Inherited predisposition to breast/ovarian cancer: the frequency and nature of BRCA*1* gene mutations in South African families

BY

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Dissertation in fulfilment of the requirements for the degree of
Magister Scientiae (M.Sc.) in Human Genetics

In the
Department of Human Genetics and Developmental Biology
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2001

ACKNOWLEDGEMENTS

There are a number of people I wish to acknowledge and thank, for helping out with this project :

- Professor Christianson and all staff and students at the Department of Human Genetics of the University of Pretoria, for making this project possible.

- Professor van Rensburg, my supervisor, for her time, support and enthusiasm throughout the duration of the project.

- All the families who volunteered to participate in the study and provided the valuable material upon which to work.

- Celmari Dorfling and Marlene de la Rey for their help and interest in the study, as well as the good times shared in the laboratory.

- I wish to thank the following organisations for financial support of this project ; The Cancer Association of South Africa (CANSA),
  The Research Committee of the Medical Faculty, University of Pretoria (NAVKOM).
  The Technology and Human Resources for Industry Programme (THRIP); a partnership programme funded by the Department of Trade and Industry (DTI) and managed by the National Research Foundation (NRF).

- Finally, my family, friends and especially Darrel for your constant support and encouragement during my years of studying.

ABSTRACT

Breast cancer is the most common cancer in South African women. Approximately 5-10% of all breast cancer cases are due to an inherited predisposition, resulting from mutations in tumour suppressor genes. The BRCA1 gene on chromosome 17q is one such tumour suppressor gene, that when mutated confers an increased risk of breast /ovarian cancer in carriers. To date, more than 500 different BRCA1 mutations have been reported worldwide. Some of these mutations are frequently reported and others occur commonly in certain population groups. These population-specific differences in mutations represent founder effects, whereby a single ancestral mutation accounts for the majority of breast cancer cases. This study was undertaken as the nature and frequency of BRCA1 mutations in South African breast/ovarian cancer families is unknown. Fifty-one breast/ovarian cancer families were screened for three commonly occurring mutations (185delAG, 4184del4 and 5382insC) using polymerase chain reaction (PCR) and allele-specific oligonucleotide (ASO) hybridisation. The protein truncation test (PTT) was utilised to detect truncating mutations in the large exon 11, and the remaining coding exons were screened for mutations using exon-by-exon PCR single strand conformation polymorphism/heteroduplex analysis (SSCP/HA).

Seven disease-causing mutations were identified in 15 families, consisting of five different frameshift mutations and two different nonsense mutations. Four Ashkenazi Jewish families were found to harbour the 185delAG mutation; the 5382insC mutation was identified in two Afrikaner families and one Ashkenazi Jewish family. Haplotype analysis revealed that the four Ashkenazi families share the common Ashkenazi Jewish haplotype, suggesting a common ancestor for these families. Similarly, the two Afrikaner families share the same haplotype as families of north and east European ancestry with the 5382insC mutation. The haplotype of the Ashkenazi Jewish family with this mutation was however different to the linked haplotype, indicating a recombination event or an independent mutation. Both these mutations are thought to have occurred in or before the medieval period. Furthermore, four Afrikaner families were found to carry the novel E881X nonsense mutation, which has not been previously described. Haplotype analysis of these families suggested that these patients share a common ancestor, and genealogic studies have identified the founding couple for this mutation, who both arrived in the Cape from France in the late 1600s. Four additional families were found to harbour BRCA1 mutations by SSCP/HA. Three of these mutations have not been previously reported – the S451X nonsense mutation (identified in a family of Scottish

origin), the 1493delC mutation identified in an Afrikaner family, and the 4957insC mutation identified in an Indian family. The 448insA mutation was identified in a family of German origin, where the patient had cancer of the fallopian tubes. A number of different described polymorphisms and variants of unknown functional significance were also identified.

This is the first study to show that BRCA1 is involved in South African breast/ovarian cancer families, to the extent that 29.4% (15/51) of families have BRCA1 mutations. Furthermore, minor founder effects in the Afrikaner population have been demonstrated. These results enable improved genetic counselling and clinical management of mutation positive families as well as subsequent testing of family members.

