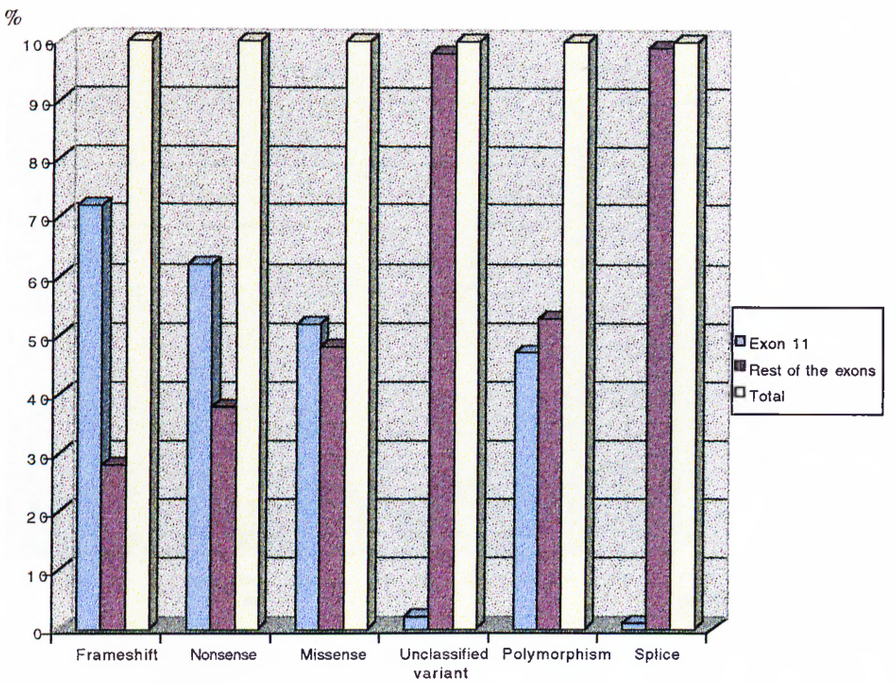


Figure 5. Frequency of mutation types in *BRCA1* exon 11 (adapted from Bic)

Population studies on *BRCA1* mutation status reflects an interesting view on population dynamics. The studies on families with three or more cases of female breast and/or ovarian cancer shows a picture with wide variations. In this group of patients which is constituted from the most susceptible individuals to breast cancer, the mutation frequency of *BRCA1* differs from 79% in Russia (Gayther *et al*, 1997) to 9% in Iceland (Thorlacius *et al*, 1996). The mutation frequencies obtained from different populations (Britain, Canada, France, Finland , France, Germany, Holland and Belgium, Hungary, Iceland, Israel, Italy, Japan, Norway, Russia, Sweden and Denmark, United States) are summarized in figure 7.

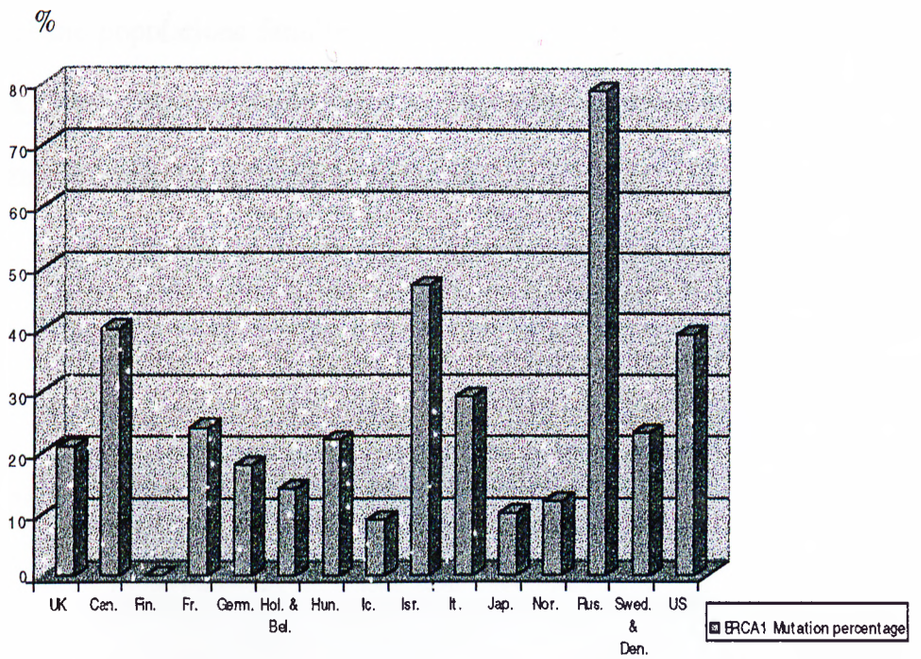


Figure 6. *BRCA1* mutation frequency in different populations in families with three or more cases of female breast and/or ovarian cancer (Adapted from Szabo and King,1997)

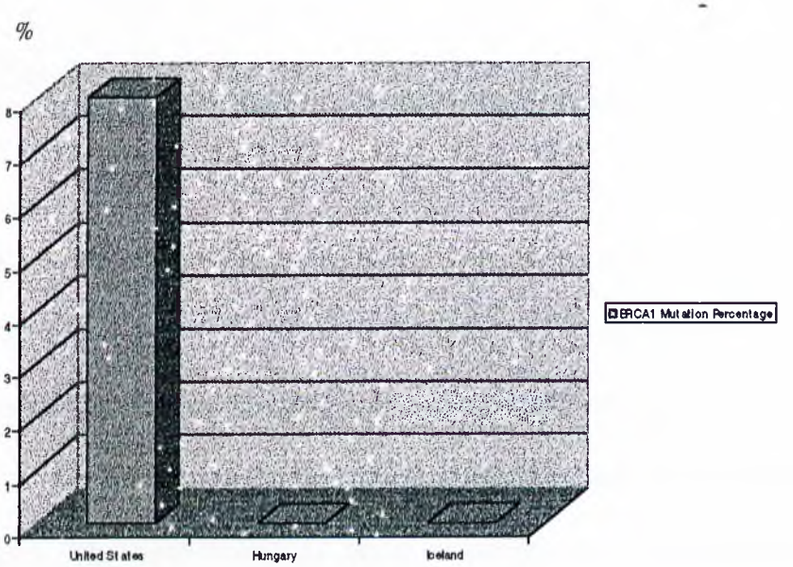


Figure 7. *BRCA1* mutation frequency in US, Hungarian and Icelandic families with male and female breast cancer.

(Adapted from Szabo and King, 1997)

In some of the populations families with male and female breast cancer and patients without a family history were studied. The data obtained from these studies are summarized in figure 7 and figure 8 respectively.

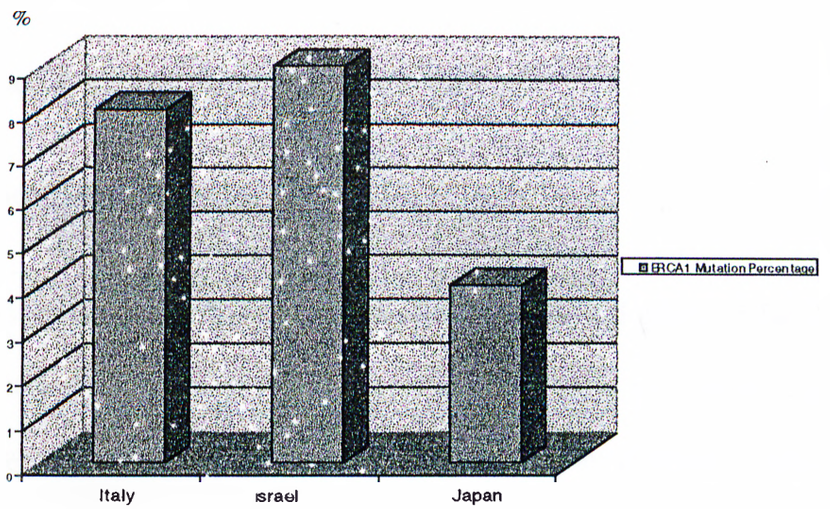


Figure 8. *BRCA1* mutation frequency in Italian, Israeli and Japanese population in breast and/or ovarian cancer patients not selected for family history.
(Adapted from Szabo and King, 1997)

These population data clearly show that *BRCA1* mutations play an important role in hereditary breast and/or ovarian cancer cases, although the frequency of mutations vary widely in different populations. However in families with male breast cancer exist, and in breast and/or ovarian cancer cases without a family history the mutation ratio of *BRCA1* decreases. Mutations in *BRCA1* occur in approximately 5% of women in whom ovarian cancer is diagnosed before the age of 70 years (Stratton *et al*, 1997)

Approximately 85% of breast cancer cases are sporadic and the remaining cases are hereditary. From the above mentioned population data it is possible to conclude that approximately 30% of hereditary breast cancer cases are linked to *BRCA1*. Thus there should be other loci that must be responsible for the remaining 70% of hereditary breast cancer cases. Four years after the localization of *BRCA1* to 17q21 the second breast cancer susceptibility gene appeared on the scene.

1.5.2. Breast Cancer susceptibility gene 2 (*BRCA2*)

1.5.2.1. Identification of *BRCA2*

In a collaborative study genomic linkage search was performed using 15 families that had multiple cases of early-onset breast cancer and that were not linked to *BRCA1*. These families were classified according to the number of cases of female breast cancer, male breast cancer, and ovarian cancer. This analysis localized the second breast cancer susceptibility locus, *BRCA2*, to a 6 centimorgan interval on chromosome 13q12-13 (Wooster *et al*, 1994). Just a few months later it was shown that the wild type allele of *BRCA2* was lost in breast cancers from families linked to chromosome 13q12-13, confirming the role of that locus in hereditary breast cancer (Collins *et al*, 1995).

A year later, *BRCA2* gene was identified with a cDNA of 10400 bp and 27 exons (Figure 9). In that study six germline mutations, each causing serious a disruption to the open reading frame of the transcriptional unit, in breast cancer families were reported indicating that this was the *BRCA2* gene (Wooster *et al*, 1995).

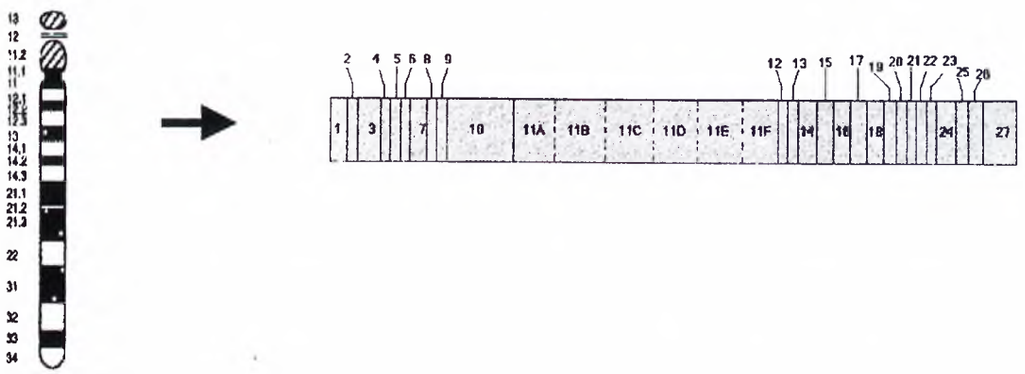


Figure 9. Chromosomal location* and exonic organization of *BRCA2*.**
*adapted from NCBI database.
**adapted from Bic database.

Ovarian cancer occurrence in the family along with breast cancer is an important point that links the breast cancer susceptibility to *BRCA1* (Miki *et al.* 1994). For *BRCA2,* the presence of male breast cancer in the family is one of the most important determining characteristic of hereditary breast cancer linkage to *BRCA2 (*Thorlacius *et al,* 1995). Breast cancer is a rare disease in men, affecting less than 0.1% of the male population (Haraldsson *et al,* 1998). The expression of hormone receptors in male breast tumors suggested a central role for endogenous hormones in male breast carcinoma. High degree of androgen receptor was detected. The high frequency of bcl-2 positivity may implicate antiapoptotic mechanisms in the carcinogenesis of male breast cancer. Besides, positive cyclin D1 expression is associated with increased PFS (progression

free survival) in male breast cancer cases, which suggested that interactions among cell cycle regulatory proteins might be important in this disease (Rayson *et al*, 1998).

In a recent study genomic alterations of sporadic and *BRCA2* positive male breast cancer tumors were compared to those of female tumors. The results suggested that despite substantial hormonal differences between females and males, similar genetic changes were occuring during tumor progression. Besides, the presence of a highly penetrant germline *BRCA2* mutation apparently leads to a characteristic somatic tumor progression pathway, again shared between affected male and female mutation carriers (Tirkkonen *et al*, 1999). Analysis of the expression of *BRCA1* and *BRCA2* genes in ovariectomized mice progesterone, shows the up-regulation of mRNA expression in the breast by ovarian hormones significantly greater for *BRCA1* than for *BRCA2*. This result suggest that the phenotype difference associated with germ-line mutations in these genes is related to the differential regulation of these genes by sex hormones (Rajan *et al*, 1997).

BRCA2 mutations is also important in pancreatic cancer. It is reported that in 41 pancreatic cancer cases not selected on the basis of family history, 5% of cases bear a *BRCA2* mutation. In the same report this ratio was determined as 10% for Jewish pancreatic cancer cases. According to statistical formulas it is calculated that having a *BRCA2* mutation leads to an increase of 3.51 fold in pancreatic cancer, 2.59 fold in stomach cancer, 4.97 fold increase in gall bladder and bile duct cancers, in 2.58 fold increase in malignant melanoma, and in 4.65 fold increase in prostate cancer (Özçelik, *et al*, 1997).

1.5.2.2. The Function of *BRCA2*

The first report about the function of *BRCA2* was about its expression through cell cycle. It was found that like *BRCA1*, *BRCA2* mRNA levels was at a maximum level during late G_0 and S phases implicating its protective role in cellular proliferation (Vaughn, *et al*, 1996; Wang *et al*, 1997; Su *et al*, 1998).

Later it was shown that *BRCA2* exon 3 sequences (residues 18-105) had a transcription activation potential (Milner *et al*, 1997). It was also found that *BRCA2* had a histone acetyl transferase activity. This result supports the previous report since there were some findings implying a possible relationship between gene transcriptional activation and histone acetylation by several enzymes involved in acetylation and deacetylation of histone residues (Siddique *et al*, 1998).

In an other study by Sharan and co-workers it was found that like *BRCA1*, *BRCA2* plays a role in DNA repair. Their results indicated that *BRCA2* interacts with DNA-repair protein Rad51 in mouse. The *BRCA2* deficient embryos showed developmental arrest and they were radiation sensitive. These observations along with the finding of Brca2 interaction with Rad51 explains tumor-suppressor function of *BRCA2* (Sharan *et al*, 1997). A report that supported this finding stated that mice bearing *BRCA2* mutations in homozygous state may survive but they have a wide range of defects, including small size, improper differentiation of tissues, absence of germ cells, and the development of lethal thymic lymphomas. They also showed that fibroblasts cultured from these embryos have a proliferation defect which can be explained by the over-expression of

p53 and p21 (Connor *et al*, 1997). These results which show the loss of p53 checkpoint caused by the defected through its role in DNA repair, clearly illustrate the gatekeeper and caretaker model explaining tumorigenesis.

1.5.2.3. Mutations in *BRCA2*

BRCA2 as *BRCA1* is a gigantic gene and the mutations are scattered along the gene. To date a total of 642 mutations have been reported for 1702 times. Approximately 50% of the mutations are frameshift and nonsense mutations resulting in a truncated product (Figure 10). Exon 11 constitutes approximately 60% of the gene and nearly 50% of the mutations in *BRCA2* resides in this exon (Breast Information Core). The mutation ratio of exon 11, according to mutation types compared to other exons is shown in figure 11.

BRCA2 mutation status was studied in different patient groups and different populations. These data revealed that *BRCA2* mutations are responsible for approximately 30% of all hereditary breast cancer cases. The results show variations among different populations. These variations are clearly seen in figure 12.

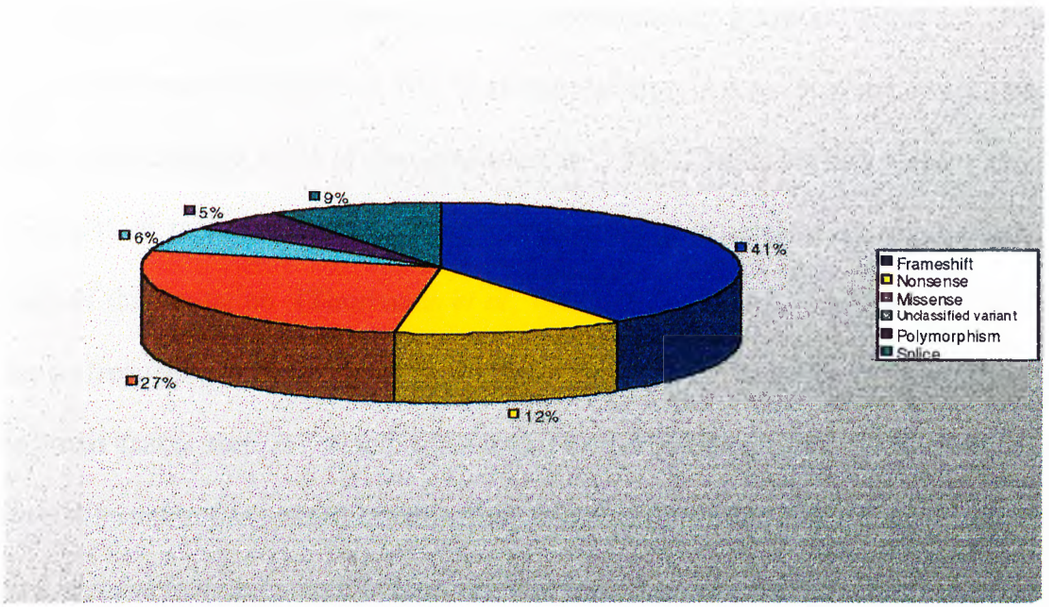


Figure 10. Mutation type ratio of *BRCA2*
(Adapted from Bic)

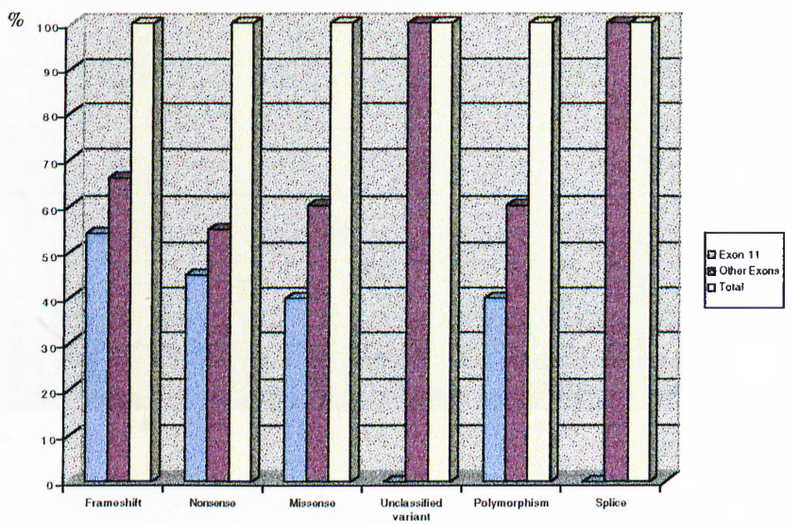


Figure 11. Frequency of mutations according to type in BRCA2 exon 11.
(Adapted from Bic)

BRCA2 mutation status variation reflects an interesting picture presenting Iceland with a more than 60% mutation frequency. A founder mutation plays an important role in breast cancer cases in Iceland. A five base-pair deletion in exon 9 of the *BRCA2* gene, 999del5, is detected in 0.6% of the population, in 7.7% of female breast cancer patients, and in 40% of males with breast cancer. Prostate and pancreatic cancer are seen in carriers of this mutation (Thorlacius *et al*, 1996; 1997). However in Finland, *BRCA2* mutation frequency was found to be only 8%. This was explained by low frequency of male breast cancer seen in Finland. Male breast cancer represents only 0.5% of all breast cancers detected in this country. In this study it is also found that *BRCA2* mutations were identified more frequently both in families with four or more cases of breast cancer an in families with both breast and ovarian cancer (See Figure 12) (Vehmanen, *et al*, 1997).

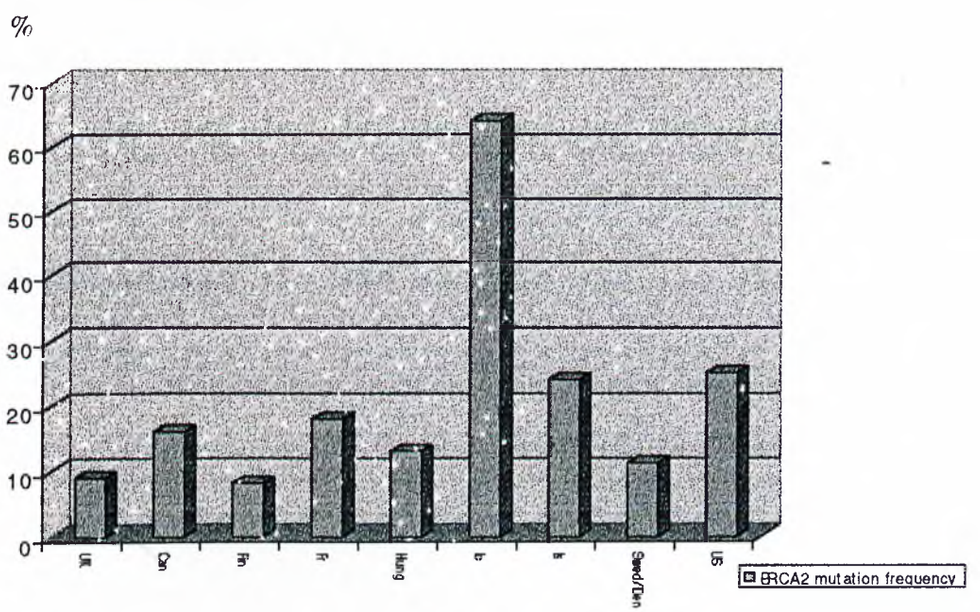


Figure 12. BRCA2 mutation frequency in English, Canadian, Finnish, French, Hungarian, Icelandic, Swedish, Danish, and US populations in families with three or more cases of female breast and/or ovarian cancer.
(Summarized from Szabo and King, 1997)

BRCA2 mutations were found to be important in male breast cancer cases. In a study performed in Sweden, it was found that 21% of male breast cancer cases without a family history bear a *BRCA2* mutation (Haroldsson, *et al.* 1998). Different results were obtained from two independent studies carried out in US. In the study of Friedman *et al*, it was found that only 4% of male breast cancer cases were detected with a *BRCA2* mutation. Besides, only the cases carrying *BRCA2* mutation had a family history of cancer. Furthermore, 89% of male breast cancers with a family history of breast/ovarian cancer in first-degree relatives remain unaccounted for by mutations in either the *BRCA1* or the *BRCA2* gene (Friedman, *et al*, 1997). Whereas in the other study, it was found that 14% of male breast cancer cases bear a *BRCA2* mutation. Besides, this ratio increase to 85% in cases with a family history of breast cancer, either male or female (Couch, *et al*, 1996). However, *BRCA1* mutations were also found in male breast cancer cases (Struewing, *et al*,1995). The mutation status of *BRCA2* in families with male breast cancer is shown in figure 13. The mutation status of BRCA2 in breast and/or ovarian cancer patients not selected for family history in Icelandic, Israeli and Japanese populations is summarized in figure 14.

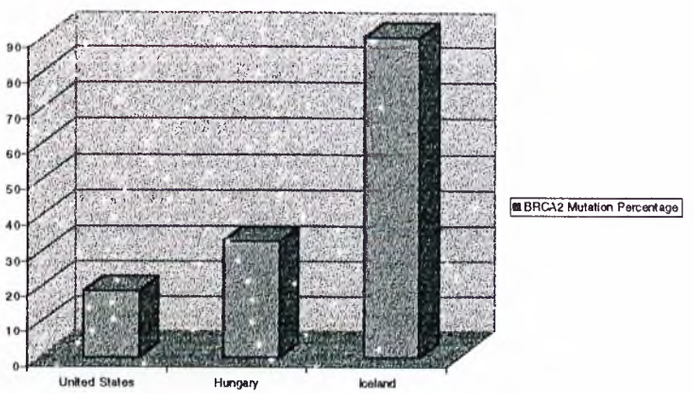


Figure 13. *BRCA2* mutation frequency in US, Hungarian and Icelandic families with male and female breast cancer cases.
(Summarized from Szabo and King, 1997)

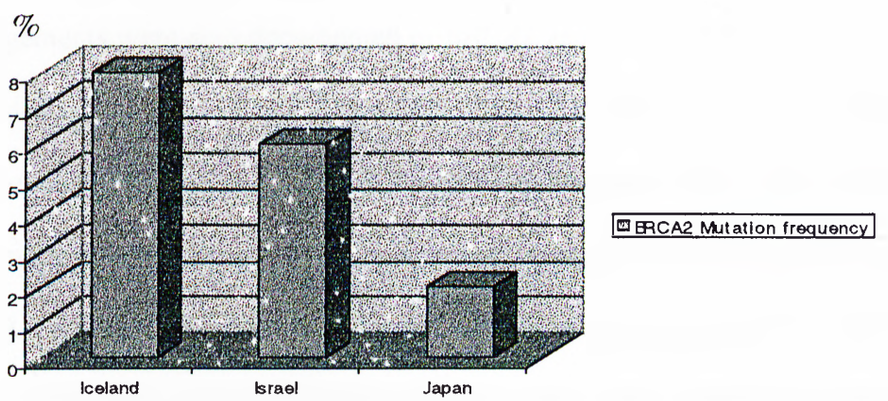


Figure 14. *BRCA2* mutation frequency in breast and/or ovarian cancer patients not selected for family history from Icelandic, Israeli and Japanese populations
(Summarized from Szabo and King, 1997)

BRCA2 mutation status was also studied in sporadic breast and ovarian cancer cases. In a study by Lancaster *et al*, it was found that only in 2.8% of breast and ovarian cancer cases a *BRCA2* mutation is detected (Lancaster, *et al*, 1996). This study and the data summarized in figure 15 show that *BRCA2* is an infrequent target for somatic inactivation in breast and ovarian carcinomas, similar to the results obtained for *BRCA1*.

Conflicting results were obtained from two studies dealing with the role of *BRCA2* mutations in pancreatic cancer in view of frequent losses of heterozygosity on chromosome 13q12-13. In the study by Teng *et al BRCA2* mutation was found in pancreatic cancer (Teng *et al*, 1996) whereas in another study by Katagiri and his coworkers, it was found that *BRCA2* mutations do not play a role in pancreatic cancer but do play a role in liver carcinogenesis (Katagiri *et al*, 1996).

Big rearrangements were also documented in *BRCA2* as in *BRCA1*. A deletion of 5068 base-pairs disrupting exon 3 which encodes the transcription activation domain of *BRCA2* gene was identified in a Swedish breast /ovarian cancer family (Nordling *et al*, 1998). A similar deletion disrupting amino-terminal transactivation domain was found in 50% of RER+ (replication errors) endometrial tumors, indicating that somatic mutations in *BRCA2* may confer a growth advantage in RER+ endometrial carcinomas (Koul *et al*, 1999).

In a study by Mazoyer and her coworkers a substitution at nucleotide 3326 causing to a stop codon was identified. This stop codon resulting in the loss of the final 93 amino acids is found to be polymorphic (Mazoyer *et al*, 1996). This interesting finding shows how variable a mutation's penetrance can be.

A similar finding implying a mutator phenotype in *BRCA2* associated tumors shows high frequency of *p53* mutations (Gretarsdottir, *et al*, 1998). This finding, although not confirmed, indicates presence of a mechanism other than mutator phenotype attributed to *BRCA2* associated tumors by Crook and coworkers (Crook *et al*, 1998).

1.5.3. Penetrance and Histoprognosis of *BRCA1* and *BRCA2* genes

Accumulation of somatic genetic changes during tumor progression may follow a unique pathway in individuals genetically predisposed to cancer, especially by the *BRCA1* gene. Activation or loss of genes in the affected chromosomal regions may be selected for during tumor progression in cells lacking functional *BRCA1* or *BRCA2* (Tirkkonen *et al*, 1997).

It was found that truncating mutations clustered in a region of approximately 3.3 kb in exon 11 are correlated with the highest risk of ovarian cancer relative to breast cancer (Gayther *et al*, 1997)

The estimated cumulative risk of breast cancer reached 28% by age 50 years and 84%

by age 70 years in women. The corresponding ovarian cancer risks were 0.4% by age 50 years and 27% by age 70 years. The lifetime risk of breast cancer appears similar to the risk in *BRCA1* carriers, but there was some suggestion of a lower risk in BRCA2 carriers <50 years of age (Ford *et al*, 1998).

When compared with non-hereditary breast cancers, *BRCA1* associated breast cancer presents a higher percentage of advers pathobiological features such as grade 3, aneuploidy, aneuploid tumor with S phase, estrogen and progesterone receptor negativity (Watson *et al*, 1998). The histology of breast cancers in predisposed women differs from that in sporadic cases, and there are differences between breast cancers in carriers of *BRCA1* and *BRCA2* mutations. This results suggest that breast cancer due to *BRCA1* has a different natural history to *BRCA2* or apparently sporadic disease, which may have implications for screening and management (Breast Ca. Link. Con., 1997).

Several studies showed that the proportion of families not linked to *BRCA1* or to *BRCA2* may be higher than previously expected suggesting the existence of at least one more major breast cancer-susceptibility gene (Sobol *et al*,1994; Serova *et al*, 1997). The attribution of approximately 60% of hereditary breast cancer cases to *BRCA1* and *BRCA2* gene lead to the studies currently in progress to identify the third locus.

1.5.4. Breast Cancer Susceptibility Genes and Risk Assesment

The observation of familial cancer syndromes opened up the possibility to make risk assesment for individuals from these families. Approximately 10% of ovarian cancer

cases and 7% of breast cancer cases in the general population are estimated to be hereditary (Claus *et al*, 1996). The identification of *BRCA1* and *BRCA2* genes, which together are thought to be responsible for nearly 60% of hereditary breast cancer cases (Miki *et al* 1994; Wooster *et al*, 1995), makes possible to define a mutation in one of these two genes segregating with the disease, in a breast cancer family, and to give genetic counseling for an unaffected individual. Although the possibility of making a prediction on cancer susceptibility, especially about a cancer which is the leading cause of deaths from cancer between ages 15 and 54 in women, is very exciting, the issue must be considered with care and caution.

Different mutations will confer different risks for breast cancer, ovarian cancer, and other cancers. Different mutations confer different likelihoods of tumor types and age of onset. Even the same mutations in the *BRCA1* and *BRCA2* genes confer different risks on different patient populations. It must be concluded that there are substantial modifying factors such as genetic, dietary, hormonal which affect the likelihood of a given *BRCA1* or *BRCA2* mutation causing cancer. Penetrance of a mutation is a very important issue in risk evaluation. Because of just a few mutations Ashkenazi Jewish and Icelandic populations are ideal models for such studies.

Three founder mutations have been described in Ashkenazi Jewish breast cancer families: 185delAG and 5382insC in *BRCA1,* and 6174delT in *BRCA2* (Simard *et al*, 1994; Friedman *et al*, 1995; Neuhausen *et al*, 1996). One of these mutations was found in 31% of Ashkenazi women with early-onset (<42 years of age) breast cancer, and

41% of Ashkenazi women with a positive family history who were diagnosed with breast cancer at 42-50 years of age (Neuhaussen *et al.* 1996; Oddoux *et al*, 1996; Offit *et al*, 1996). In a series of 220 North American Ashkenazi breast cancer families, one of the founder mutations accounted for 45% of all families, and for a significantly higher percentage (73%) of families with a history of ovarian cancer (Tonin *et al*, 1996). The combined population frequency of these mutations in a number of large series of young Ashkenazi controls approaches 2.5% in total: *BRCA1* 185delAG, 1%; *BRCA1* 5382insC, 0.1%; and *BRCA2* 6174delT, 1.4% (Streiwithg *et al*, 1995; Oddoux *et al*, 1996; Roa at al, 1996). Current estimates of penetrance of these mutations are based on the high-risk pedigrees used in the original linkage studies, where lifetime penetrance of *BRCA1* mutations was found to be >80% for breast cancer, and approached to 50% for ovarian cancer (Easton *et al*, 1993; Ford *et al*, 1994; Friedman *et al*, 1995). Penetrance of *BRCA2* mutations was estimated to be similar for breast cancer, and approached to 50% for ovarian cancer (Wooster *et al*, 1994). Families used in these studies were selected for the presence of at least four affected living relatives (for high penetrance at an early age), so it is not appropriate to apply these penetrance estimates to families with fewer affected individuals or to asymptomatic carriers without family history (Levy-Lahad *et al*, 1997).

Actually, the *BRCA2* 6174delT mutation has been inferred to have lower penetrance than the *BRCA1* 185delAG mutation, because, although their frequencies in the Ashkenazi population are similar, *BRCA2* 6174delT is significantly less common in high-risk Ashkenazi breast-ovarian cancer families. Comparisons of these frequencies

have led to the conclusion that *BRCA2* 6174delT penetrance is 25-30% that of *BRCA1* 185delAG (Oddoux *et al*, 1996; Roa *et al*, 1996). Thus, genetic testing is technically more feasible in the setting of a limited number of mutations in a defined ethnic group. However, before such testing is implemented, either in affected individuals or in the population, it is important to define the penetrance and attributable risk of these mutations for breast and ovarian cancer in the population studied (Levy-Lahad *et al*, 1997).

In a similar study performed in Iceland 10.4% of women and 13% of men diagnosed with breast cancer unselected for family history were found to carry 999del5 mutation. This mutation is present in around 0.6% of the Icelandic population. The estimated risk of breast cancer at age 50 for female carriers of this mutation was found to be 17% and 37.2% at age 70 (Thorlacius *et al*, 1998).

Overall, at present, it is thought that a woman carrying a gene mutation in *BRCA1* has a 56% risk of developing breast cancer by age 70, and a 16.5% risk of developing ovarian cancer. Higher estimates of up to 80% lifetime risk have been reported in some series. Reasons for these differences include the severity of penetrance in the distinct high-risk families that have been studied, and the technical limits of genetic analysis that may inaccurately represent the extent of BRCA mutation (Weber, 1998-online coverage). In a study by Couch and coworkers it was found that among women with breast cancer and a family history of the disease, the percentage of *BRCA1* coding-region mutations is less than 45% predicted by genetic-linkage analysis. *BRCA1* mutations were identified in

16% of women with a family history of breast cancer. Only 7% of women from families with a history of breast cancer but not ovarian cancer had *BRCA1* mutations. The rates were higher among women from families with a history of both breast and ovarian cancer (Couch *et al*, 1997). Analyzing all of these results shows that genetic counseling for hereditary breast cancer is an issue that should be evaluated carefully.

1.5.5. Homozygous germ-line *BRCA1* mutations

Heterozygous germ-line mutations in tumor suppressor genes are associated with hereditary cancer such as *p53* in Li Fraumeni syndrome, *BRCA1* and *BRCA2* in hereditary breast cancer, *RB* in retinoblastoma and *MLH1* in HNPCC. Since homozygous germ-line tumor suppressor mutations are rare its phenotypic consequences have not been studied thoroughly.