OPSOMMING

Borskanker is die mees algemene kanker in Suid Afrikaanse vroue. Ongeveer 5-10% van alle borskanker gevalle is as gevolg van 'n oorgeërfde vatbaarheid, veroorsaak deur mutasies in tumoronderdrukker gene. Die BRCA1 geen op chromosoom 17q is so 'n tumoronderdrukker geen. Persone wat 'n mutasie in BRCA1 dra het 'n verhoogde risiko vir bors/ovariële kanker. Tot op datum is daar meer as 500 verskillende BRCA1 mutasies wêreldwyd gerapporteer. Sommige van hierdie mutasies word dikwels gerapporteer, terwyl ander meer algemeen in sekere populasie groepe voorkom. Hierdie populasie-spesifieke mutasies verteenwoordig 'n stigters effek. Dit is wanneer daar 'n beperkte groep stigter voorouers is en daar dan 'n enkele persoon is met 'n mutasie wat oorgedra word en wat uiteindelik verantwoordelik is vir die meeste borskanker gevalle in daardie populasie. Hierdie studie is onderneem om die aard en frekwensie van BRCA1 mutasies in Suid-Afrikaanse bors/ovarieële kanker families te bepaal. Een-en-vyftig bors/ovariële families is deur gebruik van die polimerase kettingreaksie (PKR) en alleel-spesifieke oligonukleotied (ASO) hibridisasie getoets vir drie mutasies (185delAG, 4184del4 en 5382insC) wat gereeld voorkom. Die proteïen verkortings toets ("Protein Truncation Test", PTT) is aangewend om ekson 11 mutasies te soek wat aanleiding gee tot 'n verkorte proteïen. Die oorblywende koderende eksons is deur middel van 'n kombinasie van ekson-per-ekson PKR enkel-ketting-konformasie-polimorfisme en heterodupleks analise ("SSCP/HA") ondersoek.

Sewe siekte-veroorsakende mutasies is in 15 van die families geïdentifiseer, nl vyf verskillende raamverskuiwings mutasies en twee verskillende nonsense mutasies. Die 185delAG mutasie is in vier Ashkenazi Joodse families gevind. Terwyl die 5382insC mutasie in twee Afrikaner en een Ashkenazi Joodse familie gevind is. Haplotipe analise het aangetoon dat die vier Suid-Afrikaanse Ashkenazi Joodse families dieselfde haplotipes het as dié wat voorheen vir Ashkenazi Joodse families met dié mutasie gerapporteer is. Dit dui dus daarop dat hul dieselfde voorouer het. Die haplotipe van die twee Afrikaner families met die 5382insC mutasie stem ooreen met dié van noord en oos- Europese families met dieselfde mutasie. Die haplotipe van die Ashkenazi Joodse familie met hierdie mutasie was egter anders as die gekoppelde haplotipe, wat dui op 'n rekombinasie. Daar word gedink dat beide hierdie mutasies voor of gedurende die Middeleeue ontstaan het. Daar is verder bevind dat vier Afrikaner families 'n unieke nonsense mutasie,E881X, dra. Haplotipe analise van hierdie families het aangetoon dat hul afstam van 'n gemeenskaplike voorouer. Genealogiese studies het die stigters

paar geïdentifiseer wat van Franse afkoms is. Hulle het hul aan die Kaap gevestig in die laat 1600s. Daar is ook vier ander BRCA1 mutasies deur middel van SSCP/HA analise gevind. Drie van hierdie mutasies, nl. die S451X nonsense mutasie (geidentifiseer in 'n familie van Skotse afkoms); die 1493delC mutasie (geïdentifiseer in 'n Afrikaner familie); en die 4957insC mutasie (geïdentifiseer in 'n Indiër familie) is uniek. Die voorheen gerapporteerde mutasie, 448insA, is in 'n familie van Duitse herkoms gevind. Die indeks geval het kanker van die fallopius-buise gehad. Verskeie bekende polimorfismes en variante waarvan die funksie onbekend is, is ook geïdentifiseer.

Hierdie is die eerste studie om aan te toon dat BRCA1 by Suid-Afrikaanse bors/ovariële kanker families betrokke is, tot so 'n mate dat 29.4% (15/51) van families mutasies dra. Daar is verder ook 'n geringe stigters effek aangetoon in Afrikaners. Met behulp van hierdie resultate is toepaslike kliniese bestuur van mutasie-positiewe families nou moontlik.

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α	Alpha
ASO	Allele specific oligonucleotide
β	Beta
BAC	Bacterial Artificial Chromosome
BARD1	BRCA1 associated Ring domain protein 1
BIC	Breast cancer information core
bp	Base pairs
BRCA1	Breast Cancer 1 Gene
BRCA2	Breast Cancer 2 Gene
BRCA3	Breast Cancer 3 Gene
BRCT	BRCA1 C terminal domain
°C	Degrees Celsius
CASH	Cancer and steroid hormone
cDNA	Complementary DNA
cent	Centromere
Ci	Curies
cM	Centimorgan
cm^2	Square centimetres
conc	Concentration
dATP	Deoxyribonucleotide adenosine triphosphate
ddNTP	Dideoxyribonucleotide triphosphate
ddUTP	Deoxyribonucleotide urasil triphosphate
DIG	Digoxigenin
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediamine Tetra-Acetic Acid
ESE	Exonic splicing enhancers
EST	Expressed Sequence Tags
FISH	Fluorescent in situ hybridisation
γ	Gamma
GDB	Genome database
HCl	Hydrochloric acid

IVS	Intervening sequence
Kb	Kilobase pairs
KCl	Potassium chloride
KDa	Kilodalton
lod	Logarithm of odds
LOH	Loss-of-heterozygosity
µCi	Microcuries
µg	Micrograms
µl	Microlitres
µM	Micromolar
M	Molar
mA	Milliamps
MDE	Mutation detection enhancement
$MgCl_2$	Magnesium chloride
ml	Millilitre
mM	Millimolar
mmol	Millimole
mRNA	Messenger RNA
NaCl	Sodium chloride
$NaClO_4$	Sodium perchlorate
ng	Nanograms
nm	Nanometers
^{32}P	Phosphorus-32
PCR	Polymerase Chain Reaction
pmol	Picomoles
PTT	Protein Truncation Test
RNA	Ribose nucleic acid
rpm	Revolutions per minute
^{35}S	Sulphur-35
SDS	Sodium Dodecyl Suplhate
SNP	Single nucleotide polymorphism
SSCP	Single strand conformation polymorphism
SSCP/HA	Single strand conformation polymorphism/ Heteroduplex analysis
SSPE	Sodium Chloride Phosphate EDTA solution
SSR	Simple Sequence Repeat

CHAPTER 1
INTRODUCTION

"There are few other diseases that kill as many people as breast cancer and yet have been so badly neglected for so long..... The discovery of the BRCA1 gene is neither the first nor the last word in beating breast cancer. It is most assuredly not a cure. Nevertheless, it is already abundantly clear that the identification of this gene has brought breast cancer research the long-awaited breakthrough that is so desperately needed - and deserved! "

Breakthrough – The Race to Find the Breast Cancer Gene
Kevin Davies, Michael White. John Wiley & Sons, Inc, 1995.

Breast cancer is a complex multifactorial disease involving an interaction between both genetic and environmental factors. This chapter will introduce the topic of breast cancer, with reference to its incidence and its epidemiology within South Africa and worldwide.

1.1 INCIDENCE

Breast cancer is the third most common cancer in the world, and is the most common cancer in UK women (Key et al., 1997). The lifetime risk of developing cancer in the Western world is 1 in 12 (Wasan and Bodmer, 1997). It is estimated that the lifetime risk for the development of breast cancer in the general Caucasian population is 10% (Claus et al., 1991).

Compared to other Western countries, Taiwan is a low-incidence area for breast cancer (Yang et al., 1997). The incidence of breast cancer in Japan is three- to five fold lower than that of American women (Watanabe, 1993). Data from the United States on breast cancer incidence show that white, Hawaiian and black women have the highest incidence. The rate in white non-Hispanic women is four times higher than in Korean women. American Indian and Vietnamese women also have a low incidence (CancerNet).