There is one report on a human *BRCA1* gene knockout. The reported case was a women who had breast cancer diagnosed at age 32 and her mother and four of her maternal aunts had breast and/or ovarian cancer. She also has a first cousin on her paternal side of the family who was diagnosed with ovarian cancer at age 22. Sequencing analysis revealed that she had a deletion of two A nucleotides at position 2800, resulting in an in-frame stop codon at nucleotide 2820. This homozygous allele encodes a 900 amino acid protein (Boyd *et al*, 1995). This data implies that the role of *BRCA1* in human development is not as crucial as in mice development or *BRCA1* mutations' penetrance are rather wide. Whereas homozygous germ-line BRCA1 mutations do not change the

disease phenotype, homozygous inheritence of another hreditary cancer syndrome gene, *MLH1*, results in a different disease spectrum.Two recent reports identified human *MLH1* deficiency in two unrelated families (Ricciardone *et al*, 1999; Wang *et al*, 1999). These *MLH1* deficient cases developed hematological malignancy at a very early age and displayed signs of neurofibromatosis type 1. These studies implies that a homozygous *MLH1* mutation and consequent mismatch repair deficiency results in a mutator phenotype characterized by leukemia or lymphoma associated with neurofibromatosis type 1.

1.6. Mutation Screening

BRCA1 and *BRCA2*, responsible for breast cancer susceptibility, are gigantic genes spanning over 80 and 100 kb genomic DNA regions. They have 5500 bp and 10400 bp cDNAs respectively, and have many mutations scattered on the entire gene. This makes the mutation screening of these genes a very tedious task. To detect the mutations in a most effective and cost efficient way, an appropriate screening methodology should be chosen. Since direct sequencing is not a suitable methodology for screening, a sensitive and cost effective indirect mutation detection technique is needed. An ideal mutation screening method should :

2. screen a region up to 1 kb.
3. be 100% sensitive.
4. not give false positives and false negatives.
5. not have more than one step.

6. not need expensive equipments.
7. should not contain the usage of biohazardous reagents.
8. should not need electrophoresis.
9. should be performed in short time.
10. should be cheap.

To date, no mutation screening technique bearing all of these characterictics has been defined Many research laboratories have developed and adopted different methodologies for the screening of breast cancer susceptibility genes. The commonly used techniques used in the screening of *BRCA1* and *BRCA2* are protein truncation test (PTT), denaturing gradient gel electrophoresis (DGGE), denaturing high performance liquid chromatography (DHPLC), single strand conformation polymorphism (SSCP), heteroduplex analysis (HA), and direct sequencing (DS).

1.6.1. Protein Truncation Test

The protein truncation test (PTT) is a mutation detection method that specifically detects mutations, which lead to the termination of translation and subsequent protein truncation. Since nonsense and frameshift mutations cause premature protein products and are very frequently seen in *BRCA1* and *BRCA2* genes, PTT is one of the most popular screening techniques (Maugard *et al*,1998).

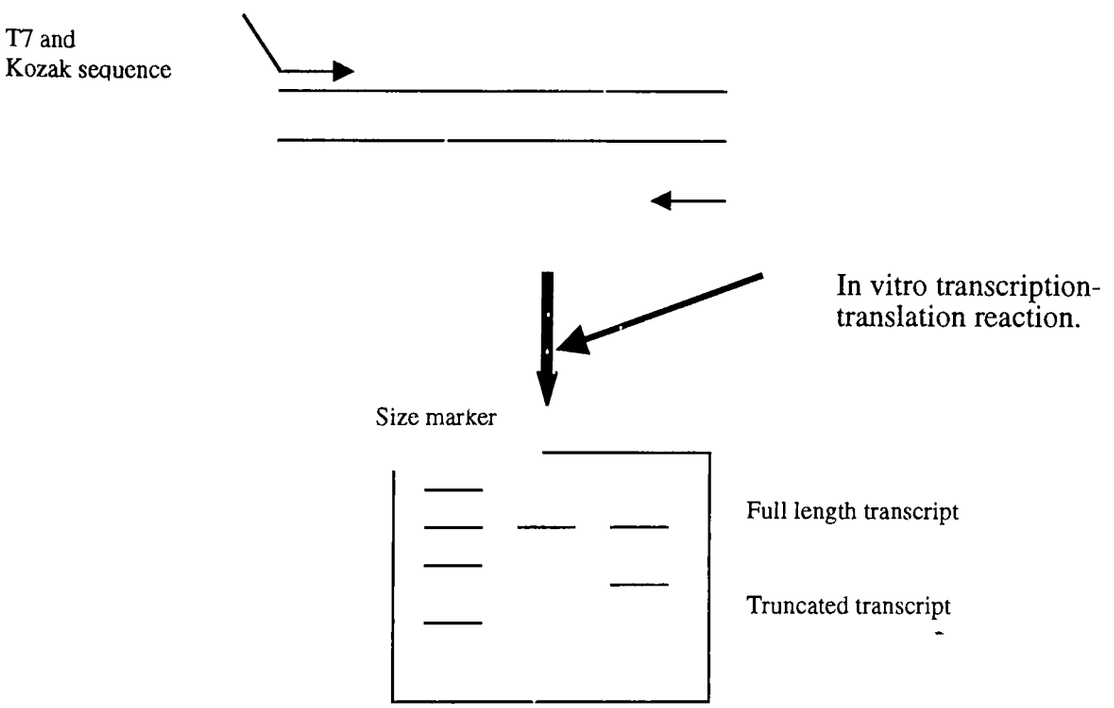


Figure 16. Schematic representation of protein truncation test.

In this method cDNA or large genomic exons are amplified via PCR using a forward primer carrying at its 5' end a T7 promoter, followed by a eukaryotic translation initiation sequence (Kozak sequence) which includes an ATG start codon. This way a synthetic transcription unit is obtained. The amplification product is then added to an in vitro transcription-translation system, which includes all the necessary components for the transcription and translation processes. For the detection of the end product, a labelled (radioactively, S^{35} or colorimetric, Biotin) amino acid (methionine) is added to the reaction.

The end product is then loaded to a SDS-PAGE along with a protein molecular weight marker. If the size of the product is lower than expected, the amplified area is sequenced to identify the mutation.

PTT ignores missense mutations; thus polymorphisms detected by other methods (SSCP, HA) do not need to be excluded as disease causing alterations by sequencing. PTT allows the analysis of large stretches of coding sequences (at least 3 kb, for example entire exon 11 of BRCA1. In addition to these advantages, one should consider that PTT does not locate a mutation precisely. It is restricted to truncating mutations. It is important to note that PTT is only suitable for large exons, unless RNA extraction and cDNA preparations are done (http://www.ich.ucl.ac. uk./cmgs/ptt98.htm).

1.6.2. Denaturing Gradient Gel Electrophoresis (DGGE)

The two strands of a DNA molecule separate, or melt, when heat or a chemical denaturant is applied. The temperature at which a DNA duplex melts is influenced by two factors:

1.The hydrogen bonds formed between complimentary base pairs, GC rich regions melt at higher temperatures than regions that are AT rich.

2.The attraction between neighbouring bases of the same strand or "stacking".

When double-stranded DNA is electrophoresed into a gradient of increasing denaturant a portion of a given strand separates but the strands are anchored together by the portion (higher melting domain) which has not melted at this point. This split in the duplex suddenly arrests the movement of the molecule in the gel. If a single-base change is present in a similar duplex in the split portion, the denaturant concentration for strand separation is usually different, thus the arrest of movement occurs at a different position in the gel, and a mutation can be detected by the differential positions of arrest (Cotton, 1993). Complete strand separation is prevented by the presence of a high melting domain, which is usually artificially created at one end of the molecule by incorporation of a GC clamp, accomplished during PCR amplification using a PCR primer with a 5' tail consisting of a sequence of 40 GC.

DGGE has a high mutation detection rate and sensitivity. It is a non radioactive method, and PCR fragments may be isolated from the gel to use in sequencing reactions. But genes which are exceptionally GC rich are not easily analyzed by DGGE. The use of special GC clamped primers is another limiting factor in this technique (http://www.ich.ucl.ac.uk/cmgs/mutdet.htm).

1.6.3. Denaturing High Performance Liquid Chromatography (DHPLC)

This method is a modified version of heteroduplex analysis. Denaturation of PCR product causes to the separation of two alleles. Renaturation by cooling the product leads to random annealing of the two alleles. If an individual is heterozygous for a

mutation, heteroduplexes will form between wild-type and mutant alleles along with homoduplexes. These homoduplexes and heteroduplexes migrate differently in a gel matrix. In DHPLC, these products are run in partially denaturing conditions to exaggerate the difference between two species. The detection is performed by an HPLC system linked to a computer. The mutation detection rate of this method is close to 100%, but it needs expensive equipment. Although single base mutations have been detected in 1.5 kb fragments, maximum sensitivity is achieved with fragments of 150-450 bp (http://www.ich.ucl.ac.uk/cmgs/mutdet.htm)

1.6.4. Single Strand Confirmation Polymorphism (SSCP)

SSCP is the simplest and most commonly used method of mutation detection. PCR is used to amplify the region of interest and the resultant DNA is separated as single stranded molecules by electrophoresis in a non-denaturing polyacrylamide gel. A strand of single-stranded DNA folds differently from another if it differs by a singly base, and it is believed that mutation-induced changes of tertiary structure of the DNA results in different mobilities for the two strands. These mutations are detected as the appearance of new bands on autoradiograms (radioactive detection), by silver staining of bands or by the use of fluorescent PCR primers which are subsequently detected with an automated DNA sequencer (non-radioactive detection).

The tertiary structure of single stranded DNA changes under different physical conditions such as temperature and ionic environment. Hence the sensitivity of SSCP

depends on these conditions. While some empirical rules have emerged for the choice of separation conditions for sequence variants in particular sequence contexts, it is not possible to predict whether a mutation can be detected under given conditions, especially when the mutation is in a new sequence context. Mutation detection for PCR-SSCP is generally over 80% in a single run for fragments shorter than 300 bp (http://www.ich.ucl.ac.uk/cmgs/mutdet.htm).

1.6.5. Heteroduplex Analysis (HA)

Heteroduplex is double-stranded DNA in which the two DNA strands do not show perfect base complementarity. When DNA is denatured, the two strands are separated. On renaturation or annealing, complimentary DNA strands reassociate and form a homoduplex. However, when there is a mutation in one of the strands then a heteroduplex is formed. The electrophoretic mobility of heteroduplex in non denaturing polyacrylamide gel is less than that of homoduplex, and they can be detected as extra slow moving bands. Resolution is better in specially formulated gel matrice (MDE). In fragments of under 200 bp insertions, deletion and most single base substitutions are detected. Several mutations in a gene can also be combined to form a multiplex heteroduplex analysis (http://www.ich.ucl.ac.uk/cmgs/mutdet.htm).

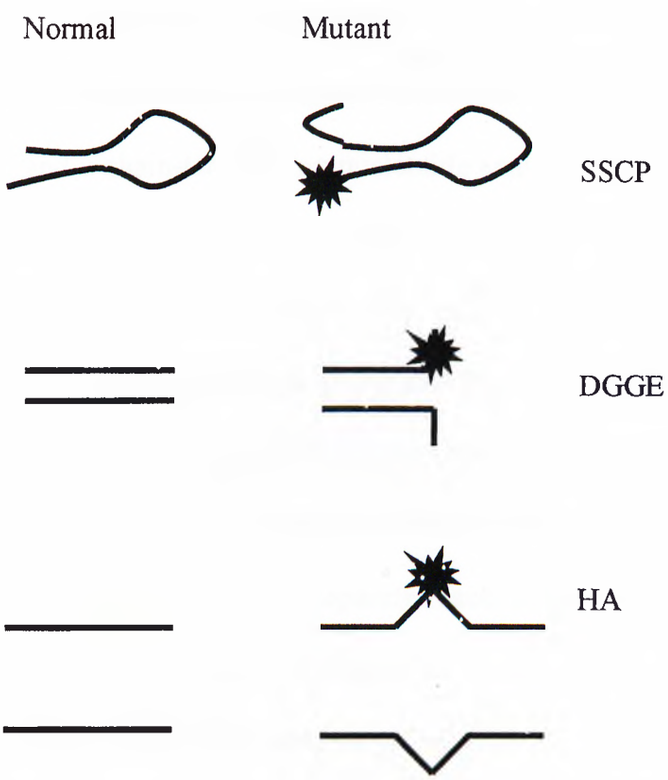


Figure 17. Comparison of SSCP, DGGE and HA in mutation detection.

1.6.6. Direct Sequencing

Dideoxy chain termination DNA sequencing method involves the in-vitro synthesis of a DNA strand by a DNA polymerase using a specifically primed single-stranded DNA template. DNA synthesis is carried out in two steps. The first is the labeling step in which the primer is extended using limiting concentrations of the deoxynucleoside

triphosphates, including radioactively labeled dATP. This step continues to complete incorporation of labeled nucleotide into DNA chains. These initial primer extensions are distributed randomly in length from several nucleotides to hundreds of nucleotides. In the second step, the concentration of all the deoxynucleoside triphosphates is increased and a chain-terminating nucleotide analog is added. These 2'3'-dideoxynucleoside-5'-triphosphates lack the 3'OH group necessary for DNA chain elongation. Processive DNA synthesis occurs, with extensions on the average of only several dozen nucleotides, until all growing chains are terminated by a ddNTP. When proper mixtures of dNTPs and one of the four ddNTPs are used, enzyme-catalyzed polymerization will be terminated in a fraction of the population of chains at each site where the ddNTP can be incorporated. Four separate reactions, each with a different ddNTP give complete sequence information (Sambrook *et al.* 1989). The reactions are then loaded on a denaturing polyacrylamide gel which is able to differentiate one base pair difference. The visualization is done by autoradiography which shows a ladder like picture containing the sequence information of the nucleotides.

In automated DNA sequencing, the principle of the reaction is also based on dideoxy chain termination reaction, but fluorescent detection is used instead of radioactive detection. Fluorescence gives the advantage of using different colors for each ddNTP thus the possibility of setting up one reaction instead of four in dye terminator chemistry. In the alternative dye primer chemistry, the fluorescent dye is on the primers used for sequencing. So in this way, four reactions must be set up. However, the advantage of

fluorescence in both cases is that the samples are loaded into one lane, since the labels of the fragments are different.

In automated DNA sequencing, in order to increase the amount of the product, the reaction is set up by cycle sequencing. In this PCR like process, repeated cycles of thermal denaturation, primer annealing, and polymerization to produce greater amounts of product is used .

The sequencing products are detected either on a polyacrylamide gel or capillary electrophoresis which has a laser detector that emits fluorescence and sends the obtained data to a collection software on a computer. The collected raw data is then analyzed by an analysis software translating this raw data to sequence data (PE, 1995.1). To date the golden standard of mutation detection and the most efficient tool of genome projects is DNA sequencing. Though the detection rate of DNA sequencing is given as 100%, to be sure of a mutation two strands of the analyzed region should be sequenced (PE, 1995.2).

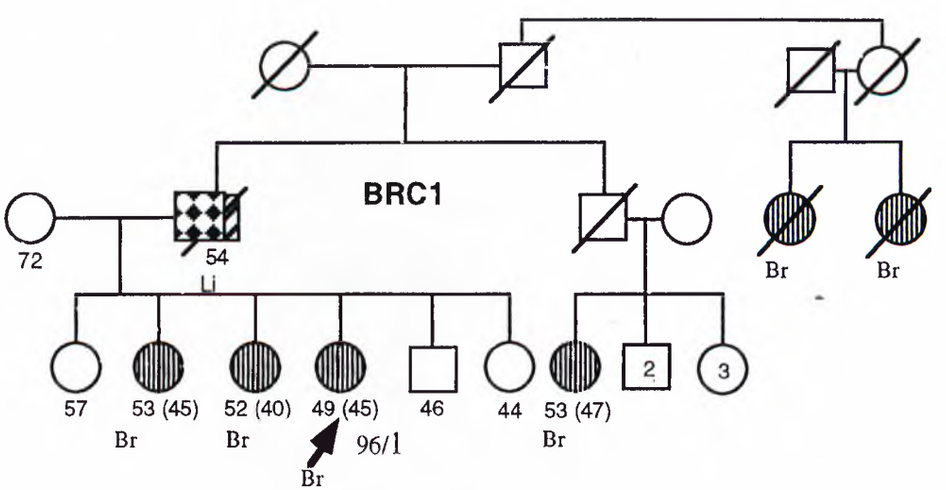
2. MATERIALS AND METHODS

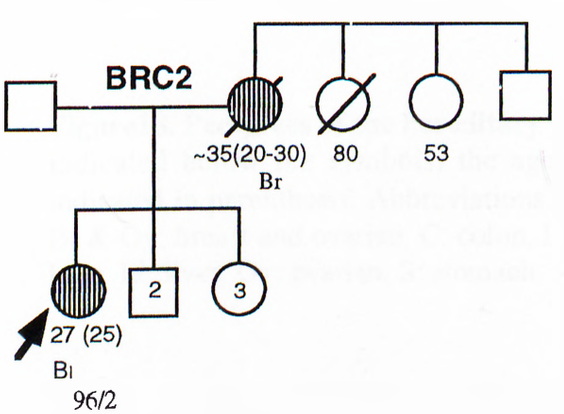
2.1. Materials

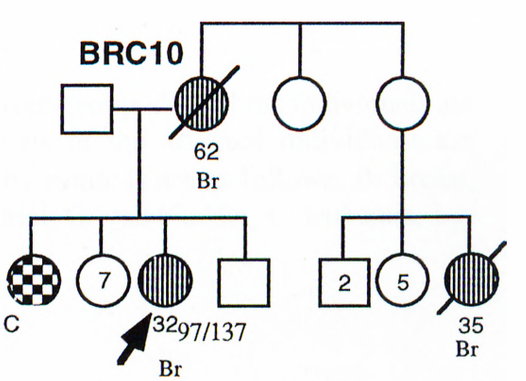
2.1.1. Families

2.1.1.1. Hereditary Breast Cancer Families

Six families are classified as hereditary breast cancer families according to the presence of at least two affected first degree relatives.







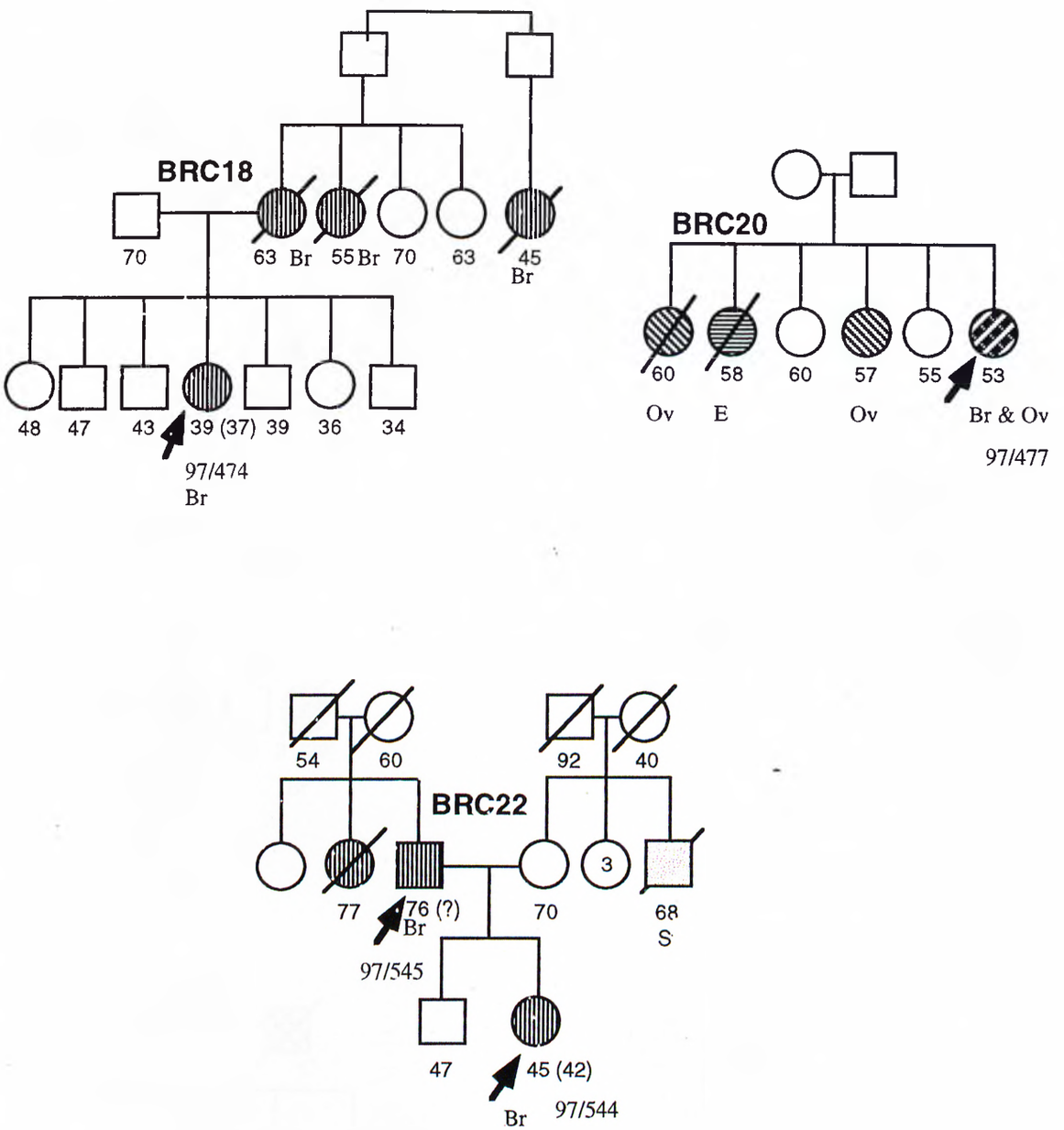
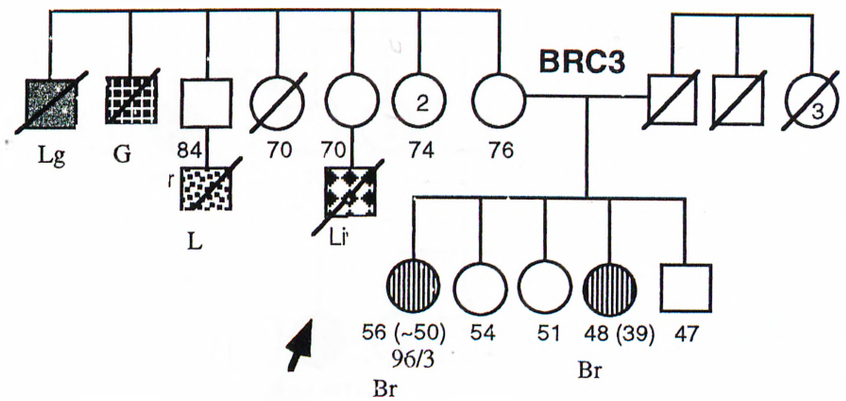
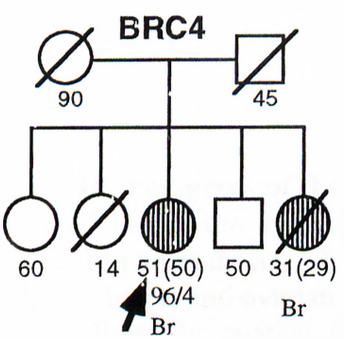
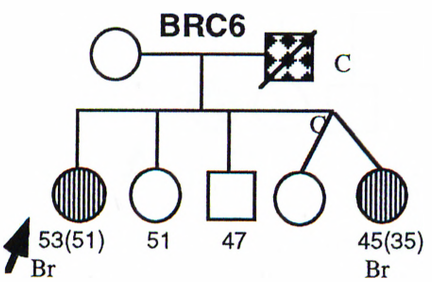


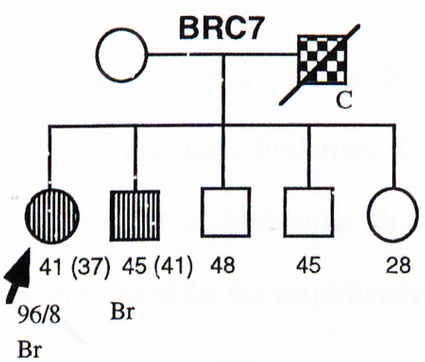
Figure18. Pedigrees of the hereditary breast cancer group. Age of the individuals are indicated below the symbols, the age at diagnosis of the affected individuals are indicated in parenthesis. Abbreviations indicated by symbols are as follows: Br:breast, Br & Ov: breast and ovarian, C: colon, E: endometrial, G: gall bladder, L: leukemia, Lg: lung, Li: liver, Ov: ovarian, S: stomach.

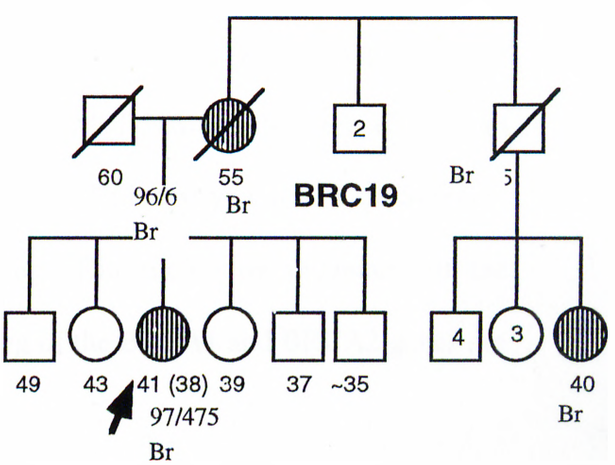
2.1.1.2. Familial.Breast Cancer Families











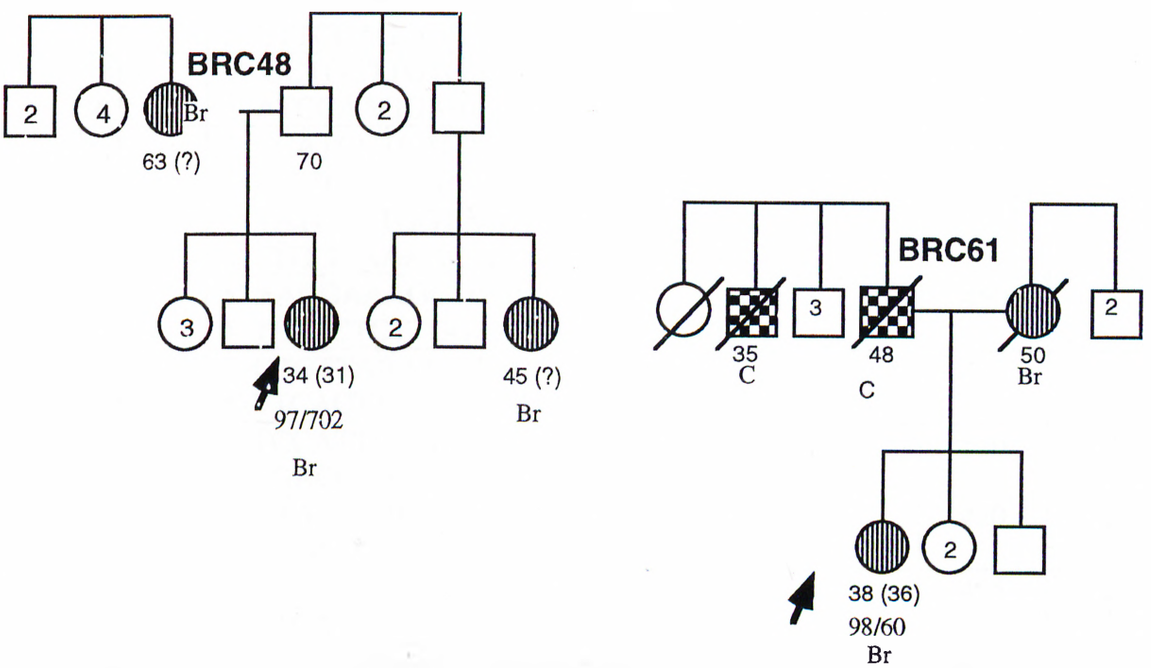


Figure 19. Pedigrees of the familial breast cancer group. Age of the individuals are indicated below the symbols, the age at diagnosis of the affected individuals are indicated in parenthesis. Abbreviations indicated by symbols are as follows: Br:breast, Br & Ov: breast and ovarian, C: colon, E: endometrial, G: gall bladder, L: leukemia, Lg: lung, Li: liver, Ov: ovarian, S: stomach.

2.1.2. Oligonucleotides

Oligonucleotides used in polymerase chain reactions and cycle sequencing reactions were synthesized on the Beckman Oligo 1000M DNA synthesizer (Beckman Instruments Inc., Fullerton, CA, USA) at Bilkent University, Faculty of Science, Department of Molecular Biology and Genetics. The nucleotide sequences of the primers used for the amplification and sequencing of the BRCA1 and BRCA2 genes are given in Table 4 and 5

Table 4. BRCA1 Primer sequences, expected fragments' sizes and optimal [$MgCl_2$]

Exon	Primer name	Primer sequence	Expected Size (bp)	[$MgCl_2$] (mM)
11	AF AR	5'-GGAATTAAATGAAAGAGTATGAGC-3' 5'-CTTCCAGCCCATCTGTTATGTTG-3'	470	2
11	BF BR	5'- GCTGCTTGTGAATTTTCTGAGACGGATG-3' 5'-ATCCAAGGAACATCTTCAGTATCTCTAGG-3'	445	2
11	CF CR	5'-CCATGCTCAGAGAATCCTAG-3' 5'-GGCTCAGTAACAAATGCTCC-3'	425	2
11	DF DR	5'-TATGCCTGGTAGAAGACTTCCTCC-3' 5'-TTGGGAAAACCTATCGGAA-3'	480	2
11	EF ER	5'-AACGAAAGCTGAACCTATAAGCAGC-3' 5'-TCTTCTCTTGGAAGGCTAGGATTG-3'	450	3
11	FF FR	5'-GTGTTAAATACCAGTGAACTTA-3' 5'-TTCACTTTCTTCCATTTCTATGC-3'	422	3
11	GF GR	5'-TCCATTGGGACATGAAGTTAACC-3' 5'-TGACCAACCACAGGAAAGCCTG-3'	310	2
11	HF HR	5'-CAAGCCTGTACAGACAGTTAATATCAC-3' 5'-GGAGCCCACTTCATTAGTAC-3'	420	2
11	IF IR	5'-CCAAGTACAGTGAGCACAATTA-3' 5'-ATCATCTAACAGGTCATCAGGT-3'	415	2
11	JF JR	5'-GGAAGTAGTCATGCATGCATCTCAGGT-3' 5'-GCTATTCTTCAATGATAATAAATTCTCC-3'	400	2
11	KF KR	5'-CGTTGCTACCGAGTGTCTGTCTAAG-3' 5'-GTGCTCCCAAAAGCATAAA-3'	450	2
2	2F 2R	5'-GAAGTTGTCATTTTATAAACCTTT-3' 5'-TGTCTTTTCTTCCCTAGTATGT-3'	250	3
5	5F 5R	5'-CTCTTAAGGGCAGTTGTGAG-3' 5'-TTCCTACTGTGGTTGCTTCC-3'	200	2
13	13F 13R	5'-AATGGAAAGCTTCTCAAAGTA-3' 5'-TGTTGGAGCTAGGTCCTTAC-3'	259	2
20	20F 20R	5'-ATATGACGTGTCTGCTCCAC-3' 5'-GGGAATCCAAATTACACAGC-3'	220	2
24	24F 24R	5'-ATGAATTGACACTAATCTCTGC-3' 5'-GTAGCCAGGACAGTAGAAGGA-3'	275	2
PTT11	11AF 11AR	5'-GGATCCTAATACGACTCACTATAGGGAGACCACCATGGCTG CTTGTGAATTTTCTG-3' 5'-GACGTCCTAGCTGTGTGAAG-3'	3000	1.5

Table 5. BRCA2 Primer sequences, expected fragments' sizes and optimal [$MgCl_2$]

Exon	Primer Name	Primer Sequence	Expected Size (bp)	[$MgCl_2$] (mM)
11	aF aR	5'-GATTGATGGTACTTTAATTTTGTCAC-3' 5'-CCTCCAAAACTGTGATTTGAAATTG-3'	1156	1.5
11	bF bR	5'-CCTCCAAAACTGTGATTTGAAATTG-3' 5'-CCTAAACCCCACTTCATTTTCATC-3'	1162	1.5
11	cF cR	5'-TGGCATTAGATAATCAAAAGAAACTG-3' 5'-TGATGGCTAAAACTGGTGATTTCAC-3'	1534	1.5
11	dF dR	5'-TGCGAAAGCTCAAGAAGCATG-3' 5'-GGTGAAGAGCTAGTCACAAGTTCCTC-3'	931	1.5
11	eF eR	5'-CTTATTCAGTCATTGAAAATTCAGCC-3' 5'-AAACCTTATGTGAATGCGTGCTAC-3'	782	1.5
11	fF fr	5'-GCCAGTATTGAAGAATGTTGAAGATC-3' 5'-CCAGCTCACAAGAGAAGAAAATACTG-3'	1253	1.5
11	gf gd	5'-GCTTTCCACTTGCTGTACTAAATCC-3' 5'-ACAGATTCTAAACTGCCAAGTCATG-3'	899	1.5
11	1F 1R	5'-GATGGTACTTTAATTTTGTCACTTTG-3' 5'-TTCCTTATTACATTTTGCTTCTTTAT-3'	220	3
11	2F 2R	5'-CTAGCTCTTTTGGGACAATTCTGAGG-3' 5'-TTTCATGATCATATAAAAGAAT-3'	300	3
11	3F 3R	5'-CAAAAGTGGAATACAGTGATAC-3' 5'-ATAATTTTCATTTAAAGCACATACAT-3'	250	3
11	4F 4R	5'-TCTAGAGGCAAAGAATCATA-3' 5'-TCATTGTCTGAGAAAAGTTC-3'	305	3
11	5F 5R	5'-TTCAAAAATAACTGTCAATCC-3' 5'-GTTGCTTGTTTATCACCTGT-3'	230	2
11	6F 6R	5'-AACCCATTTTCAAGAACTCTACCA-3' 5'-CTGAAGCTACCTCCAAAACTGTG-3'	274	3
11	7F 7R	5'-ACAAATGGGCAGGACTCTTAGG-3' 5'-CCTGCTTGGAAAATAACATCTG-3'	300	3
11	8F 8R	5'-AGTTGTTTCTGATTGTAAAAATAGTC-3' 5'-GCTGCTGTCTACCTGACCAA-3'	310	3
11	9F 9R	5'-GATGCTGATCTTCATGTCATAA-3' 5'-TACCTCTGCAGAAGTTTCCTCACTA-3'	300	3
11	10F 10R	5'-GGGTTTAGGGGCTTTTATTC-3' 5'-TATCAGTTGGCATTTATTATTTTT-3'	237	3
11	11F 11R	5'-CTTCAAGTAAATGTCATGATTCTGTC-3' 5'-CTGGCAGCAGTATATTTGTTATCT-3'	202	3
11	12F 12R	5'-ATTACTGAAAATTACAAGAGAAATA-3' 5'-AAAAAGTTAAATCTGACTCAAATCT-3'	244	3
11	13F	5'-TTTATGAAGGAGGGAAACACTCA-3'	282	3

	13R	5'-TTCAGAATTTAAGGAAAAGTTATGC-3'		
11	14F 14R	5'-AAAAATATTAGTGTCGCCAAAGAG-3' 5'-TGTATGAAAACCCAACAGAGTAGG-3'	278	2
11	15F 15R	5'-GTTAAACACAAAATACTGAAAG-3' 5'-CATTGATGGCTAAAACTGGTG-3'	233	3
11	16F 16R	5'-TCATCAAGCTAGCGGAAAAA-3' 5'-TGGCACCACAGTCTCAATAG-3'	265	2
11	17F 17R	5'-CTGCCCCAAAGTGTAAAGAAAT-3' 5'-AATGACTGAATAAGGGGACTGAT-3'	230	2
11	18F 18R	5'-GTCCTGCAACTTGTTACAC-3' 5'-GATTTTTGTCATTTTCAGC-3'	230	2
11	19F 19R	5'-AACCAGAAAGAATAAATACT-3' 5'-TCCTCAACGCAAATATCTTCAT-3'	334	3
11	20F 20R	5'-TTTCCAAAGTAATATCCAATGTA-3' 5'-ATTTTTGATTTATTCTCGTTGTT-3'	291	3
11	21F 21R	5'-AAGACATATTTACAGACAGT-3' 5'-TGAAGCTTCCCTATACTACAT-3'	300	3
11	22F 22R	5'-CACCTTGTGATGTTAGTTTG-3' 5'-TTGGGAATATAAATGTTCTGGAGTA-3'	350	3
11	23F 23R	5'-AAAGTAACGAACATTCAGACCA-3' 5'-CTGGGTTTCTCTTATCAACCGA-3'	310	2
11	24F 24R	5'-AGTCTTCACTATTCACCTACG-3' 5'-GTGAGACTTTGGTTCCTAAT-3'	280	3
11	25F 25R	5'-TTCAACAAGACAAACAACAGT-3' 5'-TGTCAGTTCATCATCTTCCATAAA-3'	270	3
11	26F 26R	5'-TTACTCCAAAGATTCAGAAAACTAC-3' 5'-AGCATACCAAGTCTACTGAATAAAC-3'	290	3

2.1.3. Chemicals and Reagents

Acetic acid	Carlo Erba, Milano, Italy	N64197
Acrylamide	Sigma, St Louis, MO, USA	A9099
Agarose	Basica LE, EU	031217PR
Ampicilline	Boehringer Mainheim	85454021
APS	Carlo Erba, Milano, Italy	7727-54-0
Bacto Agar	Difco, Michigan, USA	0140-01
Bactotryptone	Difco, Michigan, USA	0123-173
Bacto-yeast extract	Difco, Michigan, USA	0127-17-9
Bis-acrylamide	Aldrich	14607-2
Boric acid	Sigma, St. Louis, MO, USA	BO252
Bromophenol blue	Sigma, St. Louis, MO, USA	B5525
$CaCl_2$	Sigma, St. Louis,MO, USA	C3306
Chloroform	Carlo Erba, Milano, Italy	334353
Genetic Analyzer Buf.	PE, Applied Bio. CA, USA	402824
EcoRI	Sigma, St. Louis, MO, USA	R6265
EDTA pH 8.0	Carlo Erba, Milano, Italy,	303201
Ficoll type 400	Sigma, St. Louis, MO, USA	F4375
Glucose	Sigma, St. Louis, MO, USA	G7021
IPTG	MBI Fermentas	R0392
isoamyl alcohol	Merck, Schuchardt, Germany	K25713595 842

Kacetate	Merck, Schuchardt, Germany	K24483720
Met S^{35}	Amersham UK	SJ 1015
NaCl	Carlo Erba, Milano, Italy,	479687
NaOH	Carlo Erba, Milano, Italy	480507
P^{32} dATP 10 mCi/ml	Amersham UK	A 0005
Phenol	Sigma, St Louis, MO, USA	P1037/
POP6	PE, Applied Bio. CA, USA	9903020
Proteinase K	Appligene, MD, USA	130203
SDS	Merk, Schuchardt, Germany	D822050
Sodium Acetate	Merk, Schuchardt, Germany	TA6555468
TNT T7 coupled reticulocyte lysate system	Promega,	L4610
TrisHCl	Merk, Schuchardt, Germany	K21570697 627
trisodium citrate	Sigma, St. Louis, MO, USA	S4641
TSR	PE, Applied Bio. CA, USA	401674
Urea	Merck, Schuchardt, Germany,	K22063886
X Gal	Sigma, St. Louis, MO, USA	B4252
Xylene cyanol	Sigma, St. Louis, MO, USA	X4126

2.1.4. PCR Materials

Taq Polymerase 5U/µl (MBI Fermentas Inc, Amherst, NY, USA)

25 mM $MgCl_2$

10X Taq Polymerase buffer (100 mM TrisHCl, pH 8.8, 500 mM KCl, 0.8% Nonidet P40)

10 mM dNTP (10 μM each of dATP, dCTP, dGTP, dTTP)

0.2 ml Thermowell tubes Corning Costar Corp, Cambridge, MA, England

Gene Amp PCR system 9600 Perkin Elmer, Foster City, CA, USA

2.1.5. Sequencing Materials

ABI PRISM ™ Ready Reaction Dye Terminator Cycle Sequencing Kit (ABI, Perkin Elmer, Foster City, CA, USA) containing terminator premix with A-dye terminator, C-dye terminator, G-dye terminator and T-dye terminator; dITP, dATP, dCTP and dTTP; Tris-HCl (pH 9.0); $MgCl_2$; thermal stable pyrophosphatase; and AmpliTaq DNA polymerase, FS (8 U/μl).