In South Africa, breast cancer is the second most common cancer in women (Sitas et al., 1998), accounting for 16% of all reported women's cancers. However, significant

population differences are observed, it is the most common cancer in white (17.9%) and Asian women (24.4%), but ranks second in coloured (18.2%) and black women (13.4%). The lifetime risk of developing breast cancer also varies between population groups, as can be seen in table 1.1.

Table 1.1 Lifetime risk of breast cancer in South African women (Sitas et al., 1998)

Population Group	Lifetime Risk
White	1/13
Asian	1/21
Coloured	1/63
Black	1/81

It is obvious that white South African women have the highest lifetime risk (1/13) of developing breast cancer, black women have the lowest lifetime risk (1/81). At present the reason for the six-fold lower risk in black women is unclear.

1.2 EPIDEMIOLOGY

A number of different factors have been identified to be determinants of the occurrence of breast cancer, including reproductive factors, lifestyle factors, exogenous hormones, environmental exposures and heredity (Alberg and Helzlsouer., 1997). It is important to note that the development of breast cancer is multifactorial, involving the interaction of environmental factors, hormones and genes.

1.2.1 Endogenous Hormones

The fact that breast cancer in men is rare suggests that sex steroid hormones, specifically estrogen, play a role in breast cancer risk. Increased exposure to estrogen increases ones risk for developing breast cancer, therefore any factor that increases the number of menstrual cycles one is exposed to, will increase the breast cancer risk (Martin and Weber., 2000). The major risk factors for developing breast cancer are early age at menarche, late age at menopause, and older age at first birth (Hulka and Stark., 1995). An increased risk is associated with nulliparity, while high parity decreases the breast cancer risk. Women who have had an oophorectomy have a reduced risk of

breast cancer. All of these factors involve one's exposure to hormones, more specifically estrogen. The levels of estrogen continuously fluctuate during a woman's ovulation cycle, and this stimulates cell division of breast cells. The greater the number of ovulation cycles, the greater the risk. Therefore, the greater the exposure to estrogen, the greater the risk of developing breast cancer (Key et al., 1997). Cultural differences also result in differences in number of births and ages at menarche and menopause.

Cell division is an important step in the development of cancer; therefore any reproductive factor that increases mitotic activity in breast epithelium will increase the risk of breast cancer (Pike et al., 1993).

The role of breastfeeding in the risk of developing breast cancer is controversial and remains largely unresolved. It has been hypothesized that breast cancer risk is reduced by lactation (Romieu et al.; 1996; Enger et al., 1997), however other studies have identified no significant association between breastfeeding and the reduction of breast cancer risk (Katsouyanni et al., 1996; Negri et al., 1996). It is therefore likely, that if an association is found, it will be a weak protective effect offered by breastfeeding. The modest protective effect found is associated with longer duration of breastfeeding in premenopausal women.

A number of other reproductive factors have been associated with breast cancer risk, including multiple births and the "dual effect" of pregnancy. This "dual effect" is the transient increase in risk for approximately three years following birth, followed by a long-term reduction in risk (reviewed in Alberg and Helzlsouer., 1997).

1.2.2 Exogenous Hormones

Much controversy surrounds studies on the effect of oral contraceptive use and the risk of breast cancer. However, a large study has determined that the use of oral contraceptives is associated with a small increased risk of breast cancer, which is reduced after usage is stopped (Collaborative Group on Hormonal Factors in Breast Cancer, 1996). Hormone replacement therapy has been found to increase the risk of breast cancer in users (reviewed in Martin and Weber., 2000). The development of breast cancer in both oral contraceptive users and hormone replacement therapy is likely to be delayed following administration. It is for this reason that the results from studies of these associations are inconclusive, as oral contraceptives have only been in

use from the 1970s, and it is possibly too soon to see the effects (Eccles and Houlston, 1995).

1.2.3 Lifestyle Factors

Many different studies have attempted to find an association between dietary fat intake and the incidence of breast cancer. One large study, in particular found no association between adult fat intake and a risk of developing breast cancer (Hunter et al., 1996). However, other studies (reviewed in Hulka and Stark, 1995) have found no significant association. Currently work is being conducted in this field, as diet is one of the risk factors that could possibly be controlled, to reduce a risk of breast cancer.