Thermo Sequenase Dye Terminator Cycle Sequencing Pre-Mix Kit (Amersham Life Science, Inc., Cleveland, OH, USA) containing 125 mM Tris HCl, pH 9.5, 5 mM $MgCl_2$, 1.25 mM dITP, 0.25 M dATP, dCTP, dTTP, ddATP (dye-labeled), ddCTP (dye-labeled), ddGTP (dye-labeled), ddTTP (dye-labeled), Thermo Sequenase DNA polymerase, Thermoplasma acidophilam thermostable inorganic pyrophosphatase (TAP), Nonidet P40, Tween 20, 6.25% glycerol.

ABI PRISM^R BigDye^{TM} Terminator Cycle Sequencing Ready Reaction kit (ABI, Perkin Elmer, Foster City, CA, USA) containing A-DyeTerminator labeled with dichloro R6G, C-Dye Terminator labeled with dichloro ROX, G-Dye Terminator labeled with dichloro

R110, T-Dye Terminator labeled with dichloro TAMRA, dATP, dCTP, dITP, dUTP, AmpliTaq DNA polymerase, FS, with thermally stable pyrophosphatase, $MgCl_2$, Tris HCl buffer, pH 9.0.

0.2 ml Thermowell tubes Corning Costar Corp, Cambridge, MA, England

Gene Amp PCR system 9600 Perkin Elmer, Foster City, CA, USA

377 Automated Sequencer ABI, Perkin Elmer, Foster City, CA, USA

310 Genetic Analyzer ABI, Perkin Elmer, Foster City, CA, USA

41cm green labeled capillaries

2.1.6. Standard Solutions and Buffers

20XSSC

3 M	NaCl
0.3 M	Trisodium citrate

(Sterilized by autoclaving)

Extraction buffer

10 mM	TrisHCl pH 8.0
10 mM	EDTA pH 8.0
0.5%	SDS

Proteinase K 20 mg/ml

Phenol/chloroform/isoamyl alcohol 25:24:1

PTT Stop solution
1/10 2b mercaptoethanol in 2X Laemlli

Sodium Acetate (NaOAc) 3M pH 5.2

10X TBE

108 g	Tris HCl
55 g	boric acid
20 ml	0.5 M EDTA

10X Protein Buffer

144 g	Glycine
250 ml	Tris 1M pH 8.3
10 ml	10% SDS (filtered)

Complete final volume to 1 lt with ddH_2O.

2X Laemlli

4 ml	10% SDS (filtered)
2 ml	Glycerol
1.2 ml	Tris 1M pH 6.8-7.4
2.8 ml	ddH2O
10 ml	0.01% Bromphenol blue

6X Loading buffer

15%	Ficoll type 400
0.05%	Bromophenol blue
0.05%	Xylene cyanol

LB medium

10 g	Bactotryptone
5 g	Bacto-yeast extract
10g	NaCl

950 ml	ddH_2O

The solutes are dissolved by shaking. The pH is adjusted to 7.0 with 5N NaOH (0.2ml). The volume of the solution is completed to 1 lt with deionized H_2O. LB medium is sterilized by autoclaving.

LB-Agar

10 g	Tryptone
5 g	Yeast extract
10 g	NaCl
15 g	Bacto Agar

The solutes are dissolved in deionized water with a total volume of 1 lt. The solution is sterilized by autoclaving. 1 ml of ampicilline is added when its temperature decreases to 50°C.

1 M $CaCl_2$
10 ml aliquots is kept at –20°C. When preparing competent cells, an aliquot is thawed and diluted to 100 ml with deionized water. The solution is sterilized by filtration through a 45 μm filter and chilled to 0°C before using.

100 mg/ml Ampicilline is prepared and sterilized by filtrating through a 45 μm filter. The solution is aliquotted in 1.7 ml tubes and stored at –20°C.

IPTG
solution is prepared in a concentration of 0.2 M.

X Gal
solution is prepared in a concentration of 50 mg/ml.

GTE

50 mM	Glucose
25 mM	TrisCl (pH 8.0)
10 mM	EDTA

The solution is autoclaved for sterilization and stored at 4°C.

Solution II

0.2 N	NaOH (It is freshly diluted from a 10N stock)
1%	SDS

Solution III

60 ml 5 M	Kacetate
11.5 ml	Acetic acid

28.5 ml deionized water is mixed to prepare solution III. This solution is tored at 4°C.

Phenol/Chloroform 1:1

40% (39:1) Acrylamide:bis-acrylamide

39 g	Acrylamide
1g	Bis-acrylamide

Dissolve in ddH_2O, complete the final volume to 100 ml with ddH_2O. Filter and degas.

20% (19:1) Acrylamide: Bisacrylamide:

19 g	Acrylamide

1g	Bis-acrylamide

Dissolve in ddH_2O, complete the final volume to 100 ml with ddH_2O. Filter and degas.

40% (29:1) Acrylamide: Bisacrylamide

29 g	Acrylamide
1 g	Bis-acrylamide

Dissolve in ddH_2O, complete the final volume to 75 ml with ddH_2O. Filter and degas.

2.2. Methods

2.2.1.DNA isolation from peripheral blood

Blood can be stored at 4°C for a maximum of five days before aliquoting and freezing. It is frozen in 700 µl aliquots. After thawing blood add 800 µl of 1XSSC, mix by vortexing and spin at 13000 rpm for 1 minute. Remove the1.4 ml supernatant, discard into disinfectant (Chlorox). It is important not to disturb the cell pellet at this step. Add 1.4 ml of 1XSSC, briefly vortex, and spin at 13000 rpm for 1 minute. Remove all the supernatant avoiding the pellet. For cell lysis add 800 µl of extraction buffer and 10 µl of proteinase K. Briefly vortex and incubate the sample at 56°C for 3 hours. At the end of the incubation period, if the cell pellet is totally dissolved, add 400 µl phenol/chloroform/isoamyl alcohol (25:24:1) and vortex for 1 minute. This step must be carried out in a fume hood. The sample is then centrifuged for 5 minutes at maximum speed. Remove the upper aqueous layer and place in a new tube. Repeat phenol/chloroform/isoamyl alcohol extraction step till the interface becomes clear. After

the final extraction the upper aqueous layer (700 µl) is divided into two tubes. Then add 35 µl 3 M, NaOAc (pH 5.2), and 700 µl ice cold absolute ethanol into each tube. Mix the tubes by inversing and place at –20°C for 30 minutes. Spin the samples for 15 minutes at maximum speed, remove the alcohol and wash the pellet with 1 ml of 70% ethanol at room temperature. After 5 minutes of spin at 13000 rpm, remove ethanol with a micropipette. Leave the tubes open in the fume hood to evaporate the remaining ethanol. The DNA pellet can be resolubilized in 200 µl of sterile deionized water and incubated overnight at 56°C. The quality and concentration of DNA samples are determined by optical density measurement and by agarose gel electrophoresis.

2.2.2. Agarose Gel Electrophoresis

Agarose gel electrophoresis is a commonly used method for DNA analysis. The method is based on the mobility of DNA molecules in the pores of agarose, an algue derived polymer, which is dependent on the shape and weight of DNA, and also the current applied during electrophoresis and the concentration of agarose gel.

Genomic DNA samples and PCR products are routinely analyzed by agarose gel electrophoresis. Agarose gels are prepared with 1XTBE. They contain 1 µl of Ethidium bromide solution (20 mg/ml). 2 µl of genomic DNA sample with 6X loading buffer is loaded on 1% agarose whereas 5 µl of PCR product in 6X loading buffer is loaded on 2% agarose gel. Runs are performed with 1X TBE at 90 V for 30 minutes.

2.2.3. Polymerase Chain Reaction

Polymerase chain reaction is a method frequently used in the analysis of specific nucleotide sequences. PCR amplification involves two oligonucleotide primers that flank the DNA fragment to be amplified and repeated cycles of heat denaturation of the DNA, annealing of the primers to their complementary sequences, and extension of the annealed primers with heat stable Thermus aquaticus DNA polymerase. Each cycle results in the exponential accumulation of the specific target DNA segment, approximately 2^n, where n is the number of cycles (Saiki *et al*, 1998). In order to amplify the desired regions of *BRCA1* and *BRCA2* genes sets of different primer pairs is used. The primer sequences, expected fragment sizes, and optimal $MgCl_2$ concentrations are summarized in Table 4 and 5.

2.2.3.1. PCR for Heteroduplex analysis

PCR reactions are carried out in a total of 10 µl. 10 ng of genomic DNA is amplified with 2 pmols of forward and reverse primers, 1.5 U Taq Polymerase, 1X Taq Polymerase buffer, 1.5-3.0 mM $MgCl_2$, 1 µCi P^{32} dATP and 200 µM dNTP. PCR conditions are as follows; Initial denaturation is at 95°C for 5 min. Denaturation for 30 seconds at 94°C, annealing 30 sec at 55°C, 40 sec or 1 min 15 sec at 72°C for polymerization depending on the length of target fragment is repeated for 30 cycles (Except for the fragments of BRCA2 exon 11, 40 sec of extension time is used in the

amplification process). 10 min at 72°C for final extension is followed by 4°C soak. Presence of these radioactively labeled PCR products are not checked on agarose gel electrophoresis. They are then loaded on 6% native PAGE PCR products are denatured at 95°C for 10 min and left at 37°C for 2 hours for the formation of heteroduplexes.

2.2.3.2. PCR for DNA Sequencing

PCR reactions are carried out in a total volume of 50 µl. 50 ng of genomic DNA is amplified with 10 pmol of forward and reverse primers, 1.5 U Taq Polymerase, 1X Taq Polymerase buffer, 1.5-3.0 mM $MgCl_2$ and 200 µM dNTP. PCR conditions are as follows; Initial denaturation is at 95°C for 5 min. 30 cycles of denaturation for 30 sec at 94°C, annealing 30 sec at 55°C, polymerization 40 sec or 1 min 15 seconds depending on the length of target fragment. 10 min at 72°C for final extension is followed by 4°C soak. PCR products are checked by agarose gel electrophoresis before application of PCR purification columns (Qiagen) according to manufacturer's instructions in order to get rid of excess primers, and primer-dimers which may interfere with cycle sequencing. 2 µl of purified PCR products were loaded on 2% agarose gel for concentration estimation. A minimum of 40 ng PCR product is required for cycle sequencing.

2.2.3.3. PCR for PTT

PCR is performed using Expand long template PCR system which contains Taq and Pwo DNA polymerases. Two mixes are prepared to prevent primers and DNA polymerases from interacting with each other at inappropriate conditions, since Pwo DNA polymerase has 3'5' exonuclease activity.

Mix 1. 1.4 µl dNTP (5mM)

0.8 µl Forward Primer (10 pmol)

0.8 µl Reverse Primer (10 pmol)

ddH_2O to a total volume of 9 µl.

200 ng DNA

Mix 2. 6.3 µl Buffer

0.2 µl DNA pol.

ddH2O to a total volume of 8.5 µl. Total PCR volume 20 µl.

PCR conditions are as follows:

94°C	2 min		
94°C	10 sec	}	10 cycles
65°C	30 sec		
68°C	2 min		

The gel is washed 20 minutes in 10% acetic acid, 20% methanol solution and processed 30 minutes in Amplify . Dried gel is exposed to X-ray film for 12-18 hours.

2.2.5. Cloning

2.2.5.1.Ligation and Transformation

The frameshift mutations detected by sequencing of PCR products were confirmed by cloning the suspected fragment in a TA cloning vector (Promega) and sequencing it. PGEM Teasy cloning vector is used according to the manufacturer's directions. After overnight ligation reaction at 4°C, the PCR fragment inserted to the vector is transformed to E. coli HB101strain. Transformation is performed as follows: 200 µl of HB101 is incubated overnight in 5 ml of LB. 1.5 ml of grown cells is transferred into a 1.5 ml tube and spinned for 1 min at 13000 rpm. The supernatant is removed and 500 µl cold $CaCl_2$ is added. The sample is left on ice for 30 minutes and spinned for 1 min at 13000 rpm. The supernatant is removed again and this time 100 µl $CaCl_2$ is added. The pellet is dissolved gently by pipetting. The whole ligation product (10 µl) is added on the dissolved pelled and incubated on ice for 30 min. The sample is transferred into a 42°C water-bath for 90 sec. After a 2 min incubation on ice 1 ml LB is added and left 1 hour at 37°C. At the end of this incubation period the sample is centrifuged for 2 min. The supernatant is removed leaving 50 µl sample in the tube to dissolve the pellet by pipetting.The dissolved pellet is plated out on ampiciline (100 mg/ml) containing and Xgal (20 µl 50 mg/ml), IPTG (100 µl 100 mM) spreaded LB Agar plates and incubated overnight at 37°C.

The vector used in this assay contains the multiple cloning site in the lacZ gene that is used to choose the successful ligated product containing clones. The clones which contain ligated products will disrupt the lacZ gene so that galactose cannot be used by them. They will appear as white. Next day pick up at least 20 white colonies with pipette tips, put them in 15 ml LB (containing 100mg/ml ampicilline) and incubate overnight at 37°C in order to prepare their low scale DNA samples.

2.2.5.2.Low scale DNA Isolation

1.5 ml overnight grown cultures are transferred into 1.7 ml tubes and centrifuged for 3 min at 13000 rpm. The supernatant is decanted and 100 µl GTE is added on the pellet. The pellet is dissolved by vortexing. Freshly prepared 200 µl solution II is added and mixed by inverting. 150 µl solution is added and mixed by vortexing. At this step the cell pellet is lysed and the DNA becomes naked. In order to isolate and purify the DNA, phenol/chloroform extraction is performed. 500 µl phenol/chloroform is added and vortexed for 2 min. After 5 min centrifugation at 13000 rpm, the aqueous layer is transferred in a new tube. This step is repeated till the interface becomes clear. 1 ml cold absolute ethanol is added on the aqueous layer transferred into a new tube. The sample is mixed by inverting, left for 30 min at –20°C and centrifuged for 10 min at 13000 rpm. The supernatant is poured . The pellet is washed with 500 µl 70% ethanol. Ethanol is removed and the pellet is air dried. 20 µl RNAase (0.1 µg/µl) containing sterile deionized H_2O is used to dissolve the pellet.

2.2.5.3. Restriction Endonuclease Digestion

In order to confirm that the DNA samples isolated from white colonies contain the ligated PCR products, the samples were digested with EcoRI. Recognition sites of this enzyme resides just at the upstream and downstream points of the ligation products. So that the digestion reaction will give two fragments, the linearized vector and the inserted PCR fragment. In the case that the PCR fragment does not contain any EcoRI recognition site. 2 µl (approximately 100 ng) of DNA is incubated for 4 hours at 37°C with 1 U EcoRI and 10X buffer in a total of 10 µl. The digested product is loaded on 2% agarose gel and run with 1X TBE for 1 hour at 90 V. The DNA samples containing the PCR product are chosen for sequencing.

2.2.6. DNA Sequencing

2.2.6.1. Cycle Sequencing

Shifted fragments in HA are reamplified and sequenced. After PCR amplification, products are applied to PCR purification columns (Qiagen), and quantitated on agarose gel. 40 ng of PCR product (up to 7 µl) is mixed with 3.2 pmol forward or reverse primer, and 4 µl Ready Reaction Mix. The reaction volume is completed to 20 µl with sterile deionized H_2O. PCR conditions are as follows: 3 min initial denaturation at 98°C; 30 cycles of 10 sec at 96°C, 5 sec at 50°C, and 4 min at 60°C; soak at 4°C.

In order to remove unincorporated ddNTPs which may interfere with sequencing reactions, samples are precipitated with ethanol. 20 µl of total product is added on 68 µl cold absolute ethanol, and 7 µl 7,5 M ammonium acetate. After vortexing the tube is placed on ice for 15 min, and centrifuged at 13000 rpm for 30 min. Supernatant is removed, and 250 µl 70% ethanol is added to wash the pellet. An additional centrifugation for 5 min is performed. Ethanol is removed and the pellet is air dried in a fume hood keeping them in dark. Light may give harm to fluorescein tags in sequencing products.

2.2.7. Polyacrylamide Gel Electrophoresis (PAGE)

2.2.7.1 PAGE for Heteroduplex Analysis

For HA 6% polyacrylamide is prepared in a total volume of 75 ml with 11.25 ml 40%, 39:1 acrylamide:bisacrylamide, 7.5 µl 10X TBE, 5.3 ml glycerol (7%), 50.5 ml deionized H_2O 400 µl 10% APS, and 50 µl TEMED. The gel solution is filtered and degased before the addition of APS and TEMED. Glass plates are thoroughly washed and wiped with distilled water and 70% ethanol before pouring the gel. The poured gel is left on bench for 2-5 hours to polymerize.

15 µl of loading buffer is added on PCR products before loading on polyacrylamide gel. 5 ml of PCR products are loaded on polyacrylamide gel. The gel is run overnight at

400V. The next day, the gel is dried in a vacuum drier at 80°C for 45 minutes and exposed to X Ray film overnight.

2.2.7.2. PAGE for PTT

For PTT 12% polyacrylamide is prepared in a total volume of 7,5 ml with 4,5 ml 40%, 29:1 acrylamide:bisacrylamide, 1.85 ml TrisHCl 1.5 M, pH 8.8, 3.25 ml deionized H_2O, 75 µl 10% SDS, 60 µl 10% APS, and 5 µl TEMED. The gel solution is filtered and degas before the addition of APS and TEMED. Glass plates are thoroughly washed and wiped with distilled water and 70% ethanol before pouring the gel. The poured gel is left on bench for 15 minutes to polymerize. 2 ml of stacking gel with 18 µl 10%APS and 2.5 µl TEMED is poured on the running gel. The electrophoresis is performed in BIORAD protein apparatus with 1X protein buffer at 200 V for one hour.

2.2.7.2. Denaturing PAGE for DNA Sequencing

Denaturing PAGE is used for in DNA sequencing to get a resolution of 1 bp difference. 4% polyacrylamide gel is prepared from 5 ml 20% stock solution of 19:1 acrylamide:bisacrylamide, 5 ml 10X TBE, 18 g urea , 250 µl 10% APS, 35 ml TEMED is added. The volume is brought up to 50 ml with deionized H_2O.

The pellet is resuspended in 4 µl ready loading dye, heated to 70°C for 2-5 minutes and placed on ice before loading on polyacrylamide gel. Loading 2 µl of sample is enough

for PAGE on ABI 377 Automated DNA Sequencer. Sequencing data is collected by ABI Data Collection 1.0.4 software and analyzed with DNA Sequence Analysis 3.0 software. Alignment studies are done with Sequence Navigator software after the reanalysis of the data by the Factura program.

2.2.8. Capillary Electrophoresis

DNA sequencing reactions are also analyzed with capillary electrophoresis on ABI 310 Genetic Analyzer using POP6 and 41cm green labeled capillaries. For capillary electrophoresis the pellet is resuspended in 25 µl of TSR (Template Suppression Reagent). The sample is denatured at 95°C for 3 minutes before putting on autosampler tray. Sequencing data is collected by ABI Data Collection 1.0.4 software and analyzed with DNA Sequence Analysis 3.0 software. Alignment studies are done with Sequence Navigator software after the reanalysis of the data by the Factura program.

3. RESULTS

In this study germ-line *BRCA1* and/or *BRCA2* mutations were screened in 50 breast and/or ovarian cancer patients. Selected regions of *BRCA1* (exons 2, 5, 11, 13, 20, 24) and *BRCA2* (exon 11) were analyzed. Since exon 11 of both genes are too big to analyze in a single experiment, *BRCA1* exon 11 is divided to eleven and *BRCA2* exon 11 is divided to seven overlapping fragments. Both of the genes were first screened by heteroduplex analysis which is an indirect mutation screening method. Altered fragments were re-amplified and further analyzed by DNA sequencing.

3.1. DNA Isolation

Genomic DNA was isolated from patients blood cells using phenol chloroform extraction method. Isolated DNA samples were evaluted quantitatively and qualitatively by UV spectrophotometry and agarose gel electrophoresis. All DNA samples had a 260/280 optical density ratio of 1.7-1.8, and showed a single, intact, high molecular weight band on agarose gel (Figure 19). The concentrations of these DNA samples are 200, 180, 210, 260, 195, 230, 240 and 245 µg/ml respectively.

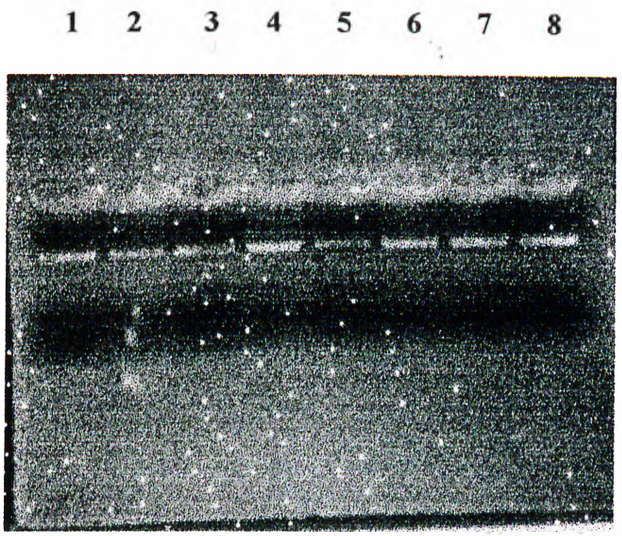


Figure 19. Analysis of extracted genomic DNA samples.
Samples were electrophoresed in a 0.8% agarose gel at 9V/cm for 45 minutes. Lanes 1, 96/1; 2, 96/2; 3, 96/3; 3, 96/4; 4, 96/5; 5, 96/6; 6, 96/7; 8, 96/8.

3.2. Polymerase chain reaction

All PCR products were checked by agarose gel electrophoresis. Figure 20 shows the amplification products of *BRCA1* exon 11B and *BRCA2* exon 11-6. The expected sizes of the PCR product was 445 bp and 274 bp respectively, which is confirmed by comparison of the observed DNA bands with the size marker.

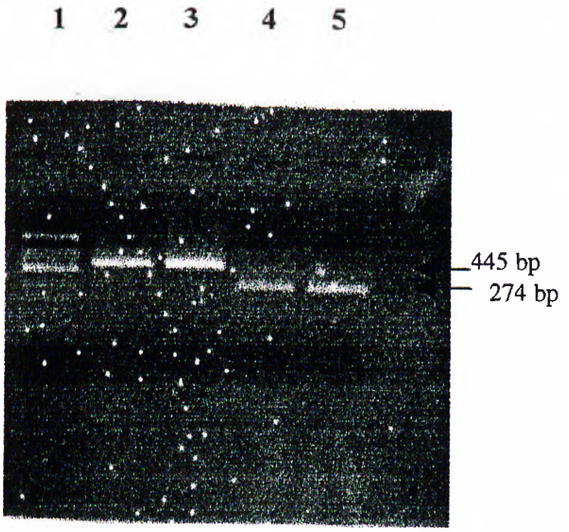


Figure 20. Analysis of PCR products.

PCR products purified from Qiagen PCR purification columns were electrophoresed in a 2% agarose gel at 9V/cm for 45 minutes. Lanes 1: ϕX174 DNA *Hinf*I marker, 2: 97/344 and 3: 97/359 *BRCA1* exon 11B; 4: 97/544 and 5: 97/545 *BRCA2* exon 11-6.

3.3. Protein Truncation Test

BRCA1 exon 11 of two hereditary (96/1, 96/2), three familial (96/3, 96/4, 97/474) and four early onset (96/5, 96/10, 97/344, 97/473) breast cancer cases was analyzed with protein truncation test. No altered size fragments were detected in any one of the samples. Figure 21 shows the *BRCA1* exon 11 PTT profile of patients 97/344, 97/473, 97/474 (lane 1, lane 2 and lane 3 respectively) and control (lane 4) sample.

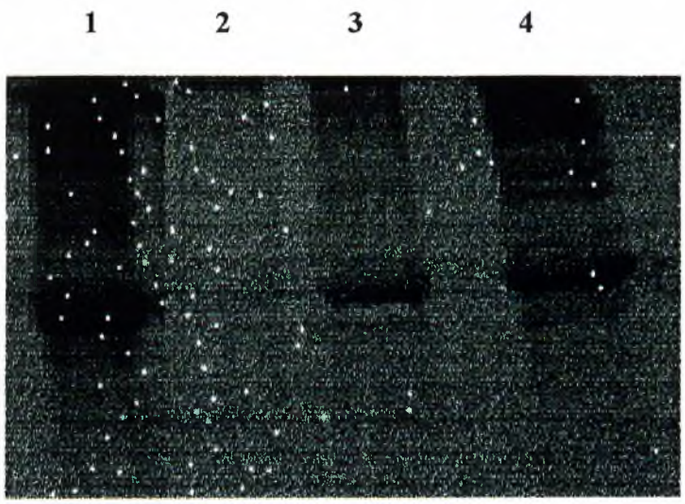


Figure 21. PTT of *BRCA1* exon 11.
Lanes 1, 97/344; 2, 97/473; 3, 97/474; 4, control.

3.4. Heteroduplex analysis

Heteroduplex analysis was performed on EC 160 vertical gel apparatus. Native 39:1 (6%) polyacrylamide gels were run at 400V overnight at room temperature. Gels were dried at 80°C for 45 minutes and exposed to Xray film overnight at –80°C.

3.3.1. *BRCA1* exon 2

BRCA1 exon 2 is amplified with 2F and 2R primers as a fragment of 250 bp. Results of one hereditary (97/137; lane 2), four early onset (97/114, 97/270, 97/274, 97/343; lanes 1, 3, 4, 5 respectively) breast cancer cases, and control (lane 6) sample is shown in figure 22. No shifts were observed in any one of the samples.

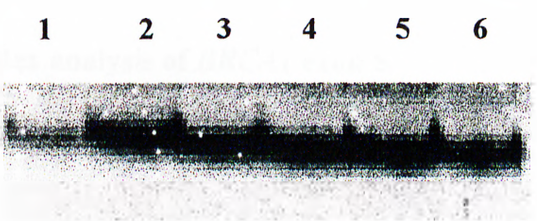


Figure 22. Heteroduplex analysis of *BRCA1* exon 2.
Lanes 1, 97/114; 2, 97/137; 3, 97/270; 4, 97/274; 5, 97/343; 6, control.

3.3.2. *BRCA1* exon 5

BRCA1 exon 5 is amplified with 5F and 5R primers as a fragment of 200 bp. Results of four hereditary (96/1, 96/2, 97/544, 97/545; lanes 1, 2, 4, 5 respectively) and two early onset (96/10, 97/702; lanes 3 and 6) breast cancer cases, and control (lane 7) sample is shown in figure 23. No shifts were observed in any one of the samples.

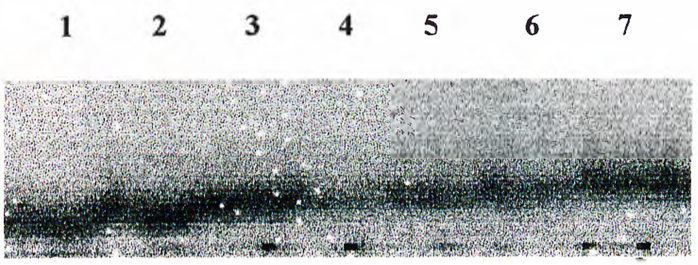


Figure 23. Heteroduplex analysis of *BRCA1* exon 5.
Lanes 1, 96/1; 2, 96/2; 3, 96/10; 4, 97/544; 5, 97/545; 6, 97/702; 7, control.

3.3.3. *BRCA1* exon 11A

BRCA1 exon 11A is amplified with AF and AR as a fragment of 470 bp. Results of one hereditary (97/137; lane 2) and three early onset (97/114, 97/359, 97/472; lanes 1, 3, 4 respectively) breast cancer cases and control (lane 5) sample is shown in Figure 24. No shifts were observed in any one of the samples.

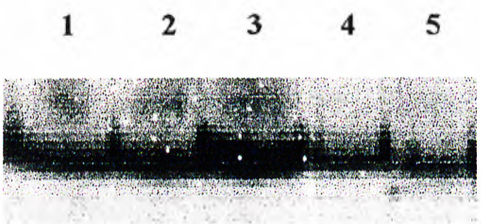


Figure 24. Heteroduplex analysis of *BRCA1* exon 11A.
Lanes 1, 97/114; 2, 97/137; 3, 97/359; 4, 97/472; 5, control.

3.3.4. *BRCA1* exon 11B

BRCA1 exon 11B is amplified with BF and BR as a fragment of 445 bp. Results of a familial (97/474; lane 2) and three early onset (97/472, 97/641, 97/656; lanes 1, 3, 4 respectively) breast cancer cases, and control (lane 5) is shown in figure 25. A heteroduplex formation resulting in a shift has been observed in patient 97/641 (lane 3). This region of the patient was later reamplified and further analyzed by DNA sequencing.

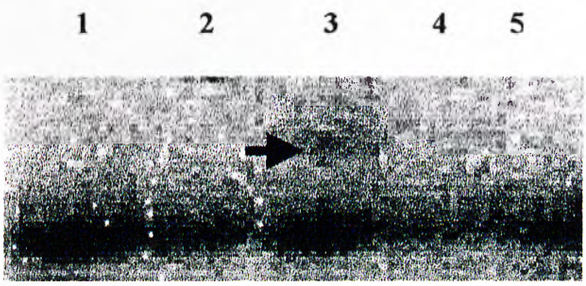


Figure 25. Heteroduplex analysis of *BRCA1* exon 11B.
Lanes 1, 97/472; 2, 97/474; 3, 97/641; 4, 97/656; 5, control; Arrow indicates the shifted fragment.

3.3.5. *BRCA1* exon 11C

BRCA1 exon 11C is amplified with CF and CR as a fragment of 425 bp. Results of four early onset (97/661, 97/662, 97/670, 97/674; lanes 1, 2, 3, and 4 respectively) breast cancer patients, and control (lane 5) sample is shown in figure 26. No shifts were observed in any one of the samples.

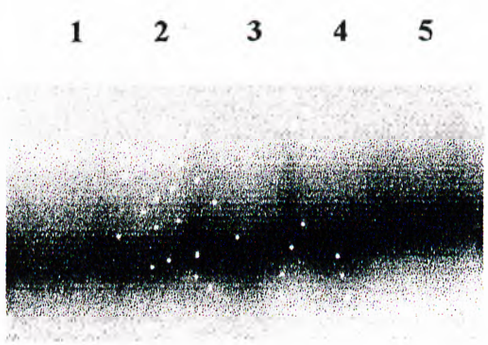


Figure 26. Heteroduplex analysis of *BRCA1* exon 11C.
Lanes 1, 97/661; 2, 97/662; 3, 97/670; 4, 97/674; 5, control.