Similarly, studies on the role of cigarette smoking and the risk of breast cancer have provided inconclusive results. Cigarette smoking has been reported to both increase and decrease the risk of breast cancer. The increased risk may be attributed directly to the carcinogens found in cigarette smoke, or indirectly by the effect of women with polymorphisms rendering them slow acetylators of carcinogens (reviewed in Hulka and Stark, 1995; Alberg and Helzlsouer, 1997).

A number of other factors have been found to influence the risk of breast cancer. Physical exercise has been associated with a reduced risk; moderate alcohol intake and stress have been shown to increase the risk (Hulka and Stark, 1995; Alberg and Helzlsouer, 1997).

1.2.4 Environmental Exposures

High-dose ionising radiation exposure is associated with an increased risk of breast cancer. This prompted the modification of mammographic equipment to reduce the exposure, as regular mammography examinations are a method of preventing breast cancer. Exposure to electromagnetic fields and pesticides has also been suggested to increase the risk of breast cancer (Hulka and Stark, 1995; Alberg and Helzlsouer, 1997).

1.2.5 Heredity

A family history of breast cancer has been shown to be associated with an increased risk of breast cancer (reviewed in Yang and Lippman., 1999). This increased risk may be due to a multifactorial effect of environmental factors as listed above and genetic background. However, there are a number of features that characterize hereditary breast cancers as such. These include an early age of onset of affected individuals, the presence of bilateral disease, the association with other malignancies including ovarian, prostate and colon cancer, and the transmission of the cancer through successive generations in an autosomal dominant pattern (reviewed in Yang and Lippman., 1999). This inherited susceptibility is usually as a result of mutations in the recently identified highly penetrant BRCA1 or BRCA2 tumour suppressor genes. However, the risk estimated for mutation carriers is probably the upper limit, as it was determined from families selected for the presence of breast and/or ovarian cancer. These risks are still higher than the general population risk of developing breast cancer. The majority of reported breast cancer cases are sporadic, i.e. no family history, it is estimated that 5-10% of cases are due to the inheritance of highly penetrant gene mutations which increase susceptibility.

CHAPTER 2
THE BRCA1 GENE AND BREAST/OVARIAN CANCER SUSCEPTIBILITY

2.1 EVIDENCE FOR GENETIC PREDISPOSITION

It wasn't until recently that the available technological advances were able to provide researchers with a clear understanding of the familial component of breast cancer. However, as early as 1866, a French physician, Paul Broca, reported ten out of twenty four women in a four-generation family to have breast cancer (reviewed in Eccles and Houlston, 1995). A large number of different case-control studies were conducted to find the familial risk of breast cancer. Of all studies, the Cancer and Steroid Hormone Study (CASH) conducted by the Centres for Disease Control was the largest and most convincing (Claus et al., 1991). The data set available for the study was the largest of its kind ever collected (population-based, case-control study), incorporating the family histories of 4730 histologically confirmed breast cancer cases between 20 and 54 years of age that were matched by age and geographic region to 4688 controls. Segregation analysis provided evidence for the existence of a highly penetrant, rare autosomal dominant allele resulting in increased susceptibility to breast cancer. It was estimated that the lifetime risk of breast cancer in carriers of the abnormal allele is 92%, compared to 10% in noncarriers. It was suggested that the majority of breast cancer cases in the general population do not have a genetic component. The pattern of breast cancer in first-degree relatives, and the age at onset were the most significant risk factors for breast cancer, with risk to relatives increasing with decreasing age at onset of the case. The risk also increases with an increasing number of affected family members with breast cancer.

Population data from the CASH were used by Schildkraut et al. (1989) to determine the relationship between breast, ovarian and endometrial cancer. Data on family history and age at onset of the disease were analysed in a set of cases and age-matched controls. Results of this study suggest that relatives of breast or ovarian cancer patients have a 2.3-fold increased risk of breast or ovarian, cancer. This was considered to be a significant genetic correlation, but the presence of genetic heterogeneity could not be ruled out. No association with breast/ovarian and endometrial cancer was found, although there was an association with colon cancer.