3.3.6. *BRCA1* exon 11D

BRCA1 exon 11D is amplified with DF and DR as a fragment of 480 bp. Results of four early onset (97/639, 97/641, 97/655, 97/656; lanes 1, 2, 3, and 4 respectively) breast cancer cases, and control (lane 5) sample is shown in figure 27. No shifts were observed in any one of samples.

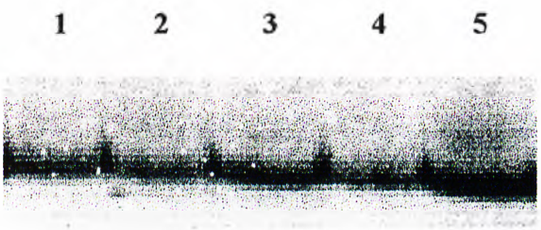


Figure 27. Heteroduplex analysis of *BRCA1* exon 11D.
Lanes 1, 97/639; 2, 97/641; 3: 97/655; 4, 97/656; 5, control.

3.3.7. *BRCA1* exon 11 E

BRCA1 exon 11E is amplified with primers EF and ER as a fragment of 450 bp. Results of six early onset (97/508, 97/639, 97/641, 97/670, 97/674, 97/681; lanes 1, 2, 3, 4, 5, and 6) breast cancer cases is shown in figure 28. A band shift has been observed in patient 97/670 (lane 4).

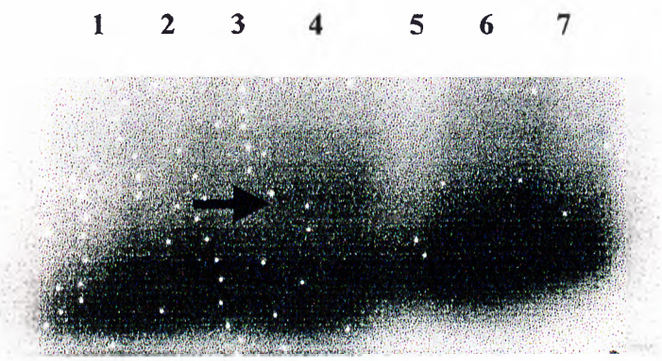


Figure 28. Heteroduplex analysis of BRCA1 exon 11F.
Lanes 1, 97/508; 2, 97/639; 3, 97/641; 4, 97/670; 5, 97/674; 6, 97/681; 7, control. Arrow indicate the shifted fragment.

3.3.8. *BRCA1* exon 11F

BRCA1 exon 11F is amplified with primers FF and FR as a fragment of 422 bp. Result of five early onset (96/10, 97/114, 97/270, 97/274; lanes 1, 2, 3, and 4 respectively) breast cancer cases, and control (lane 5) sample is shown in figure 29. No shifts were observed in any one of the samples.

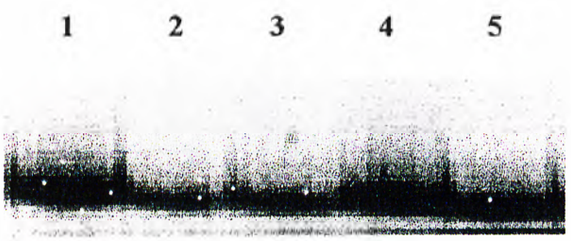


Figure 29. Heteroduplex analysis of BRCA1 exon 11F
Lanes 1, 96/10; 2, 97/114; 3, 97/270; 4, 97/274; 5, control.

3.3.8. *BRCA1* exon 11G

BRCA1 exon 11G was amplified with primers GF and GR as a fragment of 310 bp. Results of four early onset (97/343, 97/639, 97/508, 97/662; lanes 1, 2, 3, and 4 respectively) breast cancer cases, and control (lane 5) is shown in figure 30. A similar band shift was observed in two familial (97/475 and 97/702) and in an early onset breast cancer case (97/508, lane 3).

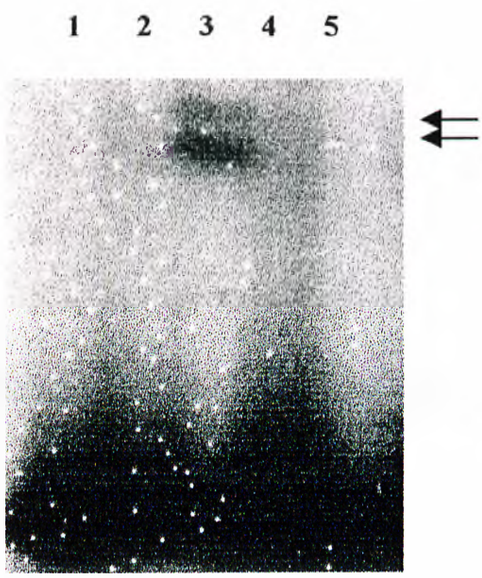


Figure 30. Heteroduplex analysis of *BRCA1* exon 11G.
Lanes 1, 97/343; 2, 97/639; 3, 97/508; 4, 97/662; 5, control. Arrows indicate the shifted fragments.

3.3.9. *BRCA1* exon 11H

BRCA1 exon 11H was amplified with primers HF and HR as a fragment of 420 bp. Results of two hereditary (96/1, 96/2; lanes 1 and 2), one familial (96/8; lane 3), one early onset (96/10; lane 4) breast cancer cases, and control (lane 5) sample is shown in figure 31. No shifts were observed in any one of the samples.

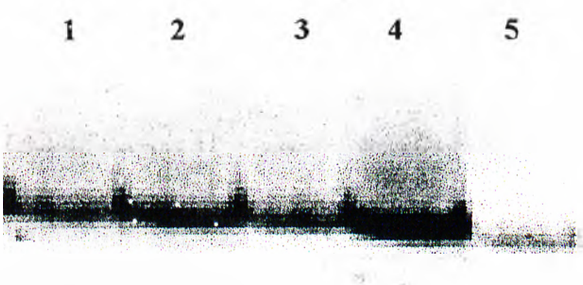


Figure 31. Heteroduplex analysis of *BRCA1* exon 11H.
Lanes 1, 96/1; 2, 96/2; 3, 96/8; 4, 96/10; 5, control.

3.3.10. *BRCA1* exon 11I

BRCA1 exon 11I is amplified wuth primers IF and IR as a fragment of 415 bp. Results of two hereditary (97/544, 97/545; lanes 2 and 3), and three familial (96/8, 97/702, 98/60; lanes 1, 4, and 5) breast cancer cases, and control (lane 6) is shown in figure 32. No shifts were observed in any one of the samples.

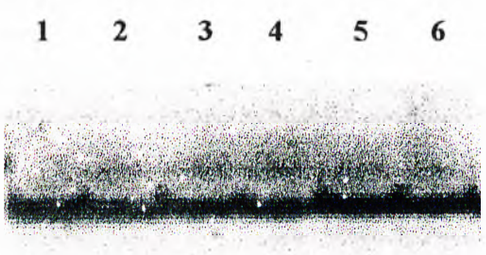


Figure 32. Heteroduplex analysis of *BRCA1* exon 11I.
Lanes 1, 96/8; 2, 97/544; 3, 97/545; 4, 97/702; 5, 98/60; 6, control.

3.3.11. *BRCA1* exon 11J

BRCA1 exon 11J was amplified with primers JF and JR as a fragment of 400 bp. Results of two hereditary (97/544, 97/545; lanes 2 and 3), two early onset (97/670, 96/10; lanes 1 and 4) breast cancer cases, and control (lane 5) sample is shown in figure 33. A similar shift has been observed in samples 97/544 and 96/10 (lanes 2 and 4 respectively) which was later found to be present in two additional hereditary (97/137, 97/477), three additional familial (96/7, 97/474, 97/475) and eight additional early onset (96/5, 97/270, 97/344, 97/632, 97/681, 97/683, 97/684, 98/18) breast cancer case. This shift was further analyzed in each of these patients by DNA sequencing.

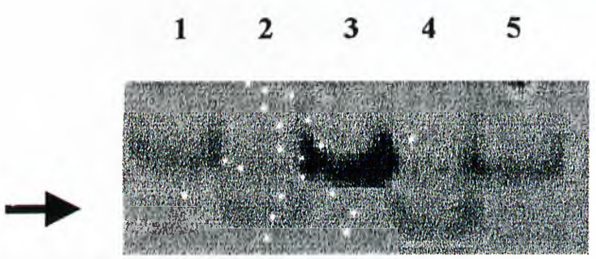


Figure 33. Heteroduplex analysis of *BRCA1* exon 11J.
Lanes 1, 97/670; 2, 97/544; 3, 97/545; 4, 96/10; 5, control. Arrow indicates the shifted fragment.

3.3.12. *BRCA1* exon 11K

BRCA1 exon 11K is amplified with primers KF and KR as a fragment of 450 bp. Results of one familial (97/702; lane 1), three early onset (97/703, 98/17, 98/18; lane 2, 3, and 4 respectively) breast cancer cases, and control (lane 5) sample is shown in figure 34. No shifts were observed in any one of the samples.

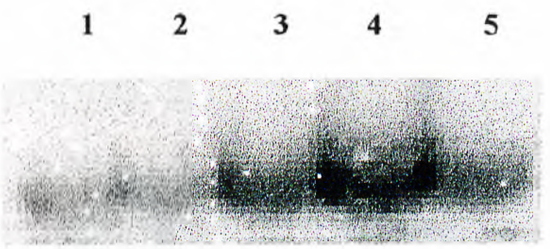


Figure 34: Heteroduplex analysis of *BRCA1* exon 11K.
Lane 1, 97/702; Lane 2, 97/703; Lane 3, 98/17; Lane 4, 98/18; Lane 5, control.

3.3.13. *BRCA1* exon 13

BRCA1 exon 13 is amplified with primers 13F and 13R as a fragment of 259 bp. Results of one familial (98/60; lane 4), three early onset (97/703, 98/17, 98/18; lanes 1, 2, and 3 respectively) breast cancer cases, and control (lane 5) sample is shown figure 35. No shifts were observed in any one of the samples.

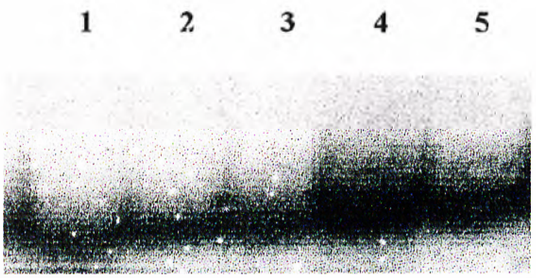


Figure 35. Heteroduplex analysis of *BRCA1* exon 13.
Lanes 1, 97/703; 2, 98/17; 3, 98/18; 4, 98/60; 5, control.

3.3.14. *BRCA1* exon 20

BRCA1 exon 20 is amplified with primers 20F and 20R as a fragment of 220 bp. Results two hereditary (96/1, 96/2; lanes 1 and 2 respectively), four familial (96/3, 96/4, 96/6, 96/7; lanes 3, 4, 6, and 7 respectively), one early onset (96/5; lane 5) breast cancer cases, and control (lane 8) sample is shown in figure 36. No shifts were observed in any one of the samples.

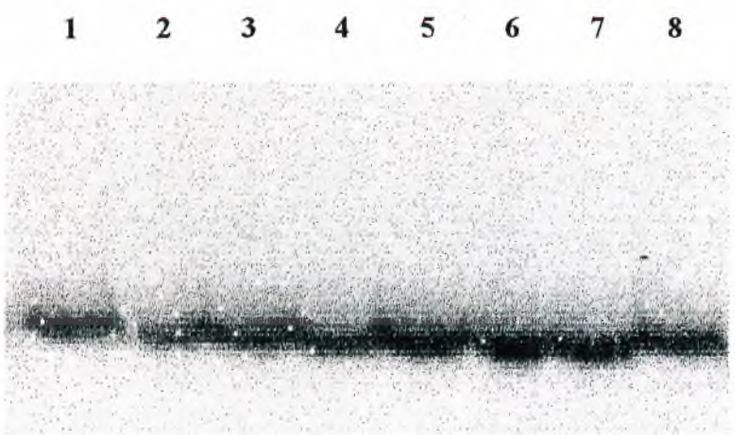


Figure 36. Heteroduplex analysis *BRCA1* exon 20.
Lanes 1, 96/1; 2, 96/2; 3, 96/3; 4, 96/4; 5, 96/5; 6, 96/6; 7, 96/7; 8: control.

3.3.15. *BRCA1* exon 24

BRCA1 exon 24 is amplified with primers 20F and 20R as a fragment of 275 bp. Results of three early onset (97/343, 97/344, 97/359; lanes 1, 2, and 3 respectively) breast cancer cancer cases, and control (lane 4) is shown in figure 37. No shifts were observed in any one of the samples.

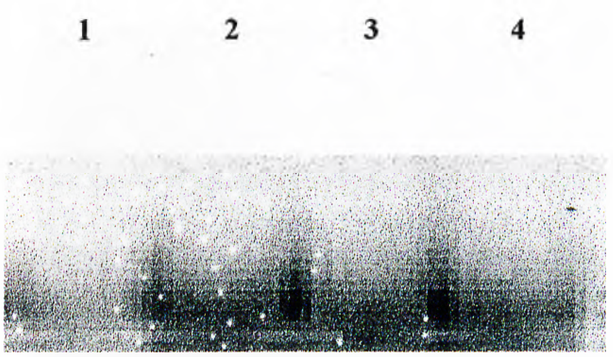


Figure 37. Heteroduplex analysis of *BRCA1* exon 24.
Lanes 1, 97/343; 2, 97/344; 3, 97/359; 4, control.

3.3.16. *BRCA2* exon 11a

BRCA2 exon 11a is amplified with primers aF and aR as a fragment of 1156 bp. Results of two hereditary (97/544, 97/545; lanes 2 and 3 respectively), one familial (96/8; lane 1) breast cancer cases, and control (Lane 4) sample is shown in figure 38. Heteroduplex formation was observed in samples 97/544 and 97/545 (lane 2 and 3) who are a father and daughter pair both affected with breast cancer (Family 22).

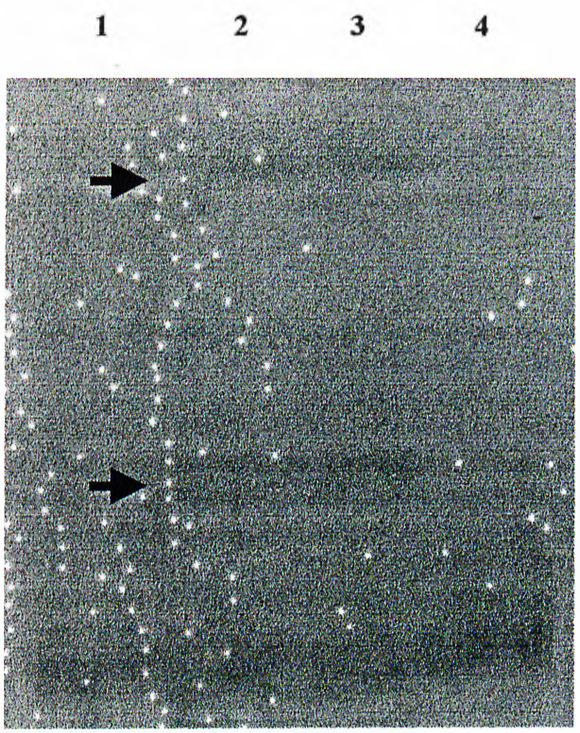


Figure 38. Heteroduplex analysis of *BRCA2* exon 11a.
Lanes 1, 96/8; 2, 97/544; 3, 97/545; 4, control. Arrows indicate shifted fragments.

3.3.17. *BRCA2* exon 11b

BRCA2 exon 11b is amplified with primers bF and bR as a fragment of 1162 bp. Results of three hereditary (96/1, 97/544, 97/545; lanes 1, 3, and 4 respectively), one familial (96/8; lane 2), two early onset (97/631, 97/694; lanes 5 and 6 respectively) breast cancer cases, and control (lane 7) is shown in figure 39. Heteroduplex formation was observed in samples 97/544 and 97/545 (lanes 3 and 4 respectively) who are a father daughter pair (Family 22).

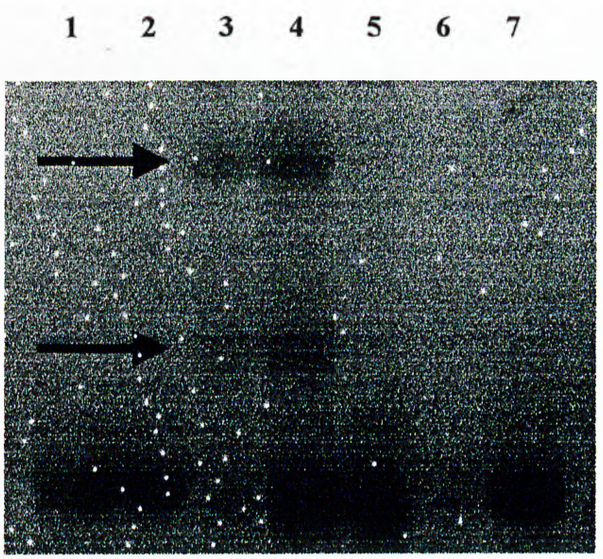


Figure 39. Heteroduplex analysis of *BRCA2* exon 11b.
Lanes 1, 96/1; 2, 96/8; 3, 97/544; 4, 97/545; 5, 97/631; 6, 97/694; 7, control.

3.3.18. *BRCA2* exon 11a-b

The overlapping region of *BRCA2* exon 11a and b is amplified as a fragment of 274 bp (Figure 40), in order to localize the exact site of the mutation causing to shifted fragments in samples of the father (97/544) and his daughter (97/545).

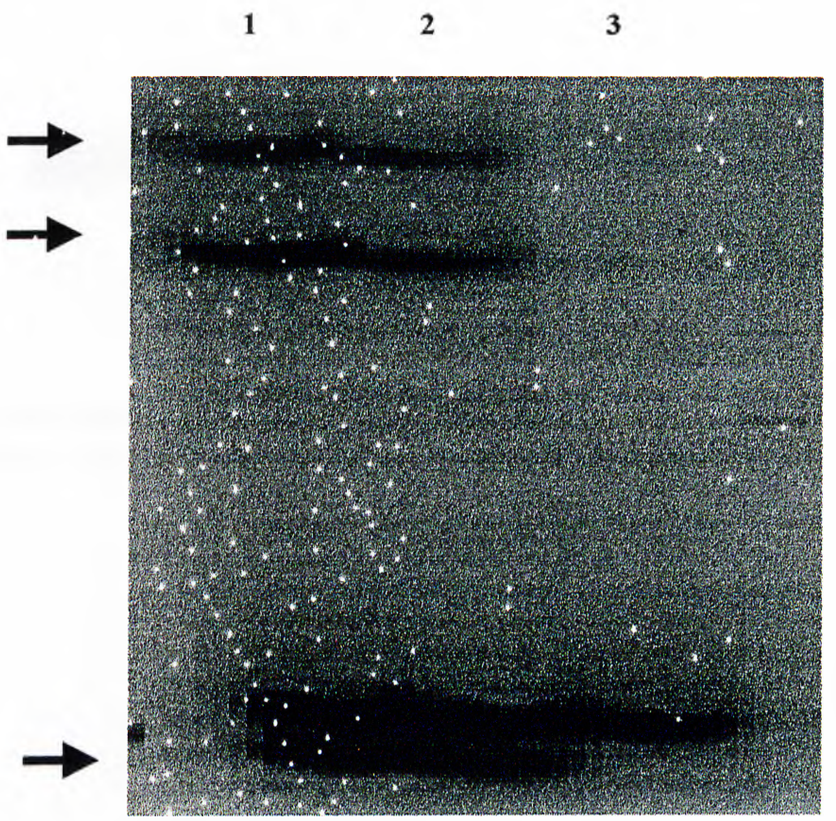


Figure 40. Heteroduplex analysis of *BRCA2* exon 11a-b.
Lanes 1, 97/544; 2, 97/545; 3, control. Arrows indicate shifted fragments.

3.3.19. *BRCA2* exon 11c

BRCA2 exon 11c is amplified with primers cF and cR as a fragment of 1534 bp. Results of five male (97/694, 97/695, 98/3, 98/15, 98/59; lanes 1, 2, 3, 4, and 5 respectively) breast cancer cases, and control (lane 6) is shown in figure 41. No shifts were observed in any one of the samples.

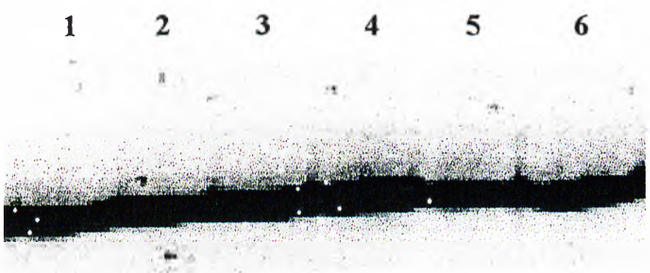


Figure 41. Heteroduplex analysis of *BRCA2* exon 11c
Lane 1, 97/694; 2, 97/695; 3, 98/3; 4, 98/15; 5, 98/59; 6, control.

3.3.20. *BRCA2* exon 11d

BRCA2 exon 11d is amplified with primers dF and dR as a fragment of 931 bp. Results of six male (97/694, 97/695, 98/3, 98/12, 98/15, 98/59; lanes 1, 2, 3, 4, 5, and 6) breast cancer cases, and control (lane 7) sample is shown in figure 42. No shifts were observed in any one of the samples.

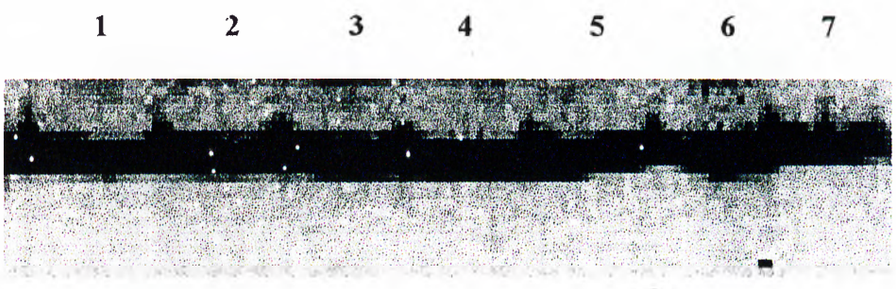


Figure 42. Heteroduplex analysis of *BRCA2* exon 11d
Lanes 1, 97/694; 2, 97/695; 3, 98/3; 4, 98/12; 5, 98/15; 6, 98/59; 7, control.

3.3.21. *BRCA2* exon 11e

BRCA2 exon 11e is amplified with primer eF and eR as a fragment of 782 bp. Results of four male (97/694, 97/695, 98/15, 98/59; lanes 1, 2, 3, and 4 respectively) breast cancer cases, and control (lane 6) sample is shown in figure 43. No shifts were observed in any one of the samples.

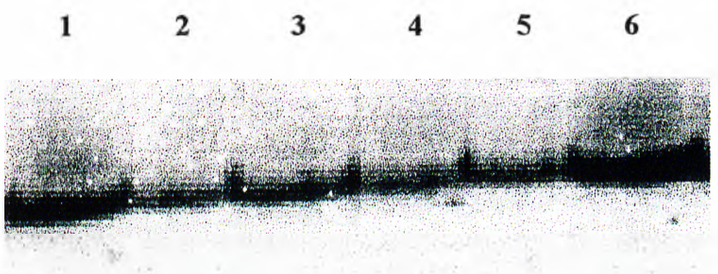


Figure 43. Heteroduplex analysis of *BRCA2* exon 11e
Lanes 1, 97/694; 2, 97/695; 3, 98/3; 4, 98/15; 5, 98/59; 6, control.

3.3.21. *BRCA2* exon 11f

BRCA2 exon 11f was amplified with fF and fR as a fragment of 1253 bp. Results of four male (97/631, 97/694, 97/695; lanes 1, 2, and 3 respectively) breast cancer cases, and control (lane 4) is shown in figure 44. No shifts were observed in any one of the samples.

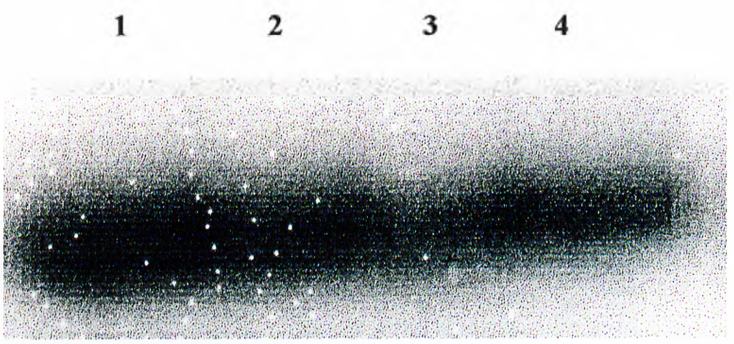


Figure 44. Heteroduplex analysis of *BRCA2* exon 11f.
Lanes 1, 97/631; 2, 97/694; 3, 97/695; 4, control.

3.3.22. *BRCA2* exon 11g

BRCA2 exon 11g was amplified with primers gF and gR as a fragment of 899 bp. Results of three hereditary (96/2, 97/544, 97/545; lanes 1, 2, and 3 respectively) breast cancer cases, and control (lane 4) sample is shown in figure 45. Heteroduplex formation has been observed in patient 96/2 (lane 1).

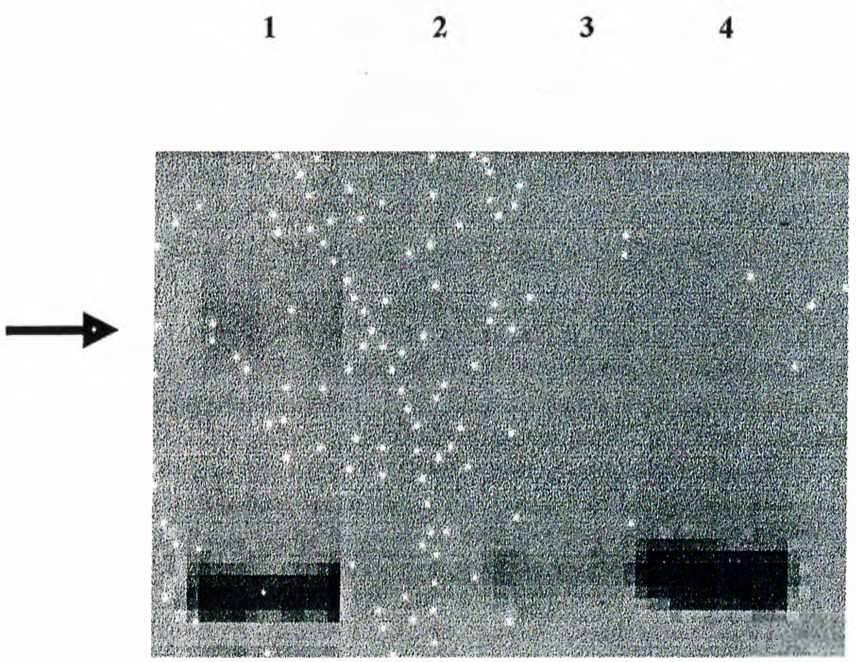


Figure 45. Heteroduplex analysis of *BRCA2* exon 11g.
Lanes 1, 96/2; 2, 97/544; 3, 97/545; 4: control. Arrow indicates the shifted fragment.

3.3.23. *BRCA2* exon 11g-26

BRCA2 exon 11g-26 was amplified with 26F and 26R as a fragment of 290 bp (Figure 46), in order to localize the exact site of the mutation causing the shifted fragment in patient 96/2.

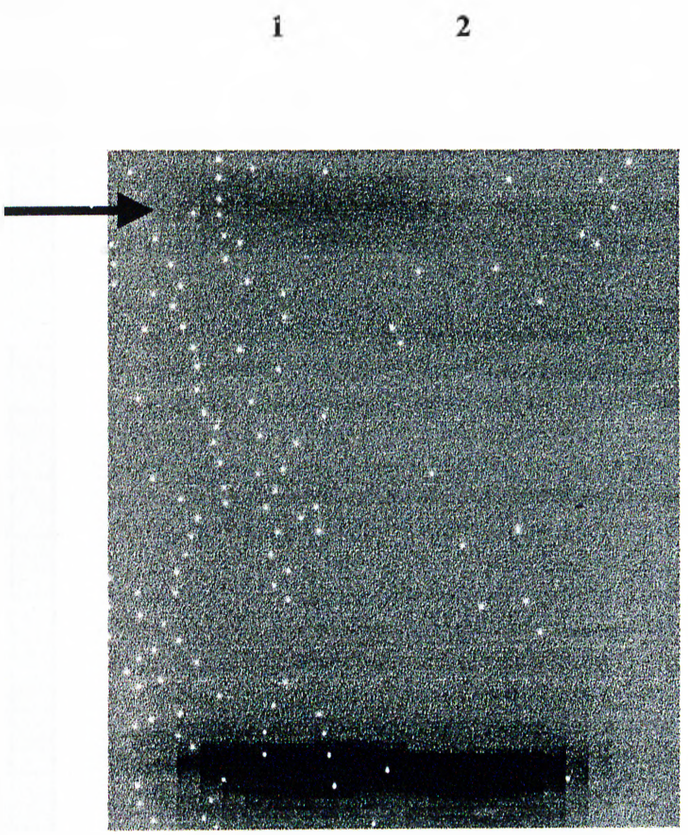


Figure 46. Heteroduplex analysis of *BRCA2* exon 11g-26.
Lanes 1, 96/2; 2, control. Arrow indicate the shifted fragment.

Table 6. Heteroduplex analysis results of BRCA1 and BRCA2 genes in Turkish breast cancer cases*

Group 1			2	5	A	B	C	D	E	F	G	H	I	J	K	13	20.	24.	a	b	c	d	e	f	g
HBC (n=5)			HEREDITARY BREAST CANCER																						
No	Fam	DNA Code																							
1	1	96/1																							
2	2	96/2																							A
3	10	97/137												A											
4	20	97/477												A											
5	22	97/544												A					A	A					
5	22	97/545																	A	A					

Group2			2	5	A	B	C	D	E	F	G	H	I	J	K	13	20.	24.	a	b	c	d	e	f	g
HBC (n=8)			FAMILIAL BREAST CANCER																						
1	3	96/3				A																			
2	4	96/4																							
3	6	96/6																							
3	6	96/7												A											
4	7	96/8																							
5	18	97/474												A											
6	19	97/475												A											
7	48	97/702									A														
8	61	98/60									A														

Group II			2	5	A	B	C	D	E	F	G	H	I	J	K	13	20.	24.	a	b	c	d	e	f	g
EBC (n=27)			EARLY ONSET BREAST CANCER (<35)																						
1	5	96/5												A											
2	8	96/10												A											
6	9	97/114																							
3	11	97/270												A											
7	13	97/343																							
4	14	97/344												A											
5	15	97/359												A											
8	16	97/472																							
9	17	97/473																							
10	21	97/508									A														
11	24	97/632												A											
12	24	97/639																							
13	26	97/641				A																			
14	28	97/655																							
15	29	97/656																							
16	30	97/661																							
17	31	97/662																							
18	34	97/670							A																
19	35	97/674																							
20	36	97/681																							
21	37	97/682				A																			
22	38	97/683																							
23	39	97/684												A											
24	49	97/703																							
25	51	98/12																							
26	56	98/17												A											
27	57	98/18												A											

Group III			2	5	A	B	C	D	E	F	G	H	I	J	K	13	20..	24..	a	b	c	d	e	f	g
MBC (n=10)			MALE BREAST CANCER																						
1	23	97/631																							
2	42	97/694																							
3	43	97/695																							
4	47	97/699																							
5	50	98/3																							
6	52	98/13																							
7	53	98/14																							
8	54	98/15																							
9	55	98/16																							
10	60	98/59																							

*Table describing heteroduplex analysis results of *BRCA1* [Exons 2, 5, 11(A, B, C, D, E, F, G, H, I, J, K), 13, 20, 24 and BRCA2 [Exon 11 (a, b, c, d, e, f, g)]genes in 60 Turkish breast cancer patients. "A" indicates altered fragments.

3.4. Cloning

Approximately 40% of *BRCA1* and *BRCA2* mutations are frameshift type. DNA sequencing of PCR products bearing frameshift mutations are difficult to analyze because of the presence of two different framed alleles. In order to facilitate the sequence analysis and to be able to clearly identify these kind of mutations most of the patients' shifted fragment were reamplified and cloned into pGEM TEAsy vector. DNA sequencing of several clones permit to identify the wild type and mutant alleles separately.

3.4.1. Mini prep DNA Isolation

Plasmid DNA samples were isolated from white colonies which are expected to have the inserted fragment. Mini prep DNA isolation was done by alkaline lysis procedure followed by phenol chloroform extraction. All extracted DNA samples were evaluated quantitatively and qualitatively by spectrophotometry and agarose gel electrophoresis. The concentrations of the mini prep DNA samples on figure 47 were 100 ng/µl, 150 ng/µl, 250 ng/µl, 215 ng/µl, 200 ng/ml, 155 ng/µl, 185 ng/µl respectively.

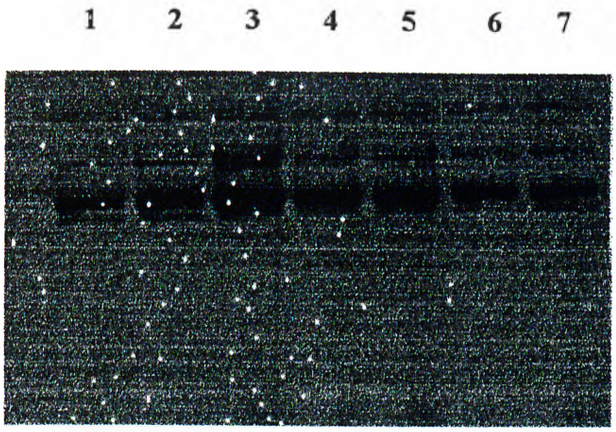


Figure 47. Agarose gel electrophoresis of extracted mini prep DNA samples of 97/641 *BRCA1* exon 11B.
Lanes 1, clone 1; 2, clone 2; 3, clone 3; 4, clone 4; 5, clone 5; 6, clone 6; 7, clone 7.

3.4.2. Restriction Endonuclease Analysis

In order to select plasmids containing the insert, mini prep DNA samples of white colonies were further digested with EcoRI restriction endonuclease from each site of the insert. Presence of the insert with an expected size fragment was confirmed by agarose gel electrophoresis of digested products (Figure 48).

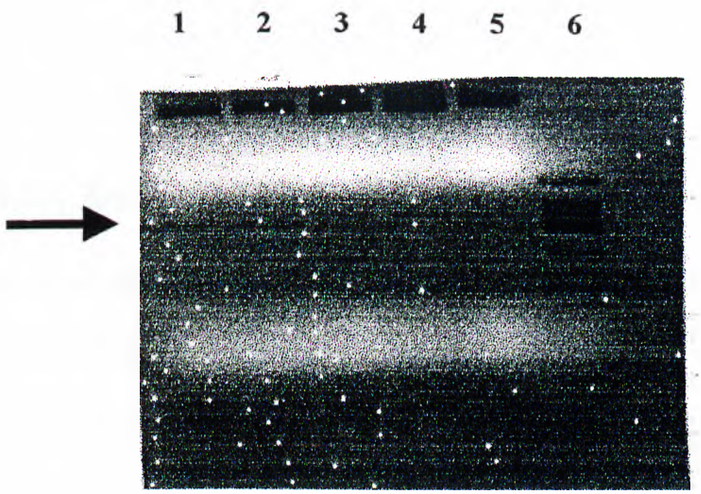


Figure 48. Restriction endonuclease analysis of 97/641 mini prep DNA samples.
Lanes 1, clone1; 2, clone 2; 3, clone 3; 4, clone 4; 5, clone 5; 6, ϕX174/*Hinf*I molecular weight marker. Arrow indicate the excised insert from pGEM TEasy vector.

3.4. DNA Sequence Analysis

3.4.1. Hereditary Breast and/or Ovarian Cancer

In this group of six patients selected on the basis of having at least two affected first degree relatives and those with bilateral breast cancer plus one affected first degree relative were studied. Two *BRCA2* alterations were found in two families.

Heteroduplex analysis of family 22 revealed shifts in both *BRCA2* exon 11 a and b. Further analysis localized this shift to the overlapping 274 bp region of these two fragments in the father (97/544) and daughter (97/545). Sequencing analysis of PCR products revealed very ambigous results indicating an insertion or deletion type mutation (Data not shown). Thus, in order to determine the exact mutation, PCR products of 97/544 and 97/545 were cloned into pGEM Teasy vector. Several clones were sequenced with both forward and reverse primers. DNA sequencing results showed a four base pair deletion (AAAC) starting at nucleotide 3034, which leads to protein truncation at codon 958 (Figure 49). This mutation which was previously reported by Phelan and co-workers (Phelan *et al*, 1996) had occurred in a repeat region frequently involved in DNA polymerase slippage errors causing frameshifts.

Heteroduplex analysis of 96/2 showed a shifted fragment in *BRCA2* exon 11g. Further analysis revealed that this shift was the result of an alteration that was present in the last three portion of this fragment. Sequencing analysis revealed that there was a G insertion

at nucleotide 6880 resulting in a premature protein product that was terminated at codon 2224 (Figure 50).

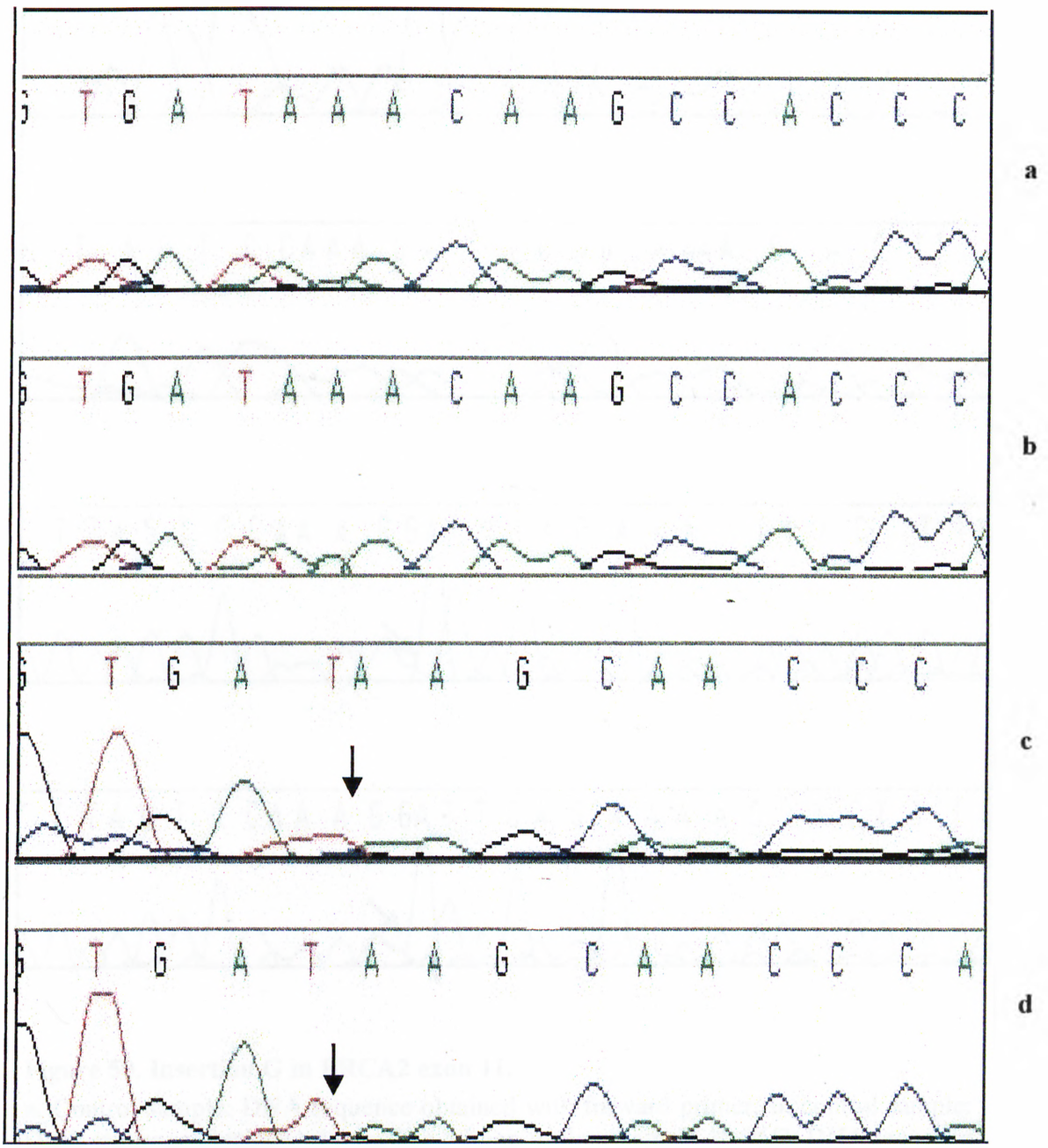


Figure 49. Deletion AAAC in BRCA2 exon 11.

a: Control sample: DNA sequence obtained with forward primer, **b:** control sample: DNA sequence obtained with reverse primer, **c:** clone sample of 97/544: DNA sequence obtained with forward primer, **d:** clone sample of 97/544: DNA sequence obtained with reverse primer. Arrows indicate the four bp deletion at nucleotide 3034.

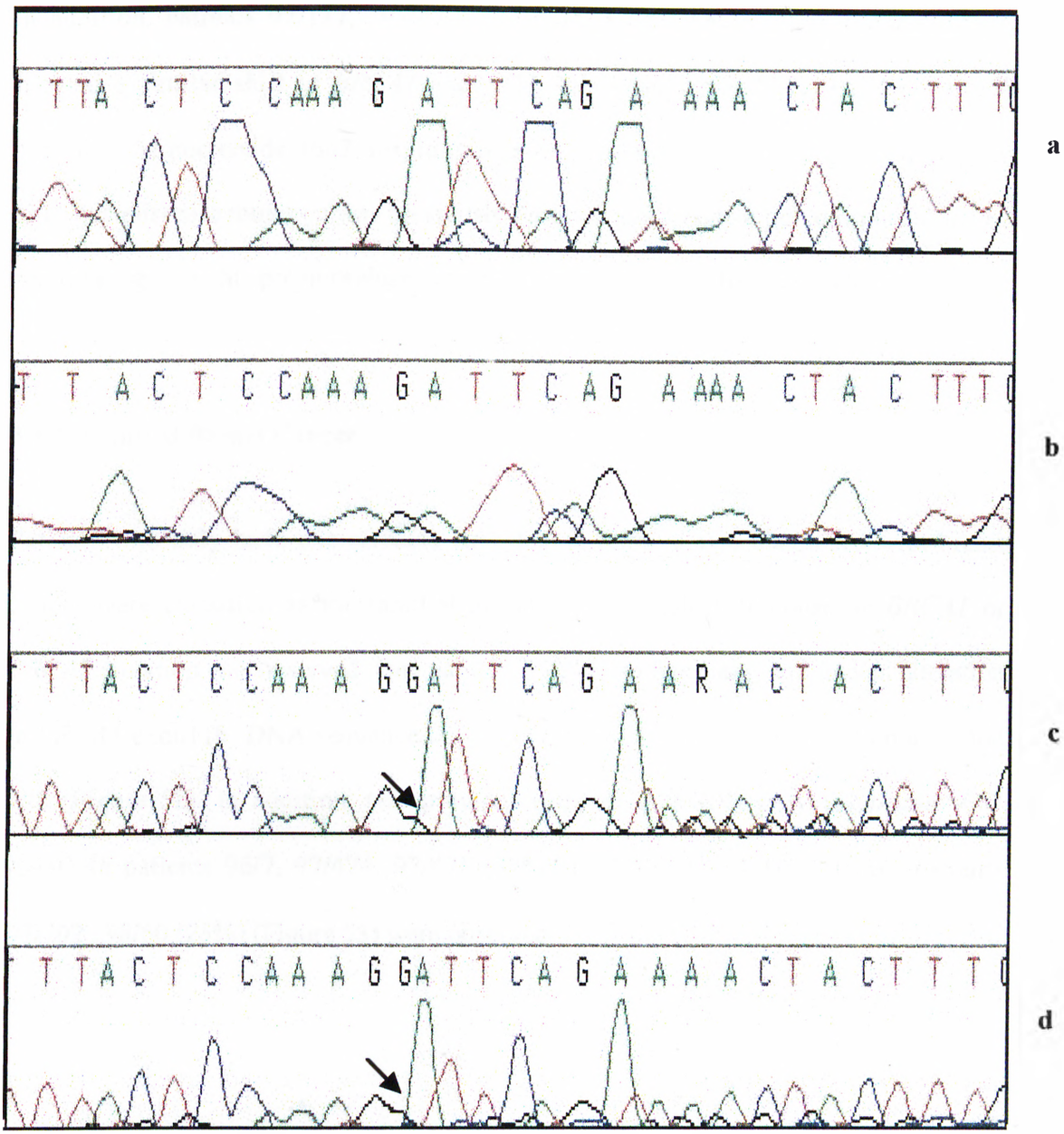


Figure 50. Insertion G in BRCA2 exon 11.

a. Control sample: DNA sequence obtained with forward primer, **b.** control sample: DNA sequence obtained with reverse primer, **c.** clone sample of 96/2: DNA sequence obtained with forward primer, **d.** clone sample of 96/2: DNA sequence obtained with reverse primer. Arrows indicate the G insertion at nucleotide 6880.

In addition, patients 97/137, 97/477, and 97/544 who belong to hereditary group showed a similar shift in *BRCA1* exon 11J. DNA sequencing revealed an A→G transition at nucleotide 3667, resulting in a substitution of lysine for arginine (Figure 51). This missense mutation was previously reported as a frequent polymorphism. In our hereditary group, this polymorphism was found in three out of five cases (60%).

3.4.2. Familial Breast Cancer

Patients who only have one affected first degree relative also diagnosed with breast cancer were classified as the familial group. No frameshift or nonsense *BRCA1* or *BRCA2* mutation was observed. One patient (96/8) from familial group had an alteration in *BRCA1* exon11b. DNA sequence analysis revealed a novel silent mutation at codon 298 (Figure 52). In addition, two previously reported *BRCA1* polymorphisms 3667 A→G in patients 96/7, 97/474, 97/475 (37%) and 2731 C→T (P871L) in patients 97/702 , 98/60 (25%) (Figure 53) were observed.

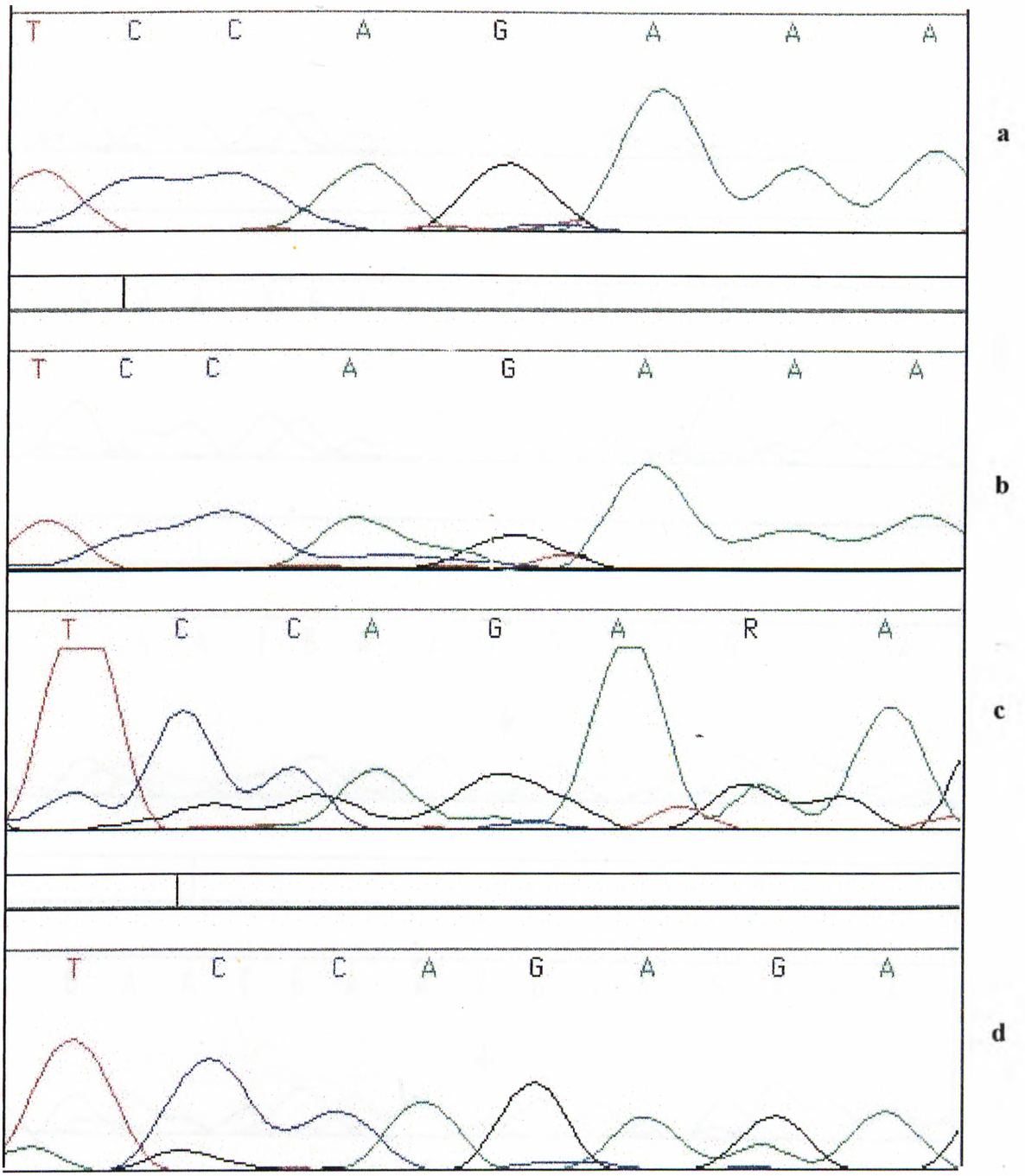


Figure 51. K1183R polymorphism in *BRCA1* exon 11.

a. Control sample: DNA sequence obtained with forward primer, **b.** control sample: DNA sequence obtained with reverse primer, **c.** sample 96/10: DNA sequence obtained with forward primer, **d.** sample 96/10: DNA sequence obtained with reverse primer. Arrows indicate A→G transition at nucleotide 3667.

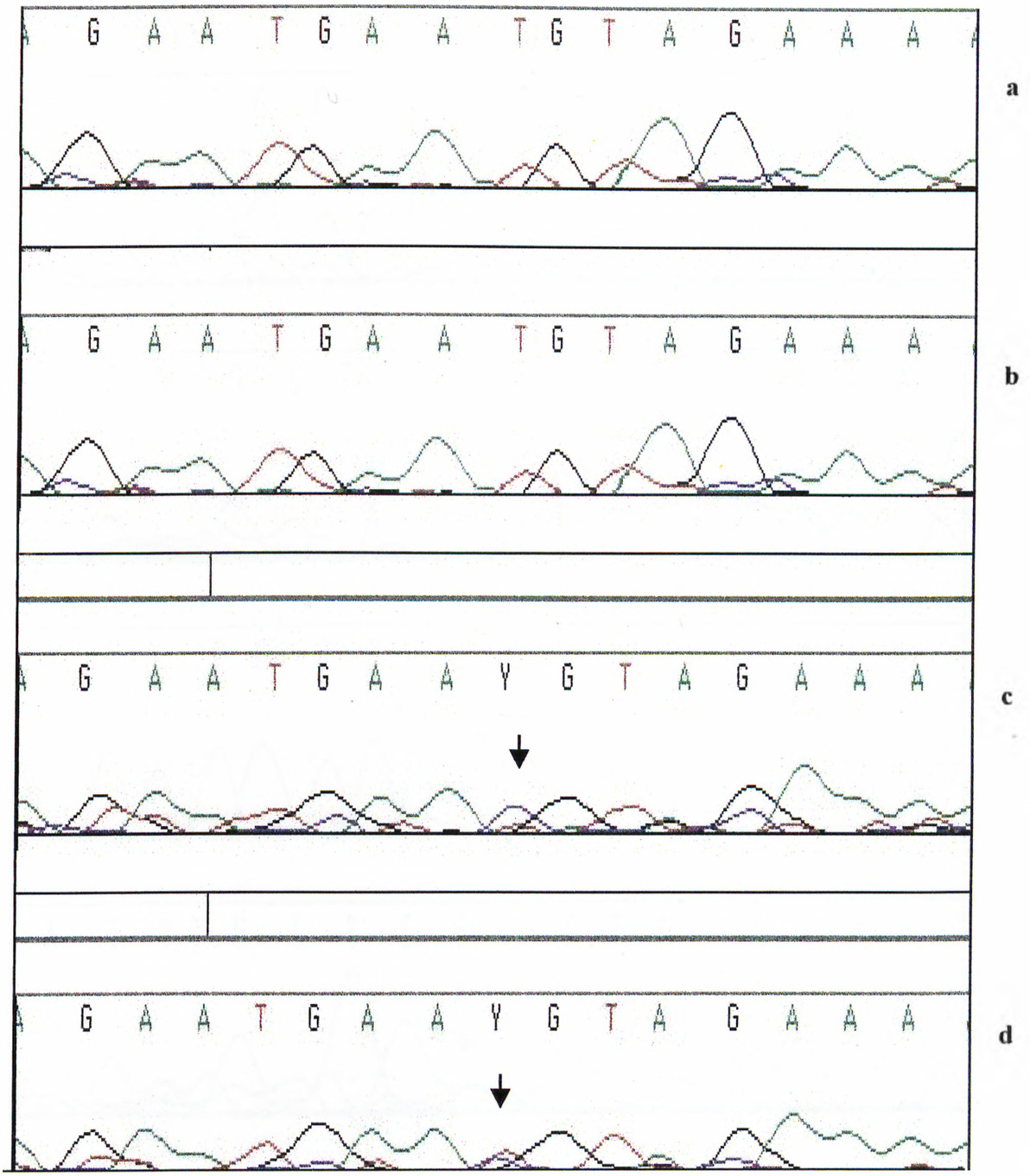


Figure 52. N298N silent mutation in *BRCA1* exon 11.

a. Control sample: DNA sequence obtained with forward primer, **b.** control sample: DNA sequence obtained with reverse primer, **c.** sample 96/8: DNA sequence obtained with forward primer, **d.** sample 96/8: DNA sequence obtained with reverse primer. Arrows indicate T→C transition at nucleotide 1013.

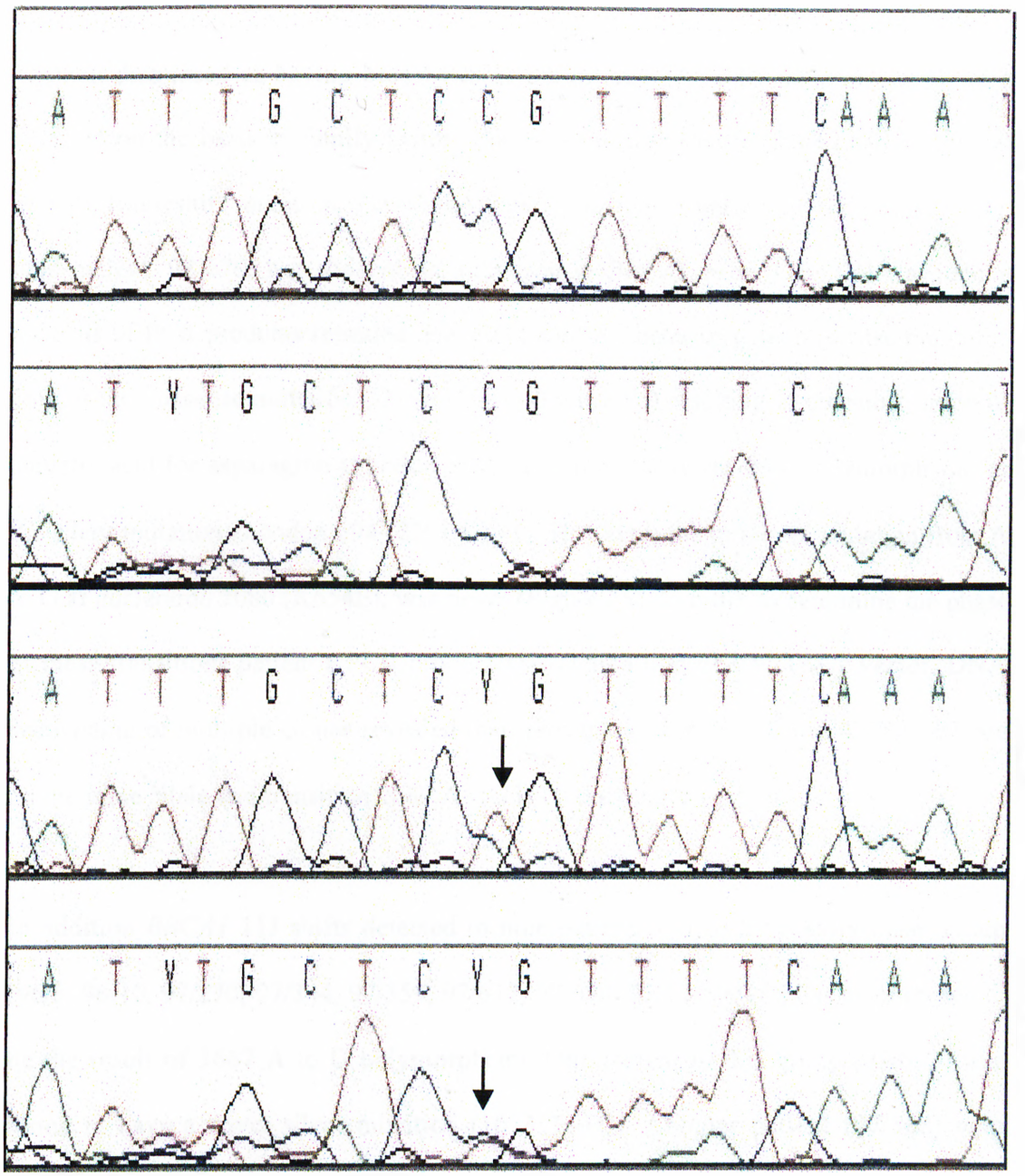


Figure 53. P871L polymorphism in *BRCA1* exon11.

a. control sample: DNA sequence obtained with forward primer, **b.** control sample: DNA sequence obtained with reverse primer. Arrows indicate C→T transition at nucleotide 2731, **c.** sample 97/359: DNA sequence obtained with forward primer, **d.** sample 97/359: DNA sequence obtained with reverse primer.

3.4.3. Early Onset Breast Cancer

Women diagnosed as having breast cancer before age 35 years, none of whom were selected on the basis of family history status were in the early onset group. A novel *BRCA1* frameshift mutation, 1200 insA was identified in patient 97/641 (Figure 54). One patient, 97/670, has showed an alteration in *BRCA1* exon 11E. DNA sequence analysis of PCR products revealed one silent and two heterozygous missense mutation. One of the missense mutations, 2196 G→A (Figure 56) resulting in the substitution of aspartic acid for asparagine at codon 693 was a previously reported polymorphism, as the silent mutation at codon 694 (2201 C→T). However, the missense mutation from A to G at nucleotide 2080 (K654E), was novel (Figure 55). In order to determine the phase of these transitions patient's PCR product was cloned into pGEM Teasy vector. DNA sequencing of multiple clones revealed that transitions 2196 G→A and 2201 C→T are on the same allele and transition 2080 A→G is on the other allele.

In addition *BRCA1* 11J shifts detected in nine patients (33%) from early onset group (96/5, 96/10, 97/270, 97/344, 97/359, 97/632, 97/684, 98/17, 98/18), has been shown to be the result of 3667 A to G polymorphism. One patient in this group (97/682) was found to have a novel silent mutation (1013 T→C). Another patient (97/508) who showed an altered fragment in *BRCA1* exon 11G, was shown to carry the 2731 C→T polymorphism.

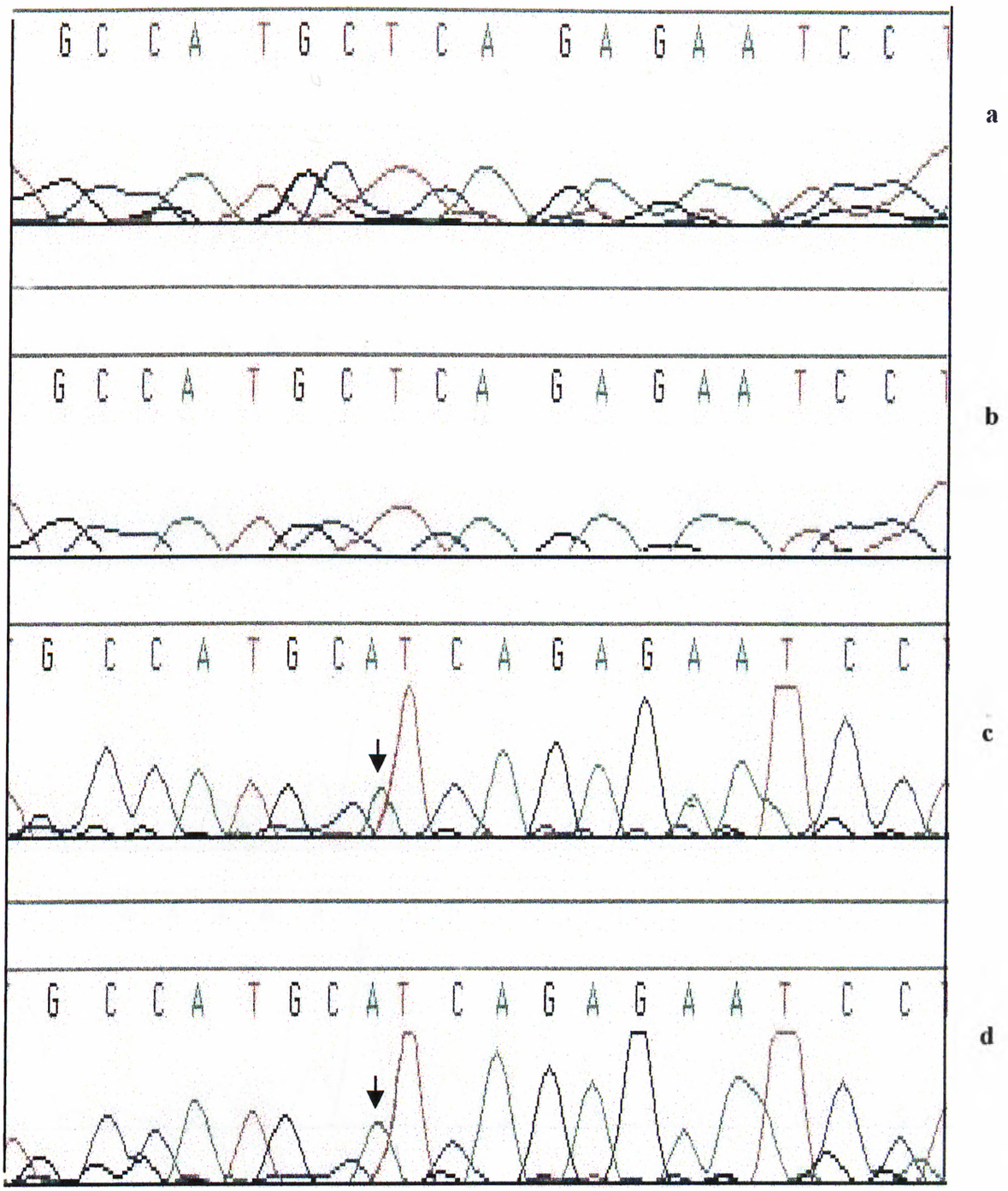


Figure 54. Insertion A in *BRCA1* exon 11.

a. Control sample: DNA sequence obtained with forward primer, **b** control sample: DNA sequence obtained with reverse primer., **c** sample 97/641: DNA sequence obtained with forward primer, **d.** sample 97/641: DNA sequence obtained with reverse primer. Arrows indicate the A insertion at nucleotide 1200.

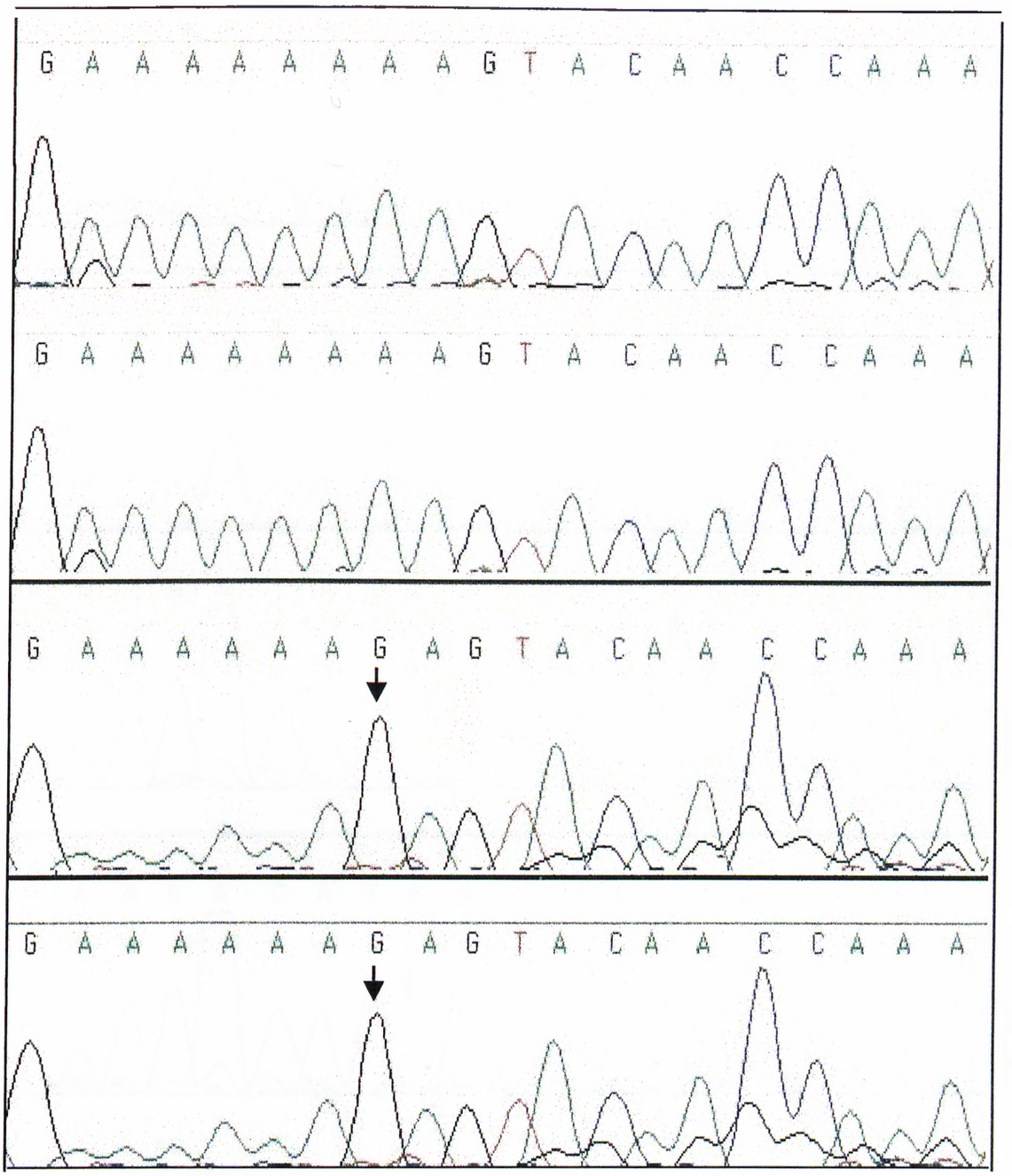


Figure 55. K654E missense mutation in *BRCA1* exon11.

a. Control sample: DNA sequence obtained with forward primer, **b.** control sample: DNA sequence obtained with reverse primer, **c.** clone sample 97/670: DNA sequence obtained with forward primer, **d.** clone sample 97/670: DNA sequence obtained with reverse primer. Arrows indicate A→ G transition at nucleotide 2080

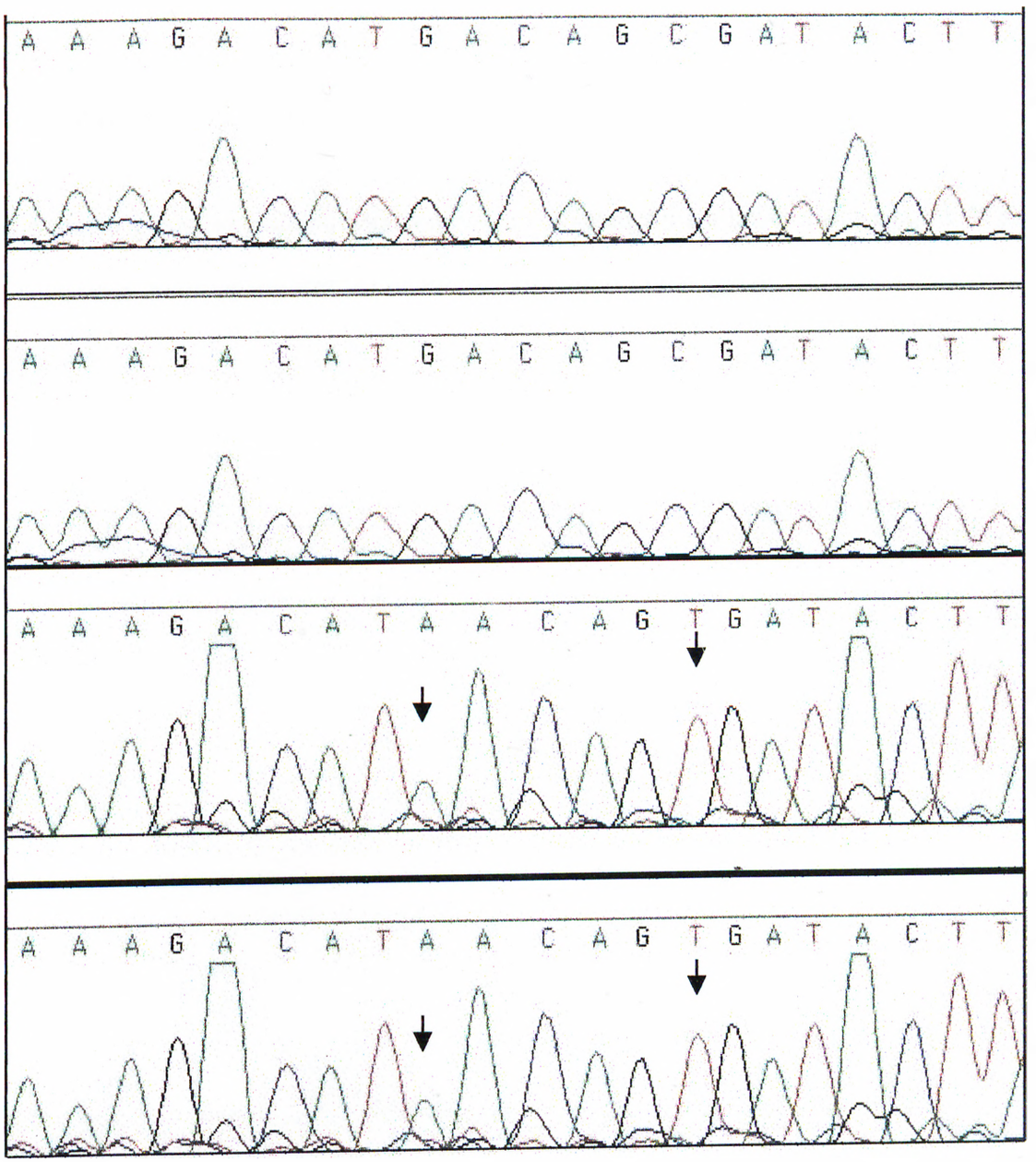


Figure 56. D693N polymorphism and S694S silent mutations in *BRCA1* exon11.

a. Control sample: DNA sequence obtained with forward primer, **b.** control sample: DNA sequence obtained with reverse primer, **c.** sample 97/670: DNA sequence obtained with forward primer, **d.** sample 97/670: DNA sequence obtained with reverse primer. Arrows indicate G→A and C→T transitions at nucleotides 2196 and 2201 respectively.