The initial studies were based on high risk families, where the clustering of breast cancer may have been the result of shared environment, culturally transmitted risk factors, polygenic effects, the effects of individual genes or a combination of all these factors (Newman et al., 1988). The large number of breast cancer cases in one family, as well as the ascertainment bias may not reflect breast cancer occurrence in the general population. However, further segregation studies by other researchers lent support to the hypothesis of the inheritance of a dominant gene responsible for familial breast cancer. Newman et al. (1988) conducted one such study, where cases were not selected for a family history. It was estimated in this study that the lifetime risk to carriers was 82% as compared to 8% of noncarriers. It was clear from this and other similar studies, that high-risk families could be used as models for breast cancer risk in the general population.

2.2 GENETIC LINKAGE

Unfortunately carriers of the mutated gene in familial breast/ovarian cancer families are not phenotypically distinguishable from non-carriers. This, together with the high incidence of sporadic breast cancer in the general population made linkage studies to identify the gene difficult. In addition to this, the presence of phenocopies within a family (sporadic breast cancers) would result in the incorrect assumption of carrier status. These individuals would have a breast cancer risk similar to that of the general population, and not elevated on the basis of carrier status. It is also possible that more than one locus is involved in the inherited susceptibility (genetic heterogeneity). It was also noted that the disease has incomplete penetrance, with its expression depending on a number of risk factors (Hall et al., 1990).

Nonetheless, a study using 23 extended families, including 146 cases of breast cancer suggested the presence of a gene for early-onset breast cancer (BRCA1) on chromosome 17q21 (Hall et al., 1990). All families used had the classic features of familial breast cancer, including early age at onset, bilateral disease and the presence of affected men in the family. The analysis revealed a lod score of 5.98 for linkage to marker D17S74 on chromosome 17q21, in families with an average age at onset less than or equal to 45 years. The lod scores obtained for families with a later age at onset were negative, indicative of evidence against linkage. The reasons for this were assumed to be one of the following - the presence of different loci being involved in those families, the chance occurrence of multiple cases of breast cancer in some

families or the prevalence of sporadic cancers in late-onset families. It was suggested that the BRCA1 gene lay within 10cM of marker D17S74.

Narod et al. (1991) confirmed these findings, and extended the linkage to include ovarian cancer families. This was done by analysing five large breast-ovarian cancer families with the D17S74 marker. Three of the families showed evidence of linkage to D17S74. These results strengthened the assignment of a breast-ovarian cancer susceptibility gene to an area on chromosome 17q12-q23. The 10cM region for the location of the gene was also confirmed, but the association between linkage and age at onset, as found by Hall et al. (1990), was not found.

Once the location was narrowed down to this region, researchers began an intensive effort to map the gene, as well as to identify mutations that result in breast cancer. A number of candidate genes in this region were investigated to determine if they were responsible for breast cancer. One such candidate gene was the EDH17B2 gene, for 17β-estradiol-dehydrogenase, which catalyses the conversion of estrone to estradiol (Hall et al., 1990).

After the initial linkage reports, an international consortium (Breast Cancer Linkage Consortium) involved in linkage analysis to find the breast cancer susceptibility gene was formed. Data from 214 families was pooled to narrow the interval where BRCA1 was expected to lie. This 14cM interval was found to be flanked by markers D17S250 and D17S588 (Easton et al., 1993). In addition to this, it was estimated that while 100% of breast-ovarian cancer families were linked to chromosome 17q12-23, only 45% of breast cancer only families were linked to chromosome 17q12-23. Subsequently, the BRCA1 locus was localized to a narrower interval flanked by the markers THRA1 and D17S181 (reviewed in Goldgar et al., 1994).

2.3 CLONING THE BRCA1 GENE

2.3.1 BRCA1 cDNA Sequence

The abundance of the well-characterised genetic markers allowed for the construction of high-density genetic maps, using somatic cell hybrids to determine the approximate positions of the chromosome 17q markers by Anderson et al. (1993). A genetic map was used as the framework for constructing YAC contigs. In addition to this, YACs, P1, BACs and cosmid clones were used to generate a physical map for the region (reviewed in Miki et al., 1994).