3.4.4. Male Breast Cancer

The fourth group we studied was composed of isolated male breast cancer cases. We were unable to detect any *BRCA1* or *BRCA2* mutations in this group.

4. DISCUSSION

Breast cancer is one of the most common malignancies affecting women. Inherited gene mutations may be responsible for 5-10% of breast cancer cases (Miki *et al*, 1994; Thorlacius *et al.*, 1998; Feunteun *et al.*, 1999). In Turkish women, breast cancer is among the most common malignancies (Karaoguz and Içli, 1993). The frequency and the types of germ-line mutations leading to breast/ovarian cancers in the Turkish population is not well known. In this study, germ-line *BRCA1* and/or *BRCA2* mutations were screened in 50 breast and/or ovarian cancer patients. We employed a *BRCA1* and *BRCA2* mutation screening strategy which is based on the analysis of exons that have been reported to harbor majority of the previously reported mutations in these genes. Selected regions of *BRCA1* (exons 2, 5, 11, 13, 20 & 24), and *BRCA2* (exon 11) were subjected to heteroduplex analysis, and altered fragments were further analyzed by DNA sequencing. According to the Breast Information Core database, these *BRCA1* and *BRCA2* regions harbor 80% and 45% of the mutations respectively. Nine different sequence alterations (seven for *BRCA1* and two for *BRCA2*) were identified in 25 patients. In *BRCA2*, one previously reported deletion (3034 delAAAC), and one novel insertion (6880 insG) – type of frameshift mutations leading to protein truncation were observed. In *BRCA1*, one novel frameshift mutation (1200 insA), one novel unclassified variant (2080 A→G; K654E), and one novel silent mutation (1013 T→C; N298N) were

identified. In addition, four previously reported *BRCA1* polymorphisms (three missense and one silent mutations) were also found in these Turkish breast cancer patients (see Table 1) (Özdag *et al.*, in press).

The patients were divided into four groups of hereditary (n=6) breast/ovarian cancer, and familial (n=7), early onset (n=27) or male (n=10) breast cancer. Both *BRCA1* and *BRCA2* genes were analyzed in all the groups.

Table 7. Mutations and polymorphisms identified in Turkish breast and breast-ovarian cancer patients.

# of time Mutation Recorded	Gene	Exon	NT	Codon	Base Change	AA Change	Designation	Mutation Type	Mutation Effect
1	BRCA2	11	3034	938	del AAAC	Stop 958	3034 delAAAC	F	F
1	BRCA2	11	6880	2218	ins G	Stop 2224	6880 insG	F	F
1	BRCA1	11	1200	361	ins A	Stop 368	1201 insA	F	F
1	BRCA1	11	2080	654	A to G	Lys to Glu	K654E	M	M
1	BRCA1	11	2196	693	G to A	Asp to Asn	D693N	M	P
3	BRCA1	11	2731	871	C to T	Pro to Leu	P871L	M	P
16	BRCA1	11	3667	1183	A to G	Lys to Arg	K1183R	M	P
2	BRCA1	11	1013	298	T to C	Asn to Asn	N298N	P	P
1	BRCA1	11	2201	694	C to T	Ser to Ser	S694S	P	P

F, frameshift; M, missense; P, polymorphism; UV, unclassified variant.

4.1. Mutation Screening

Mutation screening of *BRCA1* and *BRCA2* genes are rather difficult because of their size and the wide variety of mutations all along the exons. There are several indirect screening methods used frequently by different groups, such as HA, SSCP, PTT and DHPLC. Among them DHPLC is the most sensitive indirect screening method. Its sensitivity is almost 100%, however it needs special and expensive equipment. PTT is one of the most suitable technique for *BRCA1* and *BRCA2* genes due to a large number of protein truncating type of mutations. It is 100% sensitive in truncating mutation detection and allows to screen kilobase long fragments. The sensitivity of HA and SSCP is nearly 100% in detecting deletion-insertion type of mutations. However their sensitivity is approximately 70% in detecting point mutations. SSCP allows to screen fragments up to 300bp. Its sensitivity decreases fragments over 300bp long.

Since it is an inexpensive, rapid method and turned out to be sensitive enough for screening long fragments, HA has been used as the main indirect screening method in this study. Although its sensitivity in detecting point mutations is 70%, this technique allowed us to screen fragments upto 1250bp. We could detect deletion-insertion type mutations in fragments over one kb. Since 40% of reported mutations are deletion-insertion type mutation, HA which does not need any special equipment or expensive kits, was found to be rather efficient. In addition, with this technique it is possible to analyze up to 200 samples on a 45X38 sequencing grade polyacrylamide gel electrophoresis apparatus by loading the samples sequentially.

Following this screening, identification of mutations was done by automated DNA sequencing. We would like to note that it is rather difficult to identify frameshift mutations are hardly identified by automated DNA sequencing since the results are based on the sequencing of both alleles of an individual. Although it is possible to define the first nucleotide affected by the mutation, a very ambigous peak pattern dominates the results. In order to overcome this difficulty, we cloned and sequenced the alleles separately.

4.2.Hereditary Breast Cancer

In the hereditary group which consists of probands with at least two affected first degree relatives, and those with bilateral breast cancer plus one affected first degree relative (Table 2), we identified two *BRCA2* frameshift mutations, 6880 insG (Figure 46 & 50) and 3034 delAAAC (Figure 40 & 49), in two of the six families (33%). Interestingly no *BRCA1* mutation was found in anyone of the patients except for a previously reported frequent polymorphism which is an A to G transition at nucleotide 3667 in three (50%) (Figure 33 & 51) families.

Table 8. Phenotypes and mutations identified in Turkish Hereditary, and Familial breast and breast-ovarian cancer groups.

Group	Family	Mutation	Predicted effect	Age of proband	Relatives with breast &ovarian cancer 1st degree	2nd degree	3rd degree	Other cancers in the relatives
Hereditary	1	-	-	45	2 (52, 51)	1 (52)	2	Liver (54)
Cancer	[1]2	BRCA2 6880insG	Stop2224	25*	1 (25)	-	-	-
	10	-	-	32*	1 (62)	-	1 (35)	Colon (?)
	18	-	-	37	1 (63)	1 (55)	1 (45)	
	20	-	-	53**	3 (57, 60)	-	-	Endometrium (58)
	22	BRCA2 3034delAAAC	Stop958	76	2 (42, 77)	-	-	Stomach (68)
Familial	3	-	-	52	1 (39)	-		Lung(?). gall bladder(?), liver(?),leukemia(?)
Cancer[2]	4	-	-	50	1 (29)	-	-	-
	6	-	-	51	1 (35)	-	-	Liver (?)
	7	-	-	37	1 (41)	-	-	Colon (63)
	19	-	-	38	1 (55)	1 (40)	1 (40)	-
	48	-	-	31	1 (63)	-	1 (45)	-
	61	-	-	36	1 (50)	-	-	Colon (35, 48)

*Bilateral breast cancer. ** Breast and ovarian cancer. The numbers in parentheses indicate age at diagnosis.
[1]Hereditary group consisted of probands with at least two affected first degree relatives, and those with bilateral breast cancer and one affected first degree relative. [2]Patients who have one affected first degree relative diagnosed with breast cancer were in the familial group

One of the *BRCA2* mutations identified in this group is not reported previously. This novel insertion type mutation was found in patient 96/2 (Figure 18). The insertion of G at nucleotide 6880 causes a protein truncation signal at codon 2224. The other *BRCA2* mutation, the deletion of AAAC at nucleotide 3034, was identified in patients 97/545 (44 years old) and her father 97/545 (74 years old) (Figure 18). This frameshift mutation, which occurred in an A repeat region, was probably the result of a DNA polymerase slippage error frequently involved in such alterations. This four base pair deletion was first reported by Phelan *et al.*

4.3. Familial Breast Cancer

Patients who have one affected first degree relative also diagnosed with breast cancer constitute the familial group. No frameshift or nonsense *BRCA1* or *BRCA2* mutations was observed. However, previously reported *BRCA1* polymorphisms 3667 A→G was present in three (42%) and 2731 C→T was present in two (28%) (Figure 30 & 53) patients. In addition, a novel silent *BRCA1* mutation (1013 T→C) (Figure 52) was identified in one patient.

4.4. Early Onset Breast Cancer

Women diagnosed as having breast cancer before age 35 years, none of whom were selected on the basis of family history status, were in the early onset group. A novel *BRCA1* frameshift mutation, 1200 insA (Figure 25 & 54), was identified in one patient.

In addition, five different *BRCA1* sequence alterations were observed in 12 patients. These alterations include one novel (1013 T→C), and four previously reported polymorphisms (see Table 1). The 3667 A→G polymorphism was observed in 33% (9/27) of the patients. Interestingly each allele of *BRCA1* displayed a missense mutation in one patient: a previously described rare polymorphism (2196 G→A; D693N) (Figure 28 & 56) and a novel missense mutation (2080 A→G; K654E) (Figure 55). This patient also carried a silent mutation (2201 C→T) co-segregating with the 2196 G→A mutation. The phase of these transitions was determined by cloning of patient's PCR product and sequencing of multiple clones. These experiments established that sequence alterations 2080 A→G and 2196 G→A which lead to amino acid substitutions K654E and D693N respectively were independently inherited. Sequence analysis showed that 2080 A→G mutation was absent in one hundred independent alleles from a control population. The novel K654E substitution is not in a conserved residue but when *BRCA1* sequence is subjected to secondary structure prediction programs SOPMA and GOR4[2] this region forms a short alpha helix, carrying four consecutive lysine residues. Even though Lys to Glu substitution may increase the helix stability, it neutralizes positive charges on the helix which may have an important role for the structure and function of the protein. Occurrence of two mutant alleles in the same patient with a sporadic early onset malignancy (age 26) suggests that these mutations may be involved in the development of breast cancer.

[2] (http://pbil.ibcp.fr/cgi-bin/secpred)

Missense mutations which constitutes 27% of all *BRCA1* mutations are difficult to evaluate for their predicted effect. It is reported that although missense changes with unknown significance were seen in breast cancer cases and controls, no frameshift or nonsense mutations were observed in a group of control women with a first-degree family history (Malone *et al*, 1998). An amino acid change is considered as a missense mutation when is not present in the normal population (Roth *et al*, 1998). But even these criteria are not adequate to clarify the possible effect of a missense mutation.

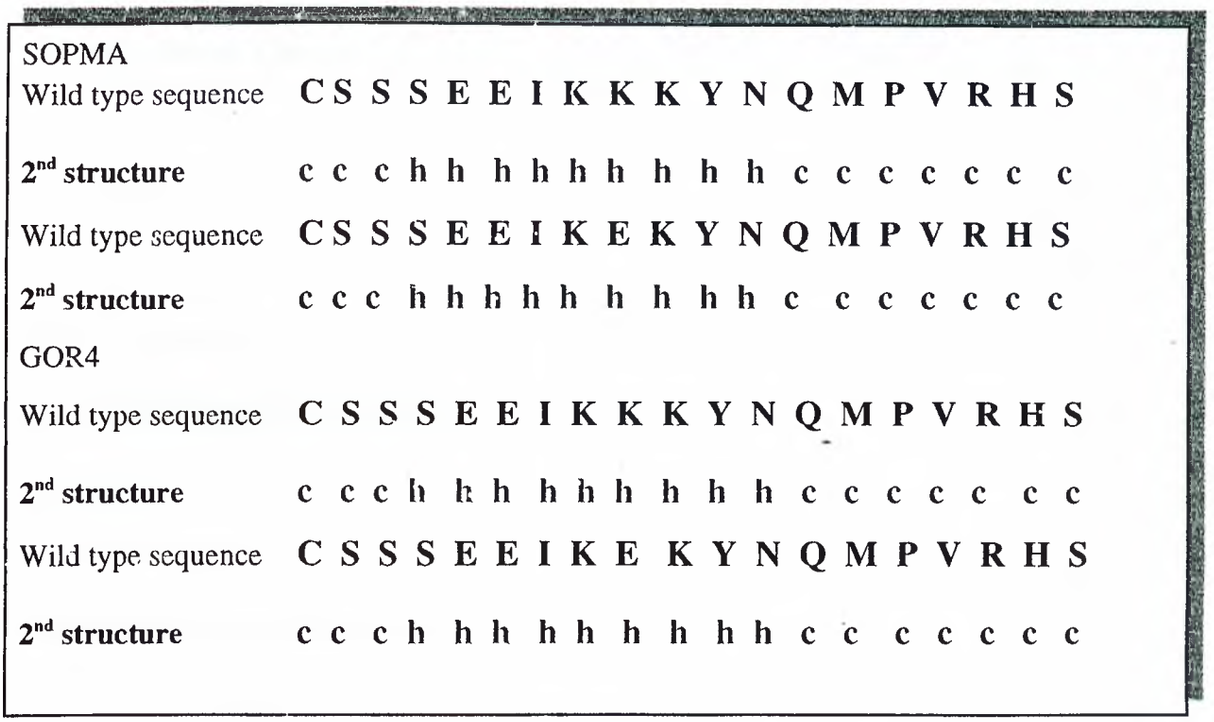


Figure 57. Secondary structure prediction of K654E substitution.

There are several controversial reports on this issue. A missense mutation, Ser1040Asn, reported as a polymorphism by Castilla *et al*, because it was found not to segregate with the disease and was also detected in three of 232 control chromosomes (Castilla *et al*, 1994). However this alteration did segregate with the disease in the family in which it

was identified and was absent in 120 control chromosomes (Friedman *et al*, 1994). Another example was provided by a collaborative survey from Shattuck-Eidens *et al*, reporting an amino acid change, Arg1347Gly, as a missense mutation in a Utah family an stating that it was absent in 156 control chromosomes. However, this same variant was found in a patient studied by the Berkeley group who also had a severe frameshift mutation implying that its functional significance is questionable (Shattuck-Eidens *et al*, 1995).

4.5. Male Breast Cancer

The fourth group we studied was composed of isolated male breast cancer cases. We were unable to detect *BRCA1* or *BRCA2* mutations in this group. In a previous study, *BRCA2* mutations were found in 2% of non-familial male breast cancer cases (Friedman *et al*, 1997). Thus *BRCA2* appears not to contribute significantly to isolated male breast cancer.

4.6. Conclusion and Perspectives

A wide variation in *BRCA1* and *BRCA2* mutation spectrum and frequency has been reported for different populations (Szabo and King, 1997). In our Turkish patients with hereditary breast cancer, *BRCA2* mutations accounted for 33% of the families. This frequency is rather high similar to the Icelandic population. Interestingly, *BRCA1* mutations in our study group appear to be rare - if not exceptional similar to patients

from Iceland, Norway, and Japan. However, in a recently published paper, Balc› *et al.* reported *BRCA1* mutations in two out of five hereditary breast cancer cases as compared to one *BRCA2* mutation (Balc› *et al*, 1999). We did not detect *BRCA1* or *BRCA2* mutations in familial breast cancer cases. Malone *et al* reported that 18% of women diagnosed as having breast cancer before age 35 years, none of whom were selected on the basis of family history status, had germline *BRCA1* mutations. Whereas they found that 7% of women diagnosed before age 45 years and had a first-degree relative with breast cancer had germline mutations in *BRCA1* (Malone *et al*, 1998). These results confirmed the report of Couch *et al* who found a *BRCA1* mutation in 7% of women with a family history of breast cancer or who had early-onset breast cancer themselves. The said group of women was broader than the studies that focus on large families containing many members with breast cancer, ovarian cancer or both. Thus these results indicate that mutation frequency decreased with increasing age of diagnosis and higher proportions of mutations were seen in cases with at least one relative diagnosed as having breast cancer before age 45, in cases with greater numbers of affected relatives, and those with ovarian cancer family history (Couch *et al*, 1997). To the best of our knowledge, *BRCA2* mutations in this group have not been reported previously. Although we did not screen all exons of *BRCA2* our observations suggest that *BRCA2* gene also is infrequently involved in familial breast cancer. The results obtained from this study indicate that *BRCA1* and *BRCA2* genes are involved in the development of some but not all hereditary breast cancers in the Turkish population as reported for other population (Sobol *et al.*, 1994; Serova *et al.*, 1997).

The extent of family history is very important in evaluating the role of *BRCA1* and *BRCA2* genes in breast cancer. In families with six or more breast cancer cases, in the presence of both male and female patients, *BRCA1* and *BRCA2* mutations account for 90% of all cases. Whereas in the presence of only male patients this ratio is 80% (20% *BRCA1* and 60% *BRCA2*). In the presence of breast and ovarian cancer cases in these families *BRCA1* mutations account for 80% and *BRCA2* mutations account for 15% of all cases. However, in families with four or less breast cancer cases *BRCA1* mutations account for 30% and *BRCA2* mutations account only for 5% of all cases (Easton, 1997).

In our hereditary and familial groups four or less breast cancer cases were documented among the family members. Although *BRCA2* mutations seem to be high, the mutation ratio found in our study groups (Table 9) is compatible with the previous reports discussed above.

Table 9. *BRCA1* and *BRCA2* mutation frequencies in different patient groups.

PATIENT GROUPS	*BRCA1*	*BRCA2*
Hereditary breast and/or ovarian cancer (n=8)	0 (0%)	2 (33%)
Familial breast and/or ovarian cancer (n=6)	0 (0%)	0 (0%)
Early onset breast cancer (n=27)	1 (4%)	0 (0%)
Male breast cancer (n=10)	0 (0%)	0 (0%)

In this study we have screened only the selected regions of both genes covering most of the mutations reported to date. Although the results obtained from this research seems to reflect the actual situation, mutation screening of remaining exons of both genes may give a more complete picture about the role of *BRCA1* and *BRCA2* genes in this group of patients. Recent data suggest that *BRCA2* exons other than exon 11 harbor an important number of mutations in males without a family history (Csokay B. *et al*, 1999). Thus screening of the remaining exons of BRCA2 will have an important value especially for the male breast cancer group.

Rearrangement type of mutations which can not be identified by PCR based methods, explain an important part of hereditary breast cancer cases. These kinds of aberrations have been reported several times for BRCA1 (Swensen *et al*, 1997; Puget *et al*, 1997; Puget *et al*, 1999a; Puget *et al*, 1999b; Mazoyer and BRCA1 exon 13 Dup. Scr. Gr., 2000). Thus in order to fully analyze BRCA1 and BRCA2 genes, rearrangement should also be screened.

Besides the arguments about the presence of other putative breast cancer susceptibility genes, the effect of endogenous and exogenous hormones, environmental factors, and genetic polymorphisms in metabolizing enzymes are also very important contributors in completing the picture of breast carcinogenesis. It is thus possible to conclude that cancer is both a multistep and a "multifactorial" disease.

REFERENCES

Alberg, A. J., Visvanathan K., Helzlsouer K. J. (1998) Epidemiology, prevention, and early detection of breast cancer. *Curr Op Oncol* **10**, 492-7.

Ambrosone, C. B., Coles, B. F., Freudenheim, J. L., Shields, P. G. (1999) Glutathione-S-transferase (GSTM1) genetic polymorphisms do not affect human breast cancer risk, regardless of dietary antioxidants. *J Nutr* **129** (2S Suppl), 565S-568S

Andersen, T. I., Borresen, A. L., and Moller, P. (1996). A common BRCA1 mutation in Norwegian breast and ovarian cancer families? [letter]. *Oncogene* **13**, 649-53.

Andersen T. I., Holm R., Nesland J. M., Heimdal K. R., Ottestad L., Borresen A. L. (1993). Prognostic significance ofTP53 gene mutations in breast cancer. *Br J Cancer* **68**:540-543.

Anderson, S. F., Schlegel, B. P., Nakajima, T., Wolpin, E. S., and Parvin, J. D. (1998). BRCA1 protein is linked to the RNA polymerase II holoenzyme complex via RNA helicase A. *Int J Cancer* 77, 354-60.

Andres, J. L., Fan, S., Turkel, G. J., Wang, J. A., Twu, N. F., Yuan, R. Q., Lamszus, K., Goldberg, I. D., and Rosen, E. M. (1998). Regulation of BRCA1 and BRCA2 expression in human breast cancer cells by DNA-damaging agents. *Oncogene* **16**, 2229-41.

Bacus S. S. Zelnick C. R., Plowman G., Yarden Y. (1994). Expression of the erb-2 family of growth factor receptors and their ligands in breast cancer. *Am J Clin Pathol* **102**, S13-16.

Balc›, A., Huusko, P., Paakkonen, K., Launonen, V., Uner, A., Ekmekci, A., and Winqvist, R. (1999). Mutation analysis of BRCA1 and BRCA2 in Turkish cancer families: a novel mutation BRCA2 3414del4 found in male breast cancer. *Eur J Cancer* **35**, 707-10.

Bertwistle, D., Swift, S., Marston, N. J., Jackson, L. E., Crossland, S., Crompton, M. R., Marshall, C. J., and Ashworth, A. (1997). Nuclear location and cell cycle regulation of the BRCA2 protein. *Science* **278**, 1874.

Bonnier, P., Romain, S., Dilhuydy, J. M., Bonichon, F., Julien, J. P., Charpin, C., Lejeune, C,. Martin, P. M,. Piana, L. (1997) Influence of pregnancy on the outcome of breast cancer: a case-control study. Societe Francaise de Senologie et de Pathologie Mammaire Study Group. *Int J Cancer* **72**(5), 720-7

Bork, P., Blomberg, N., and Nilges, M. (1996). Internal repeats in the BRCA2 protein sequence [letter]. *Nat Genet* **13**, 22-3.

Borresen, A. L., Andersen, T. I., Garber, J., Barbier-Piroux, N., Thorlacius, S., Eyjford J., Ottestad, L., Smith-Sorrensen, B., Hovig, E., Malkin, D., Friends, S. H. (1992) Scroeening for germline mutations in breast cancer patients. *Cancer Res* **52**, 3234-3236.

Boyd, M., Harris, F., McFarlane, R., Davidson, H. R., and Black, D. M. (1995). A human BRCA1 gene knockout [letter]. *Genomics* **28**, 25-31.

Burck KB, Levitt LJ (1986) Immunophenotypic transformation from acute undifferentiated leukemia to Burkitt's-like acute lymphoblastic leukemia. *Am J Med* 81(3):551-4.

Callebaut, I., and Mornon, J. P. (1997). From BRCA1 to RAP1: a widespread BRCT module closely associated with DNA repair. *FEBS Lett* **400**, 25-30.

Castilla, L. H., Couch, F. J., Erdos, M. R., Hoskins, K. F., Calzone, K., Garber, J. E., Boyd, J., Lubin, M. B., Deshano, M. L., Brody, L. C., and *et al.* (1994). Mutations in the BRCA1 gene in families with early-onset breast and ovarian cancer. *Nat Genet* **8**, 387-91.

Chen, Y., Chen, C. F., Riley, D. J., Allred, D. C., Chen, P. L., Von Hoff, D., Osborne, C. K., and Lee, W. H. (1995). Aberrant subcellular localization of BRCA1 in breast cancer [see comments] [published erratum appears in Science 1995 Dec 1;270(5241):1424]. *Science* **270**, 789-91.

Chapman, M. S., and Verma, I. M. (1996). Transcriptional activation by BRCA1 [letter; comment]. *Nature* **382**, 678-9.

Charrier, J., Maugard, C. M., Le Mevel, B., Bignon, Y.J. (1999) Allelotype influence at glutathione S-transferase M1 locus on breast cancer susceptibility. *Br J Cancer* 79(**2**):346-53

Chen, Y., Farmer, A. A., Chen, C. F., Jones, D. C., Chen, P. L., and Lee, W. H. (1996). BRCA1 is a 220-kDa nuclear phosphoprotein that is expressed and phosphorylated in a cell cycle-dependent manner [published erratum appears in Cancer Res 1996 Sep 1;56(17):4074]. *Cancer Res* **56**, 3216-9.

Clark, G. M., Osborne, C. K., Levitt, D., Wu, F., Kim, N. W. (1997) Telomerase activity and survival of patients with node-positive breast cancer. *J Natl Cancer Inst* **89**(24):1874-81.

Cleton-Jansen A. M., Moerland E. W., Kuipers-Dijkshoorn N.J., Callen D. F., Sutherland G. R., Hansen B., Devilee P., Cornelisse C. J. (1994) At least two different regions are involved in allelic imbalance on chromosome arm 16q in breast cancer. *Genes chromosom Cancer* **9**:101-4.

Coene, E., Van Oostveldt, P., Willems, K., van Emmelo, J., and De Potter, C. R. (1997). BRCA1 is localized in cytoplasmic tube-like invaginations in the nucleus [letter]. *Nat Genet* **16**, 122-4.

Collaborative Group on Hormonal Factors in Breast Cancer. (1997) Breast cancer and hormone replacement therapy: collaborative reanalysis of data from 51 epidemiologic studies of 52705 women with breast cancer and 108411 women without breast cancer. *Lancet* **350**, 1047-59.

Collins, F. S. (1996). BRCA1--lots of mutations, lots of dilemmas [editorial; comment] [see comments]. *N Engl J Med* **334**, 186-8.

Collins, N., McManus, R., Wooster, R., Mangion, J., Seal, S., Lakhani, S. R., Ormiston, W., Daly, P. A., Ford, D., Easton, D. F., and *et al.* (1995). Consistent loss of the wild type allele in breast cancers from a family linked to the BRCA2 gene on chromosome 13q12-13. *Oncogene* **10**, 1673-5.

Cotton, R. G. H. (1993)Current methods of mutation detection. *Mut Res* **285**, 125-44.

Couch, F. J., DeShano, M. L., Blackwood, M. A., Calzone, K., Stopfer, J., Campeau, L., Ganguly, A., Rebbeck, T., and Weber, B. L. (1997). BRCA1 mutations in women attending clinics that evaluate the risk of breast cancer [see comments]. *N Engl J Med* **336**, 1416-21.

Couch, F. J., Farid, L. M., DeShano, M. L., Tavtigian, S. V., Calzone, K., Campeau, L., Peng, Y., Bogden, B., Chen, Q., Neuhausen, S., Shattuck-Eidens, D., Godwin, A. K., Daly, M., Radford, D. M., Sedlacek, S., Rommens, J., Simard, J., Garber, J., Merajver, S., and Weber, B. L. (1996). BRCA2 germline mutations in male breast cancer cases and breast cancer families. *Nat Genet* **13**, 126-8.

Couch, F. J., and Hartmann, L. C. (1998). BRCA1 testing--advances and retreats [editorial; comment]. *Jama* **279**, 955-7.

Crook, T., Brooks, L. A., Crossland, S., Osin, P., Barker, K. T., Waller, J., Philp, E., Smith, P. D., Yulug, I., Peto, J., Parker, G., Allday, M. J., Crompton, M. R., and Gusterson, B. A. (1998). p53 mutation with frequent novel condons but not a mutator phenotype in BRCA1- and BRCA2-associated breast tumours. *Clin Cancer Res* **4**, 2433-7.

Cropp C. S., Champeme M. H., Lindereau R., Callahan R. (1993). Identification of three regions on chromosome 17q in primary humanbreast carcinomas which are frequently deleted. *Cancer Res* **53**, 5617-5621.

Csokay B, Udvarhelyi N, Sulyok Z, Besznyak I, Ramus S, Ponder B, Olah E. (1999) High frequency of germ-line BRCA2 mutations among Hungarian male breast cancer patients without family history. *Cancer Res* 59(5):995-8

Deng G., Chen L.C., Schott D. R., Thor A., Bhargava V., Ljung B. M., Chew K., Smith H. S. (1994). Loss of heterozygosity and p53 gene mutations in breast cancer. *Cancer Res* **54**, 499-505.

Diamandis, E. P. (1996). ... and secreted tumour suppressors [letter; comment]. *Nat Genet* **13**, 268-9; discussion 269-72.

Dorion-Bonnet F., Mautalen S., Hostein I., Longy M. (1995). Allelic imbalance study of 16q in human primary breast carcinomas using microsatellite markers. *Genes Chromosom Cancer* **14**, 171-173

Duenas-Gonzalez, A., Abad-Hernandez, M. M., Cruz-Hernandez, J. J., Gonzalez-Sarmiento, R. (1997) Analysis of bcl-2 in sporadic breast carcinoma. *Cancer***80**(11), 2100-8

Durocher, F., Shattuck-Eidens, D., McClure, M., Labrie, F., Skolnick, M. H., Goldgar, D. E., and Simard, J. (1996). Comparison of BRCA1 polymorphisms, rare sequence variants and/or missense mutations in unaffected and breast/ovarian cancer populations. *Cell Growth Differ* 7, 711-5.

Easton, D., Bishop, D. T., Ford, D., Crockford, G. P., and the Breast Cancer Linkage Consortium. (1993). Genetic linkage analysis in familial breast and ovarian cancer: Results from 214 families. *Am J Hum Genet* **52**, 678-701.

Easton, D. (1997). Breast cancer genes--what are the real risks? [news]. *Nat Genet* **16**, 210-1.

Eisen A., Weber, B. (1998) Recent advances in breast cancer biology. Curr Op Oncol 10, 486-91.

Enger, S. M., Ross, R. K., Henderson, B. E., Bernstein, L. (1997) Breastfreeding history pregnancy expand risk of breast cancer. *Br J Cancer* **76**, 118-23.

Feunteun, J. (1999). La predisposition hereditaire au cancer du sein liee a BRCA1 et BRCA2: une maladie de la reponse aux lesions genotoxique? *M/S* **15**, 38-44.

Ford, D., Easton, D. F., Stratton, M., Narod, S., Goldgar, D., Devilee, P., Bishop, D. T., Weber, B., Lenoir, G., Chang-Claude, J., Sobol, H., Teare, M. D., Struewing, J., Arason, A., Scherneck, S., Peto, J., Rebbeck, T. R., Tonin, P., Neuhausen, S., Barkardottir, R., Eyfjord, J., Lynch, H., Ponder, B. A., Gayther, S. A., Zelada-Hedman, M., and *et al.*

(1998). Genetic heterogeneity and penetrance analysis of the BRCA1 and BRCA2 genes in breast cancer families. The Breast Cancer Linkage Consortium. *Am J Hum Genet* **62**, 676-89.

Friedman, E., Bar-Sade Bruchim, R., Kruglikova, A., Risel, S., Levy-Lahad, E., Halle, D., Bar-On, E., Gershoni-Baruch, R., Dagan, E., Kepten, I., Peretz, T., Lerer, I., Wienberg, N., Shushan, A., and Abeliovich, A. D. (1998). Double heterozygotes for the Ashkenazi founder mutations in BRCA1 and BRCA2 genes [letter]. *C R Seances Soc Biol Fil* **192**, 235-40.

Friedman, L. S., Gayther, S. A., Kurosaki, T., Gordon, D., Noble, B., Casey, G., Ponder, B. A., and Anton-Culver, H. (1997). Mutation analysis of BRCA1 and BRCA2 in a male breast cancer population. *Am J Hum Genet* **60**, 313-9.

Friedman, L. S., Ostermeyer, E. A., Szabo, C. I., Dowd, P., Lynch, E. D., Rowell, S. E., and King, M. C. (1994). Confirmation of BRCA1 by analysis of germline mutations linked to breast and ovarian cancer in ten families. *Nat Genet* **8**, 399-404.

Gayther, S. A., Harrington, P., Russell, P., Kharkevich, G., Garkavtseva R. F., Ponder B. A. J. and the UKCCCR Familial Ovarian Cancer Group. (1996) Rapid detection of regionally clustered germ-line BRCA1 mutations by multiplex heteroduplex analysis. *Am J Hum Genet* **58**, 451-6.

Gayther, S. A., Mangion, J., Russell, P., Seal, S., Barfoot, R., Ponder, B. A., Stratton, M. R., and Easton, D. (1997). Variation of risks of breast and ovarian cancer associated with different germline mutations of the BRCA2 gene. *Clin Chem* **43**, 6-8.

Gayther, S. A., Harrington, P., Russell, P., Kharkevich, G., Garkavtseva, R. F., and Ponder, B. A. (1997). Frequently occurring germ-line mutations of the BRCA1 gene in ovarian cancer families from Russia [letter]. *Am J Hum Genet* **60**, 1242-6.

Glebow, O. K., McKenzie, K. E., White, C. A., Sukumar, S. (1994). Frequent p53 gene mutations and novel alleles in familial breast cancer. *Cancer Res* **54**, 3703-9.

Gowen, L. C., Avrutskaya, A. V., Latour, A. M., Koller, B. H., and Leadon, S. A. (1998). BRCA1 required for transcription-coupled repair of oxidative DNA damage. *Curr Biol* **8**, 877-80.

Gowen, L. C., Johnson, B. L., Latour, A. M., Sulik, K. K., and Koller, B. H. (1996). Brca1 deficiency results in early embryonic lethality characterized by neuroepithelial abnormalities. *Nat Genet* **12**, 191-4.

Gretarsdottir, S., Thorlacius, S., Valgardsdottir, R., Gudlaugsdottir, S., Sigurdsson, S., Steinarsdottir, M., Jonasson, J. G., Anamthawat-Jonsson, K., and Eyfjord, J. E. (1998). BRCA2 and p53 mutations in primary breast cancer in relation to genetic instability. *Cancer Res* **58**, 859-62.

Gudas, J. M., Nguyen, H., Li, T., and Cowan, K. H. (1995). Hormone-dependent regulation of BRCA1 in human breast cancer cells. *Cancer Res* **55**, 4561-5.

Gundmundsson J., Barkardottir R. B., Eirikdottir G., Baldursson T., Arason A.,EgilssonV., Ingvarsson S. (1995). Loss of heterozygosity at chromosome 11 in breast cancer. Association of prognostic facttors with genetic alterations *Br J Cancer* **72**, 696-699.

Haber, D., and Harlow, E. (1997). Tumor suppressor genes: evolving definitions in the genomic age. *Nat Genet* **16**, 320-2.

Haiman, C. A., Hankinson, S. E., Spiegelman, D., Colditz, G.A., Willett, W. C., Speizer, F. E., Kelsey, K. T. (1999) The relationship between a polymorphism in CYP17 with plasma hormone levels and breast cancer. *Cancer Res* **59**(5), 1015-20

Hall, J. M., Newman, M. K., Newman, B., Morrow, J. E., Anderson L. A., Huey B., King, M. C. (1990). Linkage of early-onset familial breast cancer to chromosome 17q21. *Science* **250**, 1684-9.

Hakansson, S., Johannsson, O., Johansson, U., Sellberg, G., Loman, N., Gerdes, A. M., Holmberg, E., Dahl, N., Pandis, N., Kristoffersson, U., Olsson, H., and Borg, A. (1997). Moderate frequency of BRCA1 and BRCA2 germ-line mutations in Scandinavian familial breast cancer [see comments]. *Am J Hum Genet* **60**, 1085-98.

Hamann, U., Becher, H., Zimmermann, T., Pella, K., Bastert, G., and Chang-Claude, J. (1996). German family study on hereditary breast-ovarian cancer. *Lancet* **348**, 408.

Hamann, U., Herbold, C., Costa, S., Solomayer, E. F., Kaufmann, M., Bastert, G., Ulmer, H. U., Frenzel, H., and Komitowski, D. (1996). Allelic imbalance on chromosome 13q: evidence for the involvement of BRCA2 and RB1 in sporadic breast cancer. *Cancer Res* **56**, 1988-90.

Haraldsson, K., Loman, N., Zhang, Q. X., Johannsson, O., Olsson, H., and Borg, A. (1998). BRCA2 germ-line mutations are frequent in male breast cancer patients without a family history of the disease. *Cancer Res* **58**, 1367-71.

Healy, B. (1997). BRCA genes--bookmaking, fortunetelling, and medical care [editorial; comment] [see comments]. *N Engl J Med* **336**, 1464-71.

Hethcote, H. W., Knudson, A. G. Jr (1978) Model for the incidence of embryonal cancers: application to retinoblastoma. *Proc Natl Acad Sci* U S A **75**(5), 2453-7

Hogervorst, F. B., Cornelis, R. S., Bout, M., van Vliet, M., Oosterwijk, J. C., Olmer, R., Bakker, B., Klijn, J. G., Vasen, H. F., Meijers-Heijboer, H., Menko, F. H., Cornelisse, C. J., Dunnen, J. T., Devilee, P., Ommen G. B. (1995). Rapid detection of BRCA1 mutations by the protein truncation test. *Jama* **273**, 1692-3.

Holt, J. T., Thompson, M. E., Szabo, C., Robinson-Benion, C., Arteaga, C. L., King, M. C., and Jensen, R. A. (1996). Growth retardation and tumour inhibition by BRCA1 [see comments] [published erratum appears in Nat Genet 1998 May;19(1):102]. *Nat Genet* **12**, 298-302.

Hulka, B. S., Stark, A. T. (1995) Breast cancer: cause and prevention. *Lancet*, **346**, 883-87.

Inoue, R., Fukutomi, T., Ushijima, T., Matsumoto, Y., Sugimura, T., and Nagao, M. (1995). Germline mutation of BRCA1 in Japanese breast cancer families. *Lancet* **346**, 583.

Jeng M. H., ten Dijke P., Iwata K. K., Jordan V. C. (1993). Regulation of the levels ofthree transforming growth factor beta mRNAs by estrogen and their effect on the proliferation of human breast cancer cells. *Mol Cell Endocrinol* **97**, 115-121.

Jensen, R. A., Thompson, M. E., Jetton, T. L., Szabo, C. I., van der Meer, R., Helou, B., Tronick, S. R., Page, D. L., King, M. C., and Holt, J. T. (1996). BRCA1 is secreted and exhibits properties of a granin [see comments]. *Nat Genet* **12**, 303-8.

Johannsson, O., Ostermeyer, E. A., Hakansson, S., Friedman, L. S., Johansson, U., Sellberg, G., Brondum-Nielsen, K., Sele, V., Olsson, H., King, M. C., and Borg, A. (1996). Founding BRCA1 mutations in hereditary breast and ovarian cancer in southern Sweden. *Am J Hum Genet* **58**, 441-50.

Johnston S. R. D., MacLennon K. A., Sacks N. P. M., Salter J., Smith I. E., Doswett M. (1994). Modulation of Bcl-2 and Ki-67 expression in oestrogen receptor human breast cancer by tamoxifen. *Eur J Cancer* **30A**, 1663-1667.

Karao¤uz H. and Içli F. (1993) Cancer problem in Türkiye.*J. Ankara Med. Sch.* **15**, 547-558.

Katagiri, T., Nakamura, Y., and Miki, Y. (1996). Mutations in the BRCA2 gene in hepatocellular carcinomas. *Cancer Res* **56**, 4575-7.

Khan, S. A., Rogers, M. A., Khurana, K. K., Meguid, M. M., Numann, P. J. (1998) Estrogen receptor expression in benign breast epithelium and breast cancer risk. *J Natl Cancer Inst* **90**(1):37-42

Kinzler, K. W., and Vogelstein, B. (1997). Cancer-susceptibility genes. Gatekeepers and caretakers [news; comment]. *Science* **276**, 531-2.

Koonin, E. V., Altschul, S. F., and Bork, P. (1996). BRCA1 protein products ... Functional motifs... [letter]. *Nat Genet* **13**, 268; discussion 269-72.

Koul, A., Nilbert, M., and Borg, A. (1999). A somatic BRCA2 mutation in RER+ endometrial carcinomas that specifically deletes the amino-terminal transactivation domain. *Genes Chromosomes Cancer* **24**, 207-12.

Krainer, M. (1994) Genetic predisposition for breast cancer: From epidemiologic studies to molecular genetics. *Onkologie* **17**, 470-7.

Lancaster, J. M., Wooster, R., Mangion, J., Phelan, C. M., Cochran, C., Gumbs, C., Seal, S., Barfoot, R., Collins, N., Bignell, G., Patel, S., Hamoudi, R., Larsson, C., Wiseman, R. W., Berchuck, A., Iglehart, J. D., Marks, J. R., Ashworth, A., Stratton, M. R., and Futreal, P. A. (1996). BRCA2 mutations in primary breast and ovarian cancers. *Nat Genet* **13**, 241-4.

Lancaster, J. M., Cochran, C. J., Brownlee, H. A., Evans, A. C., Berchuck, A, Futreal, P. A., Wiseman, R. W. (1996) Detection of BRCA1 mutations in women with early-onset ovarian cancer by use of the protein truncation test. *J Natl Can Inst* **88** (8), 552-53.

Lane, T. F., Deng, C., Elson, A., Lyu, M. S., Kozak, C. A., and Leder, P. (1995). Expression of Brca1 is associated with terminal differentiation of ectodermally and mesodermally derived tissues in mice [published erratum appears in Genes Dev 1996 Feb 1;10(3):365]. *Genes Dev* **9**, 2712-22.

Lee E. Y., To H., Shew J. Y., Bookstein R., Scully P., Lee W. H. (1988). Inactivation of the retinoblastoma susceptibility gene in human breast cancers. *Science* **241**, 218-220.

Levy-Lahad, E., Catane, R., Eisenberg, S., Kaufman, B., Hornreich, G., Lishinsky, E., Shohat, M., Weber, B. L., Beller, U., Lahad, A., and Halle, D. (1997). Founder BRCA1 and BRCA2 mutations in Ashkenazi Jews in Israel: frequency and differential penetrance in ovarian cancer and in breast- ovarian cancer families [see comments]. *Am J Hum Genet* **60**, 1068-78.

Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., Bigner, S. H., Giovanella, B. C., Ittmann, M,. Tycko, B., Hibshoosh, H., Wigler, M. H., Parsons, R. (1997) PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* **275**(5308), 1943-7

Lindblom, A., Skoog, L., Rotstein, S., Werelius, B., Larsson, C., and Nordenskjold, M. (1993). Loss of heterozygosity in familial breast carcinomas. *Tumori* **79**, 291-6.

Liu, C. Y., Flesken-Nikitin, A., Li, S., Zeng, Y., and Lee, W. H. (1996). Inactivation of the mouse Brca1 gene leads to failure in the morphogenesis of the egg cylinder in early postimplantation development. *Cancer Res* **56**, 3168-72.

Lynch, H. T., Lynch, J., Conway, T., Watson, P., Feunteun, J., Lenoir, G., Narod, S., and Fitzgibbons, R., Jr. (1994). Hereditary breast cancer and family cancer syndromes. *Oncogene* **9**, 97-102.

Malkin, D., Li, F. P., Strong, L. C., Fraumeni, J. F., Nelson, C. E., Kim, D. H., Kossel, J., Grya, M. A., Bischoff, F. Z., Tainsky, M. A., Friend, S. H. (1990) Germline p53 mutations in a familial syndrome of breast cancer, sarcomas and other neoplasms. *Science* **250**, 1233-38.

Malone, K. E., Daling, J. R., Thompson, J. D., O'Brien, C. A., Francisco, L. V., and Ostrander, E. A. (1998). BRCA1 mutations and breast cancer in the general population: analyses in women before age 35 years and in women before age 45 years with first-degree family history [see comments]. *Jama* **279**, 922-9.

Marks, J. R., Huper, G., Vaughn, J. P., Davis, P. L., Norris, J., McDonnell, D. P., Wiseman, R. W., Futreal, P. A., and Iglehart, J. D. (1997). BRCA1 expression is not directly responsive to estrogen. *Oncogene* **14**, 115-21.

Marquis, S. T., Rajan, J. V., Wynshaw-Boris, A., Xu, J., Yin, G. Y., Abel, K. J., Weber, B. L., and Chodosh, L. A. (1995). The developmental pattern of Brca1 expression implies a role in differentiation of the breast and other tissues. *R I Med* **78**, 257-60.

Mathew S., Murty V. V., Bosl G. J., Chagnati R. S. (1994). Loss of heterozygosity identifies multiple sites of allelic deletions on chromosome 1 in human male germ cell tumors. *Cancer Res* **54**, 6265-6269.

Matsushima, M., Kobayashi, K., Emi, M., Saito, H., Saito, J., Suzumori, K., and Nakamura, Y. (1995). Mutation analysis of the BRCA1 gene in 76 Japanese ovarian cancer patients: four germline mutations, but no evidence of somatic mutation. *Hum Mol Genet* **4**, 1953-6.

Maugard, C., Tuffery, S., Beaufrere, L., Bareil, C., Claustres, M. (1998) Le test de troncation des proteines (PTT): un outil pour la detection de mutations ponctuelles dans l'ADN. *M/S* **14**, 467-74.

Mazoyer, S., Dunning, A. M., Serova, O., Dearden, J., Puget, N., Healey, C. S., Gayther, S. A., Mangion, J., Stratton, M. R., Lynch, H. T., Goldgar, D. E., Ponder, B. A., and Lenoir, G. M. (1996). A polymorphic stop codon in BRCA2 [letter]. *Nat Genet* **14**, 253-4.

Mazoyer, S., Puget, N., Perrin-Vidoz, L., Lynch, H. T., Serova-Sinilnikova, O. M., and Lenoir, G. M. (1998). A BRCA1 nonsense mutation causes exon skipping [letter]. *Am J Hum Genet* **62**, 713-5.

Mazoyer, S. and exon13 Duplication screening group. (2000). The exon 13 duplication in the *BRCA1* gene is a founder mutation present in geographically diverse populations. *Am J Hum Genet*, in press

Merajver, S. D., and Petty, E. M. (1996). Risk assessment and presymptomatic molecular diagnosis in hereditary breast cancer. *Clin Lab Med* **16**, 139-67.

Meza, J. E., Brzovic, P. S., King, M. C., and Klevit, R. E. (1999). Mapping the functional domains of BRCA1. Interaction of the ring finger domains of BRCA1 and BARD1. *J Biol Chem* **274**, 5659-65.

Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P. A., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, L. M., Ding, W., and *et al.* (1994). A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Cell* **79**, 1-3.

Milner, J., Ponder, B., Hughes-Davies, L., Seltmann, M., and Kouzarides, T. (1997). Transcriptional activation functions in BRCA2 [letter] [see comments]. *Nature* **386**, 804-10.

Monteiro, A. N., August, A., and Hanafusa, H. (1996). Evidence for a transcriptional activation function of BRCA1 C-terminal region. *Nature* **384**, 297.

Murphy, M. F., Broeders, M.J., Carpenter, L. M., Gunnarskog, J., Leon, D.A. (1997) Breast cancer risk in mothers of twins. *Br J Cancer* **75**(7), 1066-8.

Musgrove E. A., Lee C. S. L., Buckley M. F., Sutherland R. L. (1991). Cyclin D1 induction in brest cancer cells shortens G1 and is sufficient for cells arrested in G1 to complete the cell cycle. *Proc Natl Acad Sci USA* **91**, 8022-8026.

Nelen, M. R., van Staveren, W. C., Peeters, E. A., Hassel, M. B., Gorlin, R. J., Hamm, H., Lindboe, C. F., Fryns, J. P., Sijmons, R. H., Woods, D. G., Mariman, E. C., Padberg, G. W,. Kremer, H. (1997) Germline mutations in the PTEN/MMAC1 gene in patients with Cowden disease. *Hum Mol Genet* **6**(8), 1383-7

Neuhausen, S. L., and Marshall, C. J. (1994). Loss of heterozygosity in familial tumors from three BRCA1-linked kindreds. *Cancer Res* **54**, 6069-72.

Nordling, M., Karlsson, P., Wahlstrom, J., Engwall, Y., Wallgren, A., and Martinsson, T. (1998). A large deletion disrupts the exon 3 transcription activation domain of the BRCA2 gene in a breast/ovarian cancer family. *Cancer Res* **58**, 1372-5.

Ozcelik, H., Schmocker, B., Di Nicola, N., Shi, X. H., Langer, B., Moore, M., Taylor, B. R., Narod, S. A., Darlington, G., Andrulis, I. L., Gallinger, S., and Redston, M. (1997). Germline BRCA2 6174delT mutations in Ashkenazi Jewish pancreatic cancer patients [letter]. *Am J Hum Genet* **60**, 1013-20.

Özda¤ H., Tez M., Sayek I., Tarcan O., Müslümano¤lu M., Içli F., Öztürk M., Özçelik T. Germline mutations in Turkish breast cancer patients. *Eur J Can,* in press.

Pavelic ZP, Pavelic L, Lower EE, Gapany M, Gapany S, Barker EA, Preisler HD. (1992) c-myc, c-erbB-2, and Ki-67 expression in normal breast tissue and in invasive and noninvasive breast carcinoma. *Cancer Res* 52(9):2597-602

Peelen, T., van Vliet, M., Petrij-Bosch, A., Mieremet, R., Szabo, C., van den Ouweland, A. M., Hogervorst, F., Brohet, R., Ligtenberg, M. J., Teugels, E., van der Luijt, R., van der Hout, A. H., Gille, J. J., Pals, G., Jedema, I., Olmer, R., van Leeuwen, I., Newman, B., Plandsoen, M., van der Est, M., Brink, G., Hageman, S., Arts, P. J., Bakker, M. M., Devilee, P., and *et al.* (1997). A high proportion of novel mutations in BRCA1 with strong founder effects among Dutch and Belgian hereditary breast and ovarian cancer families [see comments]. *Am J Hum Genet* **60**, 1059-67.

Phelan, C. M., Lancaster, J. M., Tonin, P., Gumbs, C., Cochran, C., Carter, R., Ghadirian, P., Perret, C., Moslehi, R., Dion, F., Faucher, M. C., Dole, K., Karimi, S., Foulkes, W., Lounis, H., Warner, E., Goss, P., Anderson, D., Larsson, C., Narod, S. A., and Futreal, P. A. (1996). Mutation analysis of the BRCA2 gene in 49 site-specific breast cancer families [see comments] [published erratum appears in Nat Genet 1996 Jul;13(3):374]. *Nat Genet* **13**, 123-5.

Plummer S. J., Anton-Culver, H., Webster, L., Noble, B., Liao, S., Kennedy, A., Belinson, J., Casey, G. Detection of BRCA1 mutations by protein truncation test. *Hum Mol Genet* **4** (10), 1989-91.

Puget, N., Siniinikova, O. M., Stoppa-Lyonnet, D., Audoynaud, C., Pages, S., Lynch, H. T., Goldgar, D., Lenoir, G. M., and Mazoyer, S. (1999). An Alu-mediated 6-kb duplication in the BRCA1 gene: a new founder mutation? [letter]. *Am J Hum Genet* **64**, 300-2.

Puget, N., Stoppa-Lyonnet, D., Sinilnikova, O. M., Pages, S., Lynch, H. T., Lenoir, G. M., and Mazoyer, S. (1999). Screening for germ-line rearrangements and regulatory mutations in BRCA1 led to the identification of four new deletions. *Cancer Res* **59**, 455-61.

Puget, N., Torchard, D., Serova-Sinilnikova, O. M., Lynch, H. T., Feunteun, J., Lenoir, G. M., and Mazoyer, S. (1997). A 1-kb Alu-mediated germ-line deletion removing BRCA1 exon 17. *Cancer Res* **57**, 828-31.

Rajan, J. V., Marquis, S. T., Gardner, H. P., and Chodosh, L. A. (1997). Developmental expression of Brca2 colocalizes with Brca1 and is associated with proliferation and differentiation in multiple tissues. *N Engl J Med* **336**, 1125-30.

Rajan, J. V., Wang, M., Marquis, S. T., and Chodosh, L. A. (1996). Brca2 is coordinately regulated with Brca1 during proliferation and differentiation in mammary epithelial cells. *Gene* **179**, 279-86.

Ramus, S. J., Kote-Jarai, Z., Friedman, L. S., van der Looij, M., Gayther, S. A., Csokay, B., Ponder, B. A., and Olah, E. (1997). Analysis of BRCA1 and BRCA2 mutations in Hungarian families with breast or breast-ovarian cancer [letter]. *J Nutr* **127**, 929S-932S.

Rao, V. N., Shao, N., Ahmad, M., and Reddy, E. S. (1996). Antisense RNA to the putative tumor suppressor gene BRCA1 transforms mouse fibroblasts. *Oncogene* **12**, 523-8.

Rayson, D., Erlichman, C., Suman, V. J., Roche, P. C., Wold, L. E., Ingle, J. N., Donohue, J. H. Molecular markers in male breast carcinoma. Cancer 83, 1947-55.

Rebbeck, T. R., Couch, F. J., Kant, J., Calzone, K., DeShano, M., Peng, Y., Chen, K., Garber, J. E., and Weber, B. L. (1996). Genetic heterogeneity in hereditary breast cancer: role of BRCA1 and BRCA2. *Am J Obstet Gynecol* **175**, 738-46.

Rhei, E., Kang, L., Bogomolniy, F., Federici, M. G., Borgen, P. I., Boyd, J. (1997) Mutation analysis of the putative tumor suppressor gene PTEN/MMAC1 in primary breast carcinomas.*Cancer Res* **57**(17), 3657-9

Ricciardone, M.D., Özçelik, T., Cevher, B., Özda¤, H., Tuncer, M., Gürgey, A. Uzunalimo€lu Ö., Çetinkaya, H., Tanyeli, A., Erken, E, Öztürk, M. (1999) Human MLH1 deficiency predisposes to hematological malignancy and neurofibromatosis type 1. *Cancer Res* 59 (2):290-3.

Rice, J. C., Massey-Brown, K. S., and Futscher, B. W. (1998). Aberrant methylation of the BRCA1 CpG island promoter is associated with decreased BRCA1 mRNA in sporadic breast cancer cells. *Am J Epidemiol* **148**, 623-30.

Robbins SL, Cotran RS, Kumar V. (1984). Pathologic basis of disease. W.B Saunders Company, Japan.

Rodrigo, L., Alvarez, V., Rodriguez, M., Perez, R., Alvarez, R., Coto, E. (1999) N-acetyltransferase-2, glutathione S-transferase M1, alcohol dehydrogenase, and cytochrome P450IIE1 genotypes in alcoholic liver cirrhosis: a case-control study.*Scand J Gastroenterol* **34**(3), 303-7

Roth, S., Kristo, P., Auranen, A., Shayehgi, M., Seal, S., Collins, N., Barfoot, R., Rahman, N., Klemi, P. J., Grenman, S., Sarantaus, L., Nevanlinna, H., Butzow, R., Ashworth, A., Stratton, M. R., and Aaltonen, L. A. (1998). A missense mutation in the BRCA2 gene in three siblings with ovarian cancer. *Br J Cancer* 77, 1199-202.

Ruffner, H., and Verma, I. M. (1997). BRCA1 is a cell cycle-regulated nuclear phosphoprotein. *Proc Natl Acad Sci U S A* **94**, 7138-43.

Saitoh S., Cunningham J, De Vries E. M., McGovern R. M., Schroeder J. J., Hartmann A., Blaszyk H., Wold L. E. (1994). P53 mutations in breast cancers in midwestern US women: Null as well as missense-type mutations are assocciated with poor prognosis. *Oncogene* **9**, 2869-2874.

Schatzkin, A., Longnecker, M. P. (1994) Alcohol and breast cancer: Where are we now and where do we go from here? *Cancer* **74**, 1101-10.

Scully, R., Anderson, S. F., Chao, D. M., Wei, W., Ye, L., Young, R. A., Livingston, D. M., and Parvin, J. D. (1997). BRCA1 is a component of the RNA polymerase II holoenzyme. *Proc Natl Acad Sci U S A* **94**, 5605-10.

Scully, R., Chen, J., Ochs, R. L., Keegan, K., Hoekstra, M., Feunteun, J., and Livingston, D. M. (1997). Dynamic changes of BRCA1 subnuclear location and phosphorylation state are initiated by DNA damage. *Cell* **90**, 425-35.

Scully, R., Chen, J., Plug, A., Xiao, Y., Weaver, D., Feunteun, J., Ashley, T., and Livingston, D. M. (1997). Association of BRCA1 with Rad51 in mitotic and meiotic cells. *Cell* **88**, 265-75.

Scully, R., Ganesan, S., Brown, M., De Caprio, J. A., Cannistra, S. A., Feunteun, J., Schnitt, S., and Livingston, D. M. (1996). Location of BRCA1 in human breast and ovarian cancer cells [letter; comment]. *Science* **272**, 123-6.

Serova, O., Montagna, M., Torchard, D., Narod, S. A., Tonin, P., Sylla, B., Lynch, H. T., Feunteun, J., and Lenoir, G. M. (1996). A high incidence of BRCA1 mutations in 20 breast-ovarian cancer families. *Am J Hum Genet* **58**, 42-51.

Serova, O. M., Mazoyer, S., Puget, N., Dubois, V., Tonin, P., Shugart, Y. Y., Goldgar, D., Narod, S. A., Lynch, H. T., and Lenoir, G. M. (1997). Mutations in BRCA1 and BRCA2 in breast cancer families: are there more breast cancer-susceptibility genes? *Am J Hum Genet* **60**, 486-95.

Serova-Sinilnikova, O. M., Boutrand, L., Stoppa-Lyonnet, D., Bressac-de-Paillerets, B., Dubois, V., Lasset, C., Janin, N., Bignon, Y. J., Longy, M., Maugard, C., Lidereau, R., Leroux, D., Frebourg, T., Mazoyer, S., and Lenoir, G. M. (1997). BRCA2 mutations in hereditary breast and ovarian cancer in France [letter]. *Am J Hum Genet* **60**, 1242-6.

Shao, N., Chai, Y. L., Shyam, E., Reddy, P., and Rao, V. N. (1996). Induction of apoptosis by the tumor suppressor protein BRCA1. *Genes Dev* **10**, 1835-43.

Sharan, S. K., Morimatsu, M., Albrecht, U., Lim, D. S., Regel, E., Dinh, C., Sands, A., Eichele, G., Hasty, P., and Bradley, A. (1997). Embryonic lethality and radiation hypersensitivity mediated by Rad51 in mice lacking Brca2 [see comments]. *Science* **276**, 531-2.

Shattuck-Eidens, D., McClure, M., Simard, J., Labrie, F., Narod, S., Couch, F., Hoskins, K., Weber, B., Castilla, L., Erdos, M., and *et al.* (1995). A collaborative survey of 80 mutations in the BRCA1 breast and ovarian cancer susceptibility gene. Implications for presymptomatic testing and screening. *Jama* **273**, 577-85.

Sobol, H, Birnbaum, D, Eisinger F. (1994) Evidence for a third breast-cancer susceptibility gene. *Lancet* **344**, 1151-1152.

Somasundaram, K., Zhang, H., Zeng, Y. X., Houvras, Y., Peng, Y., Wu, G. S., Licht, J. D., Weber, B. L., and El-Deiry, W. S. (1997). Arrest of the cell cycle by the tumour-suppressor BRCA1 requires the CDK-inhibitor p21WAF1/CiP1. *Gynecol Oncol* **66**, 378-87.

Steck, P. A., Pershouse, M. A., Jasser, S. A., Yung, W. K., Lin, H., Ligon, A, H., Langford, L. A., Baumgard, M. L., Hattier, T., Davis, T., Frye, C., Hu, R., Swedlund, B., Teng, D. H., Tavtigian, S. V. (1997) Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat Genet* **15**(4):356-62

Steeg, P. S. (1996). Granin expectations in breast cancer? [news; comment]. *Nat Genet* **12**, 223-5.

Stoppa-Lyonnet, D., Fricker, J. P., Essioux, L., Pages, S., Limacher, J. M., Sobol, H., Laurent-Puig, P., and Thomas, G. (1996). Segregation of two BRCA1 mutations in a single family [letter]. *Am J Hum Genet* **59**, 486-7.

Stoppa-Lyonnet, D., Laurent-Puig, P., Essioux, L., Pages, S., Ithier, G., Ligot, L., Fourquet, A., Salmon, R. J., Clough, K. B., Pouillart, P., Bonaiti-Pellie, C., and Thomas, G. (1997). BRCA1 sequence variations in 160 individuals referred to a breast/ovarian family cancer clinic. Institut Curie Breast Cancer Group [see comments]. *Am J Hum Genet* **60**, 1031-40.

Stratton, J. F., Gayther, S. A., Russell, P., Dearden, J., Gore, M., Blake, P., Easton, D., and Ponder, B. A. (1997) Contribution of BRCA1 mutations to ovarian cancer *N Eng J Med* **336**, 1125-30.

Struewing, J. P., Brody, L. C., Erdos, M. R., Kase, R. G., Giambarresi, T. R., Smith, S. A., Collins, F. S., and Tucker, M. A. (1995). Detection of eight BRCA1 mutations in 10 breast/ovarian cancer families, including 1 family with male breast cancer. *Am J Hum Genet* **57**, 189.

Swensen, J., Hoffman, M., Skolnick, M. H., and Neuhausen, S. L. (1997). Identification of a 14 kb deletion involving the promoter region of BRCA1 in a breast cancer family. *Hum Mol Genet* **6**, 1513-7.

Szabo, C. I., and King, M. C. (1997). Population genetics of BRCA1 and BRCA2 [editorial; comment]. *Am J Hum Genet* **60**, 1021-30.

T'ang A., Varley J. M., Chakraborty S., Murphree A. L., Fung Y. K., (1988). Structural rearrangement of he retinoblastoma susceptibility gene in human breast cancers. *Science* **241**, 218-221.

Teng, D. H., Bogden, R., Mitchell, J., Baumgard, M., Bell, R., Berry, S., Davis, T., Ha, P. C., Kehrer, R., Jammulapati, S., Chen, Q., Offit, K., Skolnick, M. H., Tavtigian, S. V.,

Jhanwar, S., Swedlund, B., Wong, A. K., and Kamb, A. (1996). Low incidence of BRCA2 mutations in breast carcinoma and other cancers. *Nat Genet* **13**, 245-7.

Teng, D. H., Hu, R., Lin, H., Davis, T., Iliev, D., Frye, C,. Swedlund, B., Hansen, K. L., Vinson, V. L., Gumpper, K. L., Ellis, L., El-Naggar, A., Frazier, M,. Jasser, S,. Langford, L. A., Lee, J., Mills, G. B., Pershouse, M. A., Pollack, R. E., Tornos, C., Troncoso, P., Yung, W. K., Fujii, G., Berson, A., Steck, P. A., *et al* (1997) MMAC1/PTEN mutations in primary tumor specimens and tumor cell lines. *Cancer Res* **57**(23), 5221-5

Thompson, M. E., Jensen, R. A., Obermiller, P. S., Page, D. L., and Holt, J. T. (1995). Decreased expression of BRCA1 accelerates growth and is often present during sporadic breast cancer progression. *Int J Fertil Menopausal Stud* **40**, 117.

Thompson, P. A., Shields, P. G., Freudenheim, J. L., Stone, A., Vena, J. E., Marshall, J. R., Graham, S., Laughlin, R., Nemoto, T., Kadlubar, F.F., Ambrosone, C. B. 1998 Genetic polymorphisms in catechol-O-methyltransferase, menopausal status, and breast cancer risk.*Cancer Res* **58**(10), 2107-10

Thorlacius, S., Olafsdottir, G., Tryggvadottir, L., Neuhausen, S., Jonasson, J. G., Tavtigian, S. V., Tulinius, H., Ogmundsdottir, H. M., and Eyfjord, J. E. (1996). A single BRCA2 mutation in male and female breast cancer families from Iceland with varied cancer phenotypes [see comments]. *Nat Genet* **13**, 117-9.

Thorlacius, S., Sigurdsson, S., Bjarnadottir, H., Olafsdottir, G., Jonasson, J. G., Tryggvadottir, L., Tulinius, H., and Eyfjord, J. E. (1997). Study of a single BRCA2 mutation with high carrier frequency in a small population [see comments]. *Am J Hum Genet* **60**, 1085-98.

Thorlacius, S., Struewing, J. P., Hartge, P., Olafsdottir, G. H., Sigvaldason, H., Tryggvadottir, L., Wacholder, S., Tulinius, H., and Eyfjord, J. E. (1998). Population-based study of risk of breast cancer in carriers of BRCA2 mutation [see comments]. *Lancet* **352**, 1359-60.

Thorlacius, S., Tryggvadottir, L., Olafsdottir, G. H., Jonasson, J. G., Ogmundsdottir, H. M., Tulinius, H., and Eyfjord, J. E. (1995). Linkage to BRCA2 region in hereditary male breast cancer. *Lancet* **346**, 544-5.

Tirkkonen, M., Johannsson, O., Agnarsson, B. A., Olsson, H., Ingvarsson, S., Karhu, R., Tanner, M., Isola, J., Barkardottir, R. B., Borg, A., and Kallioniemi, O. P. (1997). Distinct somatic genetic changes associated with tumor progression in carriers of BRCA1 and BRCA2 germ-line mutations. *Cancer Res* **57**, 1222-7.

Tirkkonen, M., Kainu, T., Loman, N., Johannsson, O. T., Olsson, H., Barkardottir, R. B., Kallioniemi, O. P., and Borg, A. (1999). Somatic genetic alterations in BRCA2-associated and sporadic male breast cancer. *Genes Chromosomes Cancer* **24**, 56-61.

Vile RG (1994) Tumor-specific gene expression. *Semin Cancer Biol* 5(6):429-36

Vaughn, J. P., Cirisano, F. D., Huper, G., Berchuck, A., Futreal, P. A., Marks, J. R., and Iglehart, J. D. (1996). Cell cycle control of BRCA2. *Cancer Res* **56**, 4590-4.

Vaurs-Barriere, C., Vidal, V., Penault-Llorca, F., Kwiatkowski, F., Maugard, C., and Bignon, Y. (1998). Pathology of sporadic breast tumors with LOH at the BRCA1 locus: correlation with histopathological features specific to familial BRCA1 tumors and absence of microsatellite instability. *Int J Oncol* **12**, 1373-8.

Vehmanen, P., Friedman, L. S., Eerola, H., McClure, M., Ward, B., Sarantaus, L., Kainu, T., Syrjakoski, K., Pyrhonen, S., Kallioniemi, O. P., Muhonen, T., Luce, M., Frank, T. S., and Nevanlinna, H. (1997). Low proportion of BRCA1 and BRCA2 mutations in Finnish breast cancer families: evidence for additional susceptibility genes. *Hum Mol Genet* **6**, 2309-15.

Vehmanen, P., Friedman, L. S., Eerola, H., Sarantaus, L., Pyrhonen, S., Ponder, B. A., Muhonen, T., and Nevanlinna, H. (1997). A low proportion of BRCA2 mutations in Finnish breast cancer families [see comments]. *Am J Hum Genet* **60**, 1059-67.

Vogelstein, B., and Kinzler, K. W. (1994). Has the breast cancer gene been found? *Science* **266**, 120-2.

Vogelstein, Bert. (1998). The genetic basis of human cancer McGraw-Hill, Health Profession New York

Wang, S. C., Lin, S. H., Su, L. K., and Hung, M. C. (1997). Changes in BRCA2 expression during progression of the cell cycle. *Jama* **277**, 1421-2.

Watson, P., Marcus, J. N., and Lynch, H. T. (1998). Prognosis of BRCA1 hereditary breast cancer. *Lancet* **351**, 316-21.

Wilson, C. A., Payton, M. N., Elliott, G. S., Buaas, F. W., Cajulis, E. E., Grosshans, D., Ramos, L., Reese, D. M., Slamon, D. J., and Calzone, F. J. (1997). Differential subcellular localization, expression and biological toxicity of BRCA1 and the splice variant BRCA1-delta11b. *Oncogene* **14**, 1-16.

Wilson, C. A., Payton, M. N., Pekar, S. K., Zhang, K., Pacifici, R. E., Gudas, J. L., Thukral, S., Calzone, F. J., Reese, D. M., and Slamon, D. I. (1996). BRCA1 protein products: antibody specificity... [letter; comment]. *Nat Genet* **13**, 266-8.

Wilson, C. A., Ramos, L., Villasenor, M. R., Anders, K. H., Press, M. F., Clarke, K., Karlan, B., Chen, J. J., Scully, R., Livingston, D., Zuch, R. H., Kanter, M. H., Cohen, S., Calzone, F. J., and Slamon, D. J. (1999). Localization of human BRCA1 and its loss in high-grade, non-inherited breast carcinomas. *Nat Genet* **21**, 236-40.

Wolff, M. S., Weston, A. (1997) Breast cancer risk and environmental exposures. *Environ Health Perspect* **105** Suppl 4:891-6

Wooster, R., Bignell, G., Lancaster, J., Swift, S., Seal, S., Mangion, J., Collins, N., Gregory, S., Gumbs, C., and Micklem, G. (1995). Identification of the breast cancer susceptibility gene BRCA2 [see comments] [published erratum appears in Nature 1996 Feb 22;379(6567):749]. *Nature* **378**, 789-92.

Wooster, R., Neuhausen, S. L., Mangion, J., Quirk, Y., Ford, D., Collins, N., Nguyen, K., Seal, S., Tran, T., Averill, D., and *et al.* (1994). Localization of a breast cancer susceptibility gene, BRCA2, to chromosome 13q12-13. *Br J Surg* **81**, 1512-5.

Wu, L. C., Wang, Z. W., Tsan, J. T., Spillman, M. A., Phung, A., Xu, X. L., Yang, M. C., Hwang, L. Y., Bowcock, A. M., and Baer, R. (1996). Identification of a RING protein that can interact in vivo with the BRCA1 gene product. *Nat Genet* **14**, 441-7.

Xu X, Wagner KU, Larson D, Weaver Z, Li C, Ried T, Hennighausen L, Wynshaw-Boris A, Deng CX. (1999) Conditional mutation of Brca1 in mammary epithelial cells results in blunted ductal morphogenesis and tumour formation. *Nat Genet* 22(1):37-43

Yao R, Cooper GM (1995) Regulation of the Ras signaling pathway by GTPase-activating protein in PC12 cells. *Oncogene*, 11(8):1607-14.

Zhang, H., Somasundaram, K., Peng, Y., Tian, H., Bi, D., Weber, B. L., and El-Deiry, W. S. (1998). BRCA1 physically associates with p53 and stimulates its transcriptional activity. *Oncogene* **16**, 1713-21.