Simultaneously, recombinants in pedigrees were examined to narrow down the region linked to the candidate gene. This resulted in the localisation of BRCA1 to a region on chromosome 17q flanked by markers D17S250 and D17S588. Further typing of markers delineated the region distal to markers D17S776 and D17S701, and proximal to markers D17S183 and D17S78 (reviewed in van Rensburg and Ponder, 1995).

Albertsen et al. (1994) also constructed a high-density map using radiation hybrids and fluorescent in situ hybridisation (FISH). Polymorphic markers flanking BRCA1 were used to screen yeast artificial chromosome (YAC) and P1 libraries, the clones that were identified were used to form overlapping DNA fragments. The YAC and P1 inserts were cloned and sequenced, and a contig assembled. The resulting map was ordered by overlapping YAC and P1 clones, and defined as an area between markers D17S579 and THRA1. Critical recombination events in some families narrowed the BRCA1 gene proximal to the D17S78 marker, and distal to D17S776 (Albertsen et al., 1994).

A single large family linked to 17q was studied by Goldgar et al. 1994, whose 195 members were genotyped for four highly polymorphic probes, spanning the BRCA1 regions in 1- to 2- cM intervals. The presence of an affected individual with a key recombination event allowed for the further isolation of BRCA1 to be distal to marker D17S776. In addition to this, the dense map of marker loci surrounding BRCA1 allowed for the identification of a rare haplotype that cosegregated with breast and ovarian cancers. Gradually, the BRCA1 region was narrowed down to a 600Kb region between markers D17S1321 and D17SS1325. At this stage, the candidate genes could be ruled out, because they fell outside the critical region identified.

In 1994, Miki et al. cloned the BRCA1 gene, using the physical map comprising of overlapping YACs, P1, bacterial artificial chromosome (BACs), and cosmid clones that had been generated for this region. Within this region, candidate expressed sequences were identified. These were obtained by screening cDNA libraries and random genomic DNA sequencing, as well as predicting coding exons. The expressed sequences were assembled into contigs, and characterized in a number of different ways. When expressed sequence tags (ESTs) were probed onto RNA blots, a 7.8Kb transcript was observed in normal breast mRNA. The transcript found was most abundant in testis and thymus, but also found to be present in breast and ovary. The BRCA1 gene was found to span the D17S855 marker (Miki et al., 1994).

In order to prove that this was indeed the breast/ovarian cancer susceptibility gene, it was necessary to show that in families linked to chromosome 17q, mutations in this candidate gene were segregating with the disease. Miki et al. (1994) used eight families; four showing evidence for linkage and four had negative lod scores. These families were used as they all had early onset breast cancer, and four of the families had the presence of ovarian cancer. In comparing the BRCA1 sequence in these individuals to the wild-type sequence, four of the families were found to harbour heterozygous sequence variants, including two frameshift mutations, one nonsense mutation and a putative missense mutation. The frameshift and nonsense mutations were likely to disrupt the function of the BRCA1 protein, by premature truncations at various points in the protein. These mutations were not found in a control population sample and were found to cosegregate with the disease in the family, both requirements for the classification of these variants as disease-causing mutations. The mutations are also loss-of-function mutations, where the normal function of one copy is knocked out, consistent with a tumour suppressor gene.

Futreal et al. (1994) typed mainly sporadic breast and ovarian cancers with three highly polymorphic markers to detect loss of heterozygosity (LOH). Fifty percent of the breast cancers and 57% of the ovarian cancers showed LOH, and these tumours were subsequently screened for BRCA1 mutations. Four germline mutations (one nonsense and three missense mutations) were detected, and the mutations met the criteria for being cancer susceptibility alleles. However, no somatic mutations were detected, which was unexpected. These results supported the role for BRCA1 in early-onset breast/ovarian cancer, but raised the issue of a possible difference in the etiology of sporadic breast/ovarian cancers.