Zheng, W., Deitz, A. C., Campbell, D. R., Wen, W. Q., Cerhan, J. R., Sellers, T. A., Folsom, A.R., Hein, D. W. (1999) N-acetyltransferase 1 genetic polymorphism, cigarette smoking, well-done meat intake, and breast cancer risk. *Cancer Epidemiol Biomarkers Prev* **8**(3) 233-9

Date: Fri, 23 Jun 2000 12:16:42 +0100
From: "Cannell, Emma (ELSLON)" <Emma.Cannell@ejcancer.co.uk>
To: "'tozcelik@fen.bilkent.edu.tr'" <tozcelik@fen.Bilkent.EDU.TR>

[The following text is in the "iso-8859-1" character set.]
[Your display is set for the "US-ASCII" character set.]
[Some characters may be displayed incorrectly.]

Dear Dr. Ozcelik,
I have just edited your paper and before your paper is typeset I would appreciate it if you could provide a fig legend for the 2 pedigree analyses explaining the symbols for those of us unfamiliar with this type of analysis. Please do not send a new manuscript but could you email the fig legend as soon as possible so that your paper can be amended before it is sent to the publisher. Your paper is therefore now accepted and as soon as you send the fig legend we will send out a letter to that effect.

I look forward to hearing from you in the near future.
Yours sincerely,

Emma Cannell PhD (Scientific Editor)
N.B. We have moved - our new address and contact details are above.

GERM-LINE *BRCA1* and *BRCA2* GENE MUTATIONS in TURKISH BREAST CANCER PATIENTS

Hilal Özdag[1], Mesut Tez[2], Iskender Sayek[2], Mahmut Müslümanoglu[3], Oguz Tarcan[4], Fikri Içli[5], Mehmet Öztürk[1], Tayfun Özçelik*,[1]

[1]Department of Molecular Biology and Genetics, Bilkent University, Ankara 06533, Turkey; [2]Department of Surgery, Hacettepe University Medical School, Ankara 06100, Turkey; [3]Department of Surgery, Istanbul University, Istanbul Medical School, Istanbul 34080, Turkey; [4]Ankara Oncology Hospital, Ankara 06200, Turkey; [5]Department of Medical Oncology, Ankara University Medical School, Ankara 06100, Turkey

* To whom correspondence should be addressed.

For editorial correspondence:

Tayfun Özçelik, M.D.

Department of Molecular Biology and Genetics

Bilkent University

06533 Bilkent ANKARA TURKEY

Phone: +90-312-266-5081

Fax: +90-312-266-5097

e-mail: tozcelik@fen.bilkent.edu.tr

ABSTRACT

Germ-line *BRCA1* and/or *BRCA2* mutations were screened in 50 Turkish breast and/or ovarian cancer patients composed of hereditary, familial, early onset, and male cancer groups. Genomic DNA samples were tested by heteroduplex analysis and DNA sequencing. Two truncating *BRCA2* mutations, one novel (6880insG) and one previously reported (3034delAAAC), were found in two out of six (33%) hereditary breast and/or ovarian cancer patients. A novel truncating (1200insA) and a missense (2080A→G) *BRCA1* mutation was found in two of 27 (8%) individuals in the early onset group. A total of four (8%) disease causing mutations in 50 breast cancer patients were identified in *BRCA1* and *BRCA2* genes. In addition, five *BRCA1* sequence variants have been identified in 23 patients. These results indicate that *BRCA1* and *BRCA2* genes are involved in some but not all forms of hereditary predisposition to breast cancer in the Turkish population.

Key words: Hereditary breast/ovarian cancer; *BRCA1; BRCA2;* germ-line mutation

INTRODUCTION

Breast cancer is one of the most common malignancies affecting women[1]. Inherited gene mutations may be responsible for 5-10% of breast cancer cases (1, 2,3). Ovarian cancer is also known to have a familial component (4).

Two genes associated with inherited predisposition to breast and/or ovarian cancer have been identified. These are *BRCA1* on chromosome 17q12-21 (1), and *BRCA2* on chromosome 13q12-13 (5). Population genetics studies aiming to determine the relative contributions of these genes in hereditary breast and/or ovarian cancer have shown a wide variation among different populations (6). For example, in families with three or more cases of female breast and/or ovarian cancer, *BRCA1* mutations are as low as 9% in Iceland and as high as 79% in Russia. A similar variation has been documented for *BRCA2* mutations as well, which can be examplified by 8% in Finland and 64% in Iceland. In affected women before age 45 years with a first-degree relative, *BRCA1* mutations account for 7% of the families (7). In families with both male and female breast cancer, *BRCA2* mutations were documented in 19% of North American, 33% of Hungarian, and 90% of Icelandic populations (6). In isolated male breast cancer cases, a very low frequency of *BRCA2* mutations has been reported (8). In early onset breast and/or ovarian cancer patients not

[1] http://www.nci.nih.gov/public/factbook98/incidence.htm

selected for family history, 4-9% have *BRCA1*, and 2-8% have *BRCA2* mutations (6, 7).

Breast cancer is among the most common malignancies in Turkish women (9). The frequency and the types of germ-line mutations involved in Turkish breast/ovarian cancers are not well known. In order to determine the contributions of *BRCA1* and *BRCA2* mutations to the development of breast and/or ovarian cancer in the Turkish population, we screened pre-selected regions of these genes in four groups of patients composed of hereditary and familial cancer, as well as early onset and male breast cancer.

PATIENTS AND METHODS

Families

We analyzed a total of 50 genomic DNA samples isolated from white blood cells of breast cancer patients. The samples were collected from the Medical Schools of Hacettepe, Istanbul, Ankara Universities, and Ankara Oncology Hospital. Informed consent was obtained from all participants. Each proband was interviewed for pedigree construction including information concerning the family history of breast, ovarian, and other cancers. Based on pedigree analysis, patients were divided into four groups. Available medical and pathological records were reviewed to verify the diagnoses. The phenotypical characteristics of the families included in the study are summarized in Table 1.

PCR and heteroduplex analysis

DNA isolation was performed from 1 ml peripheral blood by phenol/chloroform extraction (10). Exons 2, 5, 11 (10 overlapping fragments), 13, 20, 24 of *BRCA1*, and exon 11 (7 overlapping fragments) of *BRCA2* were screened for mutations by heteroduplex analysis using previously reported primers (11, 12). PCR was performed in a total volume of 10 µl, with 10X buffer, 1.5 U Taq polymerase, 1.5 mM $MgCl_2$, 200 µM dNTP, 10 pmol of each primer, and 1µCi of [^{32}P] dCTP (specific activity 3000 Ci/mmol). PCR conditions were as follows, 94°C for 3 min, 30 cycles of 30 sec at 94°C, 30 sec at 55°C, and 30 sec at 72°C. 10 min of 72°C was added as

the extension step. PCR products were denatured at 95°C for 10 min, and left at 37°C for 2 hours. 5 µl of PCR products were loaded on to 6% native polyacrylamide gels, and run at 400V for 16 hours. The gels were then dried and exposed to X ray films O/N at -80°C.

Sequencing

Fragments that showed an alteration in heteroduplex analysis were reamplified for sequencing. The primer pairs for *BRCA1* did not change. However, *BRCA2* exon 11 was subdivided into 26 overlapping fragments for DNA sequencing reactions (12). PCR was performed in a total volume of 50 µl, with 10X buffer, 1.5 U Taq polymerase, 1.5 mM $MgCl_2$, 200 µM dNTP, and 10 pmol of each primer. PCR conditions were as follows, 94°C for 3 min, 30 cycles of 30 sec at 94°C, 30 sec at 55°C, and 30 sec at 72°C. 10 min of 72°C was added as the extension step. PCR products were sequenced using Amersham Thermosequenase Dye Terminator cycle sequencing kit, according to the manufacturer's instructions, and analyzed on an ABI 377 automated DNA sequencer or ABI 310 Genetic Analyzer.

Mutations identified by direct sequencing were subjected to confirmatory analysis. For this purpose, PCR products were cloned into Promega pGEM TEasy TA cloning kit, according to the manufacturer's directions. Several mini prep plasmid DNA samples for each patient were isolated by phenol chloroform extraction and sequenced using Perkin Elmer Big Dye cycle sequencing kit.

RESULTS

Germ-line *BRCA1* and/or *BRCA2* mutations were screened in 50 breast and/or ovarian cancer patients from Turkey. We employed a *BRCA1* and *BRCA2* mutation screening strategy which is based on the localization of previously reported mutations in these genes. Selected regions of *BRCA1* (exons 2, 5, 11, 13, 20 & 24), and *BRCA2* (exon 11) were subjected to heteroduplex analysis, and altered fragments were further analyzed by DNA sequencing. According to the BIC database[2], these *BRCA1* and *BRCA2* regions harbor 80% and 45% of the mutations respectively.

The patients were divided into four groups of hereditary (n=6) breast/ovarian cancer, and familial (n=7), early onset (n=27) or male (n=10) breast cancer. The selected regions of both *BRCA1* and *BRCA2* genes were analyzed in all four groups.

Nine different sequence alterations (seven for *BRCA1* and two for *BRCA2*) were identified in 25 patients. In *BRCA2*, one previously reported deletion (3034 delAAAC), and one novel insertion (6880 insG) (Figure 1A) – type of frameshift mutations leading to protein truncation were observed. In *BRCA1*, one novel frameshift mutation (1200 insA) (Figure 1B), one novel missense mutations (2080 A→G; K654E) (Figure 2A), and one novel silent mutation (1013 T→C; N298N) were identified. In addition, four previously

[2] (http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/index.html

reported *BRCA1* polymorphisms (one silent and three missense mutations) were also found in these Turkish breast cancer patients (see Table 2).

Hereditary group consisted of patients with at least two affected first degree relatives, and those with bilateral breast cancer plus one affected first degree relative (Table 1). We identified two *BRCA2* frameshift mutations, 6880 insG (Figure 1A) and 3034 delAAAC, in two of the six families (33%) in this group. Interestingly no *BRCA1* mutation was found except for a previously reported frequent polymorphism which is an A to G transition at nucleotide 3667 in three (50%) families.

Patients who have one affected first degree relative also diagnosed with breast cancer constitute the familial group. No frameshift or nonsense *BRCA1* or *BRCA2* mutation was observed. However, previously reported *BRCA1* polymorphisms 3667 A→G in three (42%), and 2731 C→T in two (28%) patients were present. In addition, a novel silent *BRCA1* mutation (1013 T→C) was identified in one patient.

Women diagnosed as having breast cancer before age 35 years, none of whom were selected on the basis of family history status were in the early onset group. A novel *BRCA1* frameshift mutation, 1200 insA (Figure 1B), was identified in one patient. In addition, five different *BRCA1* sequence alterations were observed in 12 patients. These alterations include one

novel (1013 T→C), and four previously reported polymorphisms (see Table 2). The 3667 A→G polymorphism was observed in 33% (9/27) of the patients. Interestingly each allele of *BRCA1* displayed a missense mutation in one patient (Figure 2A and B): a previously described rare polymorphism (2196 G→A; D693N) and a novel missense mutation (2080 A→G; K654E). This patient also carried a silent mutation (2201 C→T) co-segregating with the 2196 G→A mutation. The phase of these transitions was determined by cloning of patient's PCR product and sequencing of multiple clones. This finding establishes that sequence alterations 2080 A→G and 2196 G→A, which lead to amino acid substitutions K654E and D693N respectively, were independently inherited. Sequence analysis showed that 2080A→G mutation was absent in one hundred independent alleles from a control population. The novel K654E substitution is not in a conserved residue but when *BRCA1* sequence is subjected to secondary structure prediction programs SOPMA and GOR4[3] this region forms a short alpha helix, carrying four consecutive lysine residues. Even though Lys to Glu substitution may increase the helix stability, it neutralizes positive charges on the helix, which may have an important role for the structure and function of the protein. Occurrence of two mutant alleles in the same patient with a sporadic early onset malignancy (age 27) suggests that these mutations may be involved in the development of breast cancer.

[3] (http://pbil.ibcp.fr/cgi-bin/secpred)

The fourth group we studied was composed of isolated male breast cancer cases. We were unable to detect mutations in the selected regions of *BRCA1* and *BRCA2* genes in this group.

In conclusion, among 50 Turkish breast cancer cases, we detected two (4%) *BRCA2* and one (2%) *BRCA1* disease-causing frameshift mutations. In addition, one (2%) missense *BRCA1* mutation, and five *BRCA1* polymorphisms in 23 patients (46%) was identified.

DISCUSSION

A wide variation in *BRCA1* and *BRCA2* mutation spectrum and frequency has been reported for different populations (6). The results of our Turkish data is summarized in Table 3. In the hereditary breast cancer group, *BRCA2* mutations accounted for 33% of the cases. This frequency is rather high similar to the Icelandic population. Interestingly, *BRCA1* mutations in our study group appear to be rare - if not exceptional similar to patients from Iceland, Norway, and Japan. However, in a recently published paper, Balcı *et al.* reported *BRCA1* mutations in two out of five hereditary breast cancer cases as compared to one *BRCA2* mutation (13). We did not detect *BRCA1* or *BRCA2* mutations in familial breast cancer cases. Our *BRCA1* data confirm the observations of Malone *et al.* who identified *BRCA1* mutations in only 7% of the cases (7). To the best of our knowledge, *BRCA2* mutations in this group have not been reported previously. Although we did not screen all exons of *BRCA2* our observations suggest that *BRCA2* gene is also infrequently involved in familial breast cancer. The results obtained from this study indicate that *BRCA1* and *BRCA2* genes are involved in the development of some but not all hereditary breast cancers in the Turkish population as reported for other populations (14, 15).

Acknowledgements

We thank Ms Lütfiye Mesci for oligonucleotide synthesis, and Dr. Rengül Çetin Atalay for help in secondary structure prediction. This work was supported by grants from Bilkent Holding, TTGV, and TWAS. Hilal Özdag is a recipient of TÜBITAK-BDP fellowship.

REFERENCES

1. Miki Y., Swensen J., Shattuck-Eidens D., et al. A strong candidate for the breast and ovarian cancer susceptibility gene *BRCA1*. *Science* 1994, **266**, 66-71.

2. Thorlacius S, Struewing JP, Hartge P, et al. Population-based study of risk of breast cancer in carriers of *BRCA2* mutation. *Lancet* 1998, **352**, 1337-1339 .

3. Feunteun J. La predisposition hereditaire au cancer du sein liee a *BRCA1* et *BRCA2*: une maladie de la reponse aux lesions genotoxiques. *Medicine et Sciences* 1999, **15**, 38-44.

4. Easton DF, Bishop DT, Ford D. Genetic linkage analysis in familial breast and ovarian cancer: results form 214 families. *Am. J. Hum. Genet.*, 1993 **52**, 678-701.

5. Wooster R, Bignell G, Lancaster J. Identification of the breast cancer susceptibility gene *BRCA2*. *Nature* 1995, **378**, 789-792.

6. Szabo CI and King M-C. Population genetics of *BRCA1* and *BRCA2*. *Am. J. Hum. Genet.* 1997, **60**, 1013-1020.

7. Malone KE, Daling JR, Thompson JD. *BRCA1* mutations and breast cancer in the general population. *JAMA* 1998, **279**, 12, 922-929.

8. Friedman LS, Gayther SA, Kurosaki T. Mutation analysis of *BRCA1* and *BRCA2* in a male breast cancer population. *Am. J. Hum. Genet.* 1997, **60**, 313-319.

9. Karaoguz H. and Içli F. *J. Ankara Med. Sch.* 1993, **15**, 547-558.

10. Sambrook J, Fritsch E, Maniatis T. *Molecular Cloning* 1989, Cold Spring Harbor Laboratory Press: NY.

11. Friedman LS, Ostermeyer EA, Szabo CI. Confirmation of *BRCA1* by analysis of germ-line mutations linked to breast and ovarian cancer in ten families. *Nature Genet.* 1994, **8**, 399-404.

12. Lancester JM, Wooster R, Mangion J. *BRCA2* mutations in primary breast and ovarian cancers. *Nature Genet.* 1996, **13**, 238-240.

13. Balcı A, Huusko P, Pääkkönen K. Mutation analysis of *BRCA1* and *BRCA2* in Turkish cancer families: a novel mutation *BRCA2* 3414del4 found in male breast cancer. *Eur .J. Cancer* 1999, **35**, 5, 707-710.

14. Serova OM, Mazoyer S, Puget N. Mutations in *BRCA1* and *BRCA2* in breast cancer families: Are there more breast cancer-susceptibility genes? *Am. J. Hum. Genet.* 1997, **60**, 486-495.

15. Sobol H, Birnbaum D, Eisinger F. Evidence for a third breast-cancer susceptibility gene. *Lancet* 1994, **344**, 1151-1152.

Am. J. Hum. Genet. 67:207–212, 2000

Report

The Exon 13 Duplication in the *BRCA1* Gene Is a Founder Mutation Present in Geographically Diverse Populations

The *BRCA1* Exon 13 Duplication Screening Group*

Recently, a 6-kb duplication of exon 13, which creates a frameshift in the coding sequence of the *BRCA1* gene, has been described in three unrelated U.S. families of European ancestry and in one Portuguese family. Here, our goal was to estimate the frequency and geographic diversity of carriers of this duplication. To do this, a collaborative screening study was set up that involved 39 institutions from 19 countries and included 3,580 unrelated individuals with a family history of the disease and 934 early-onset breast and/or ovarian cancer cases. A total of 11 additional families carrying this mutation were identified in Australia (1), Belgium (1), Canada (1), Great Britain (6), and the United States (2). Haplotyping showed that they are likely to derive from a common ancestor, possibly of northern British origin. Our results demonstrate that it is strongly advisable, for laboratories carrying out screening either in English-speaking countries or in countries with historical links with Britain, to include within their *BRCA1* screening protocols the polymerase chain reaction–based assay described in this report.

Methods used to screen for mutations in the *BRCA1* gene (MIM 113705) focus mainly on genomic DNA, and, being PCR based, they do not enable the detection of large DNA rearrangements. This may explain why only 12 large germline insertions or deletions have been described (Petrij-Bosch et al. 1997; Puget et al. 1997, 1999*a*, 1999*b*; Swensen et al. 1997; Montagna et al. 1999; Rohlfs et al. 2000), compared with ~400 point mutations, small insertions, and deletions scattered across the whole coding sequence and over the splice junctions (Breast Cancer Information Core). However, in two independent studies performed on Dutch (Petrij-Bosch et al. 1997) and U.S. (Puget et al. 1999*a*) families with breast and/or ovarian cancer, rearrangements have been found to represent 36% and 15% of all mutations, respectively.

Recently, a 6-kb duplication of exon 13, ins6kbEx13, which creates a frameshift in the coding sequence, has been identified in the *BRCA1* gene (Puget et al. 1999*b*). It was initially found in three, apparently unrelated, U.S. families of European ancestry and in one Portuguese family. Haplotype data suggested a common founder for all these families.

To estimate the frequency and geographic diversity of carriers of this duplication, which will not be identified if not specifically sought, a collaborative study was set up. This involved 39 institutions in which the screening for ins6kbEx13 was done by PCR (fig. 1) using specific primers and a positive control. A total of 3,580 unrelated individuals with a family history of breast and/or ovarian cancer who were from 19 different countries (Australia, Austria, Belgium, Canada, Finland, France, Germany, Great Britain, Hungary, Ireland, Israel, Italy, the Netherlands, Norway, Spain, Sweden, Switzerland, Turkey, and the United States) were screened. To be enrolled, the family histories had to fulfill at least one of the following criteria: (1) at least three cases of female breast and/or ovarian cancer; (2) at least one case of female breast cancer and one case of male breast cancer; (3) one case of female breast cancer diagnosed at age <50 years and one case of ovarian or female breast cancer; (4) one case of female breast cancer and one case of ovarian cancer in first-degree relatives; (5) one case of female breast cancer and one case of female bilateral breast cancer; or (6) two cases of ovarian cancer. This heterogeneous sample is representative of the diversity of patients who attend clinics for individuals at high risk for cancer. At the time of the analysis, the whole coding sequence and the splice sites of the *BRCA1* and *BRCA2* genes had been screened for the presence of mutations

Received March 29, 2000; accepted for publication May 9, 2000; electronically published May 25, 2000.

Address for correspondence and reprints: Dr. S. Mazoyer, UMR CNRS-UCBL 5641, Faculté de Médecine, 8 avenue Rockefeller, 69373 Lyon Cedex 08, France. E-mail: smazoyer@rockefeller.univ-lyon1.fr

* The complete list of group members can be found in the Appendix.

0002-9297/2000/6701-0024$02.00

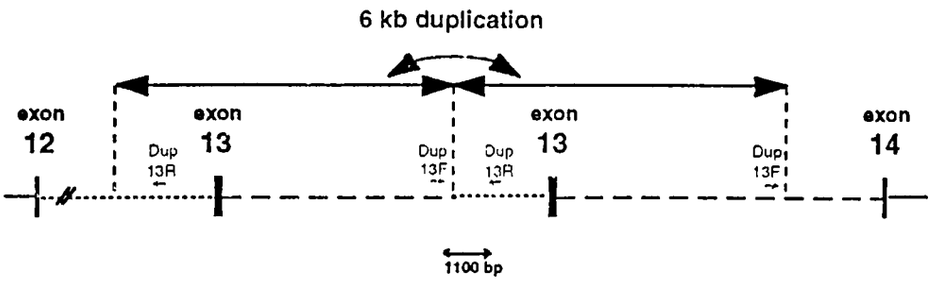


Figure 1 Schematic representation of *BRCA1* exon 13 duplication. The location and orientation of the dup13F (GAT TAT TTC CCC CCA GGC TA) and dup13R (AGA TCA TTA GCA AGG ACC TGT G) primers are represented, along with the position of the 6-kb duplicated region and of the 1.1-kb PCR fragment generated with dup13F/R. Blackened boxes denote exons; dotted lines denote intron 12; and broken lines denote intron 13.

in 61% and 37%, respectively, of the families; the screening had not been completed in 31% (*BRCA1*) and 43% of them (*BRCA2*); it had not been attempted in 8% (*BRCA1*) and 20% (*BRCA2*).

From this series of families, 1,831 were recruited and screened in English-speaking countries, and 10 apparently independent families were found to carry the duplication in Australia (1), Canada (1), Great Britain (6), and the United States (2) (table 1). No duplications were found in the 1,749 families who were recruited and screened in non–English-speaking countries (table 2). Ten of the institutions involved in this project also sought the presence of the duplication in 934 additional samples, mainly breast cancer cases selected for an early age at onset (table 3). One non–English-speaking duplication carrier was found in this second series: a Belgian woman with breast cancer diagnosed at age 34 years whose mother also developed breast cancer. If we also include the 4 families previously identified (Puget et al. 1999*a*), a total of 15 apparently unrelated families have been found to carry ins6kbEx13 (table 4). As seen with the other recurrent mutations (Tonin et al. 1996), these 15 families display a spectrum of different breast and ovarian cancer phenotypes.

Haplotype analysis was conducted with seven polymorphic short-tandem-repeat markers within or flank-

Table 1

BRCA1 Exon 13–Duplication Screening in English-Speaking Countries

Country and Town	No. of Positive Families	No. of Screened Families[a]			
		Total	Breast Cancer Only	Breast and Ovarian Cancer	Ovarian Cancer Only
Australia:					
Westmead	1	40	33	7	0
Canada:					
Montreal	0	5[b]	1	4	0
Toronto	1	504	319	174	11
Ireland:					
Dublin	0	73	15	50	8
United Kingdom:					
Cambridge	0	56	0	15	41
St Andrews	1	150	112	38	0
Leeds	4	44	0	44	0
London	1	278	210	68	0
Sutton	0	94	NC	NC	NC
United States:					
Chapel Hill	0	61	39	21	1
New York	0	92	64	27	1
Philadelphia	1	110	65	45	0
Rochester	1	257	206	51	0
Salt Lake City	0	67	43	24	0
Total	10	1,831	>1,107	>568	>62

[a] NC = not communicated.

[b] No French Canadian or Jewish Ashkenazi families were included.

Table 2

***BRCA1* Exon 13–Duplication Screening in Non–English-Speaking Countries**

Country and Town	No. of Positive Families	No. of Screened Families			
		Total	Breast Cancer Only	Breast and Ovarian Cancer	Ovarian Cancer Only
Austria:					
Vienna	0	238	177	52	9
Belgium:					
Gent	0	30	26	3	1
Canada:					
Quebec	0	45	40	5	0
Finland:					
Helsinki	0	161	126	35	0
France:					
Clermont-Ferrand	0	36	24	9	3
Lille	0	115	103	5	7
Nantes	0	48	39	8	1
Villejuif	0	70	46	24	0
Paris	0	51	44	7	0
St. Cloud	0	117	96	20	1
Germany:					
Dusseldorf	0	45	36	8	1
Heidelberg	0	75	52	22	1
Hungary:					
Budapest	0	25	24	1	0
Israel:					
Tel Hashomer	0	76	62	14	0
Italy:					
Milan	0	48	35	13	0
Padua	0	96	54	33	9
Pisa	0	17	3	14	0
Netherlands:					
Leiden	0	30	26	4	0
Utrecht	0	200	168	27	5
Norway:					
Oslo	0	19	1	13	5
Spain:					
Barcelona	0	85	58	27	0
Madrid	0	76	60	16	0
Sweden:					
Lund	0	29	0	20	9
Switzerland:					
Geneva	0	8	6	2	0
Turkey:					
Ankara	0	9	8	0	1
Total	0	1,749	1,314	382	53

ing the *BRCA1* locus and spanning ~1.9 Mb (from centromere to telomere, these markers are D17S1185, D17S1321, D17S1323, D17S1322, D17S855, D17S1326, and D17S1325; data not shown). In 8 of the 15 families, a single sample was available; thus, it was not possible to determine phase. Nevertheless, all exon 13–duplication carriers had genotypes compatible with their sharing the same haplotype. Because the particular alleles shared by duplication carriers at D17S1185 and D17S855 have population frequencies <15%, we can conclude that this duplication has most likely spread from a common ancestor. Unfortunately, because of an insufficient number of families in which haplotypes could be determined, it was not possible to accurately date this mutation.

We then tried to determine where the duplication originated. The origins of three of the families positive for the duplication could not be traced (table 4). However, the remaining ones are, in all but one case, compatible with the assumption that the ancestor for this mutation was British, because they are either of British descent or from countries—Ireland, Portugal, and Belgium—that have trading or other historical links with Britain. Five of the British families are from northern England, mainly Yorkshire, suggesting a northern British origin for the ancestor of this mutation. The exception is a U.S. family

Table 3

***BRCA1* Exon 13–Duplication Screening in Breast and/or Ovarian Cancer Cases**

Country and Town	No. of Positive Cases	No. (Age [Years]) of Screened Cases		
		Breast Cancer	Ovarian Cancer	Breast and Ovarian Cancer
Canada:				
Toronto	0	24 (<45)		5
Ireland:				
Dublin	0	14 (<50)		
United Kingdom:				
London	0	59 (<35)		2
Sutton	0	617 (<45)		...
Austria:				
Vienna	0	14 (<35)	4 (<40)	
Belgium:				
Gent	1	16 (<35)	1 (<35)	
Germany:				
Dusseldorf	0	2 (<40)		
Heidelberg	0	70 (<40)		
Spain:				
Barcelona	0	90 (<35)		
Turkey:				
Ankara	0	16 (<32)	...	...
Total	1	922	5	7

of Norwegian and Swedish ancestries. Another possibility is that the duplication could be of Viking origin, since all the countries mentioned above also had contact with Vikings. However, against this possibility, no duplication carrier has been identified in Sweden or in Norway; but the number of families screened was low in both countries (29 and 19, respectively; table 2). Alternatively, this U.S. carrier may not be fully aware of all her ancestors.

Eleven more families have been identified as carrying the exon 13 duplication, either in different series or after this study: two from Australia, one from New Zealand (E. Edkins, personal communication), five from Canada (S. Narod and C. Phelan, personal communication; N. Carson, personal communication), and three from Great Britain (A. Haworth, personal communication). In conclusion, the *BRCA1* exon 13 duplication is most likely a founder mutation distributed mainly in English-speaking countries or in countries with historical links with Britain. It would be strongly advisable, for laboratories

Table 4

Origin and Phenotype of the Families Carrying the *BRCA1* Exon 13 Duplication

Town Where Screening Was Done	Nationality	Origin	No. of Cases	
			Breast Cancer	Ovarian Cancer
Lyon[a] (France)	American	Irish/Dutch	1	4
Lyon[a](France)	American	English	7	3
Lyon[a](France)	American	Unknown	7	0
Lyon[a](France)	Portuguese	Portuguese	3	0
Gent (Belgium)	Belgian	Unknown	2	0
Perth (Australia)	Australian	Unknown	4	1
Toronto (Canada)	American	Irish/Scottish/English/German	4	0
St. Andrews (Great Britain)	British	English/Polish	3	0
Leeds (Great Britain)	British	English	3	3
Leeds (Great Britain)	British	English	2	3
Leeds (Great Britain)	British	French/English	4	1
Leeds (Great Britain)	British	English	5	1
London (Great Britain)	British	English	1	2
Philadelphia (United States)	American	Dutch/German/French/English	8	3
Rochester (United States)	American	Norwegian/Swedish	4	0
Total			58	21

[a] Families reported by Puget et al. (1999*a*).

carrying out screening for *BRCA1* mutations in these countries, to include within their protocols the PCR-based assay described here. This study emphasizes once more the necessity of screening not only for mutations in the coding sequence and splice sites but also for gene rearrangements, when one is analyzing for the presence of disease-causing mutations in the *BRCA1* gene.

Acknowledgments

The authors would like to thank the many family members who participated in the research study described in this report. Le Comité Départemental du Rhône de La Ligue contre le Cancer is acknowledged for constant funding, and Dr. Alison M. Dunning is acknowledged for helpful comments.

Appendix

The *BRCA1* Exon 13 Duplication Screening Group

Coordinator—S. Mazoyer (Université Claude Bernard, Lyon, France); Australia—J. Leary and J. Kirk (University of Sydney, Westmead); Austria—E. Fleischmann and T. Wagner (University of Vienna, Vienna); Belgium—K. Claes and L. Messiaen (University Hospital, Gent); Canada—W. Foulkes (McGill University, Montreal), M. Desrochers and J. Simard (Centre Hospitalier de l'Université Laval Research Center and Laval University, Quebec), and C. M. Phelan, E. Kwan, and S. A. Narod (Women's College Hospital, Toronto); Finland—P. Vahteristo and H. Nevanlinna (Helsinski University Central Hospital, Helsinski); France—X. Durando and Y. J. Bignon (Centre Jean Perrin, Clermont-Ferrand), J. P. Peyrat (Centre Oscar Lambret, Lille), C. Bonnardel, O. M. Sinilnikova, N. Puget, G. M. Lenoir, and S. Mazoyer (Université Claude Bernard, Lyon), C. Audoynaud and D. Goldgar (International Agency for Research on Cancer, Lyon), C. Maugard (Centre René Gauducheau, Nantes), V. Caux, S. Gad, and D. Stoppa-Lyonnet (Institut Curie, Paris), C. Noguès and R. Lidereau (Centre René Huguenin, St. Cloud), and C. Machavoine and B. Bressac-de Paillerets (Institut Gustave Roussy, Villejuif); Germany—B. Kuschel, B. Betz, D. Niederacher, and M. W. Beckmann (Heinrich-Heine-Universität, Düsseldorf) and U. Hamann (Deutsches Krebsforschungszentrum, Heidelberg); Great Britain—S. A. Gayther and B. A. P. Ponder (Strangeways Research Centre, Cambridge), M. Robinson, G. R. Taylor, and T. Bishop (St. James's University Hospital, Leeds), A. Catteau and E. Solomon (Guy's Hospital, London), B. Cohen and M. Steel (University of St. Andrews, St. Andrews, Scotland), and N. Collins and M. Stratton (Institute of Cancer Research, Sutton); Hungary—M. van der Looij and E. Oláh (National Institute of Oncology, Budapest); Ireland—N. J. Miller and D. E. Barton (Our Lady's Hospital for Sick Children, Dublin); Israel—R. S. Sverdlov and E. Friedman (Chaim Sheba Medical Center, Tel Hashomer); Italy—P. Radice (Istituto Nazionale Tumori, Milano), M. Montagna (University of Padova, Padova), and E. Sensi and M. Caligo (University of Pisa, Pisa); the Netherlands—R. van Eijk and P. Devilee (University of Leiden, Leiden) and R. van der Luijt (Universitair Medisch Centrum Utrecht, Utrecht); Norway—families contributed by K. Heimdal and P. Møller (The Norwegian Radium Hospital, Oslo) and screened by Å. Borg (University Hospital, Lund, Sweden); Spain—O. Diez, J. Cortes, M. Domenech, and M. Baiget (Hospital de la Santa Creu i Sant Pau, Barcelona) and A. Osorio and J. Benítez (Fundación Jiménez Díaz, Madrid); Sweden—Å. Borg (University Hospital, Lund); Switzerland—P. Maillet and A. P. Sappino (Centre Médical Universitaire, Geneva); Turkey—H. Özdag, T. Özçelik, and M. Ozturk (Bilkent University, Ankara); and the United States—E. M. Rohlfs (University of North Carolina, Chapel Hill), J. Boyd, D. McDermott, and K. Offit (Memorial Sloan-Kettering Cancer Center, New York), M. Unger, K. Nathanson, and B. L. Weber (University of Pennsylvania, Philadelphia), T. A. Sellers, E. Hampton, and F. J. Couch (Mayo Clinic, Rochester), and S. Neuhausen (University of Utah, Salt Lake City).

Electronic-Database Information

The accession number and URLs for data in this article are as follows:

Breast Cancer Information Core (BIC), http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omin (for *BRCA1* [MIM 113705])

References

Montagna M, Santacatterina M, Torri A, Menin C, Zullato D, Chieco-Bianchi L, D'Andrea E (1999) Identification of a 3 kb Alu-mediated *BRCA1* gene rearrangement in two breast/ovarian cancer families. Oncogene 18:4160–4165

Petrij-Bosch A, Peelen T, van Vliet M, van Eijk R, Olmer R, Drusedau M, Hogervorst FBL, et al (1997) *BRCA1* genomic deletions are major founder mutations in Dutch breast cancer patients. Nat Genet 17:341–345

Puget N, Sinilnikova OM, Stoppa-Lyonnet D, Audoynaud C, Pagès S, Lynch HT, Goldgar D, et al (1999*a*) An *Alu*-mediated 6-kb duplication in the *BRCA1* gene: a new founder mutation? Am J Hum Genet 64:300–302

Puget N, Stoppa-Lyonnet D, Sinilnikova OM, Pagès S, Lynch HT, Lenoir GM, Mazoyer S (1999*b*) Screening for germline

rearrangements and regulatory mutations in *BRCA1* led to the identification of four new deletions. Cancer Res 59: 455–461

Puget N, Torchard D, Serova-Sinilnikova OM, Lynch HT, Feunteun J, Lenoir GM, Mazoyer S (1997) A 1-kb Alu-mediated germ-line deletion removing *BRCA1* exon 17. Cancer Res 57:828–831

Rohlfs EM, Puget N, Graham ML, Silverman LM, Garber JE, Skrzynia C, Halperin JL, et al (2000) An Alu-mediated 7.1 kb deletion of *BRCA1* in breast and ovarian cancer families with evidence for a founder effect. Genes Chromosomes Cancer 28:300–307

Swensen J, Hoffman M, Skolnick MH, Neuhausen SL (1997) Identification of a 14 kb deletion involving the promoter region of *BRCA1* in a breast cancer family. Hum Mol Genet 6:1513–1517

Tonin P, Weber B, Offit K, Couch F, Rebbeck TR, Neuhausen S, Godwin AK, et al (1996) Frequency of recurrent *BRCA1* and *BRCA2* mutations in Ashkenazi Jewish breast cancer families. Nat Med 2:1179–1183