Further confirmation for BRCA1 as the breast/ovarian cancer susceptibility gene came from work by Friedman et al. (1994). The BRCA1 gene was screened for germline mutations using single strand conformation polymorphism (SSCP) analysis in 20 families linked to chromosome 17q12-21. Ten families were found to harbour nine different mutations (five frameshift, one splice-site, one nonsense and two missense mutations). Polymorphisms were also detected.

Additional work by Castilla et al. (1994) identified eight different germline BRCA1 mutations (four frameshift, two missense and two nonsense mutations) in high-risk individuals using SSCP analysis. The frameshift and nonsense mutations would clearly be disease causing as they result in a loss-of-function of the BRCA1 protein product. By now, it was apparent that BRCA1 was more than a strong candidate, and indeed the gene involved in hereditary breast/ovarian cancer.

2.3.2 BRCA1 Genomic Sequence

The complete BRCA1 genomic sequence was published in 1996 (Smith et al., 1996). The gene (5592bp) consists of 22 coding exons (24 in total – exons 1 and 4 are non-coding) distributed over 84kb of genomic DNA, and codes for a 1863 amino acid protein. The gene is conserved in mammals, but shows little homology to other known genes (Miki et al., 1994). The exon lengths range from 40bp to 3425bp and the intron lengths (which comprise 90.9% of the sequence) range from 403bp to 9193bp (Smith et al., 1996). A zinc finger domain containing cystine and histidine residues (CH3CH4) was found in the amino terminus. These domains are present in nucleic acid binding proteins, suggesting a transcription factor function for BRCA1.

The 7.8Kb transcript was expressed in abundance in testis and thymus, and also breast and ovary. The gene was found to display alternative splicing, which results from the initiation of transcription from one of two sites, 277bp apart. The resultant exons are therefore numbered 1a and 1b. GC boxes, which bind the Sp1 transcription factor are found 5' of exon 1a. Other transcription factor binding sites were identified 5' to exon 1a.

In analysing the BRCA1 gene, ~47% of the sequence was found to consist of *Alu* elements (138), and simple sequence repeats (SSRs - 68) or microsatellites of at least 10 nucleotides in length were detected (Smith et al., 1996). Initially, Miki et al. (1994) misidentified an inserted *Alu* element as exon 4. This is a non-coding exon, and the inserted *Alu* element, not normally present, would introduce a stop codon. The BRCA1

gene has the highest reported density of *Alu* repeats to date. These elements may have a significant effect, as they have been reported to serve as templates for recombination as well as the introduction of sequence errors by slippage of polymerases. These may represent hotspots for mutations within the gene, as there are diseases that are associated with *Alu*-mediated rearrangements and deletions.

Within the BRCA1 gene two complete genes (*Rho7* and *VAT1*), one incomplete gene (*IFP 35*) and two pseudogenes (*rpL21* and 1A1-3B) have been identified (Smith et al., 1996). An unusually low CpG dinucleotide content is found within the BRCA1 gene, except for three regions including the promoter region and the 5'UTR of the *Rho7* and *VAT1* genes. This pattern is not unexpected, as these short stretches of unmethylated GC-rich DNA are usually found in front of housekeeping genes.

The 5' end of the BRCA1 gene was found to lie head to head with the 5'end of the 1A1-3B gene less than 295bp apart (Brown et al., 1994). It was suggested that the two genes were co-regulated by a promoter that worked bidirectionally. The potential involvement of the 1A1-3B gene in breast and ovarian cancer was investigated. The 1A1-3B gene was renamed NBR1, and a new gene related to it, NBR2 was also found to lie head to head with BRCA1 (Xu et al., 1997a). The transcription start site of this gene was found to be in close proximity to that of BRCA1, and it was suggested that the regulation of both genes was under the control of a bi-directional promoter.

2.3.3 Function of the BRCA1 Protein

The BRCA1 gene product was shown to be a 220kDa phosphoprotein, and found to be present in normal breast epithelial cells and breast cancer cell lines (Chen et al., 1995). It was localized in normal cells in the nucleus, but in the cytoplasm of the breast and ovarian cancer cells. The mislocation of the BRCA1 gene product was shown to be more common in end-stage breast cancer.

Initially, clues to the function of the BRCA1 gene came from the presence of the zinc finger motif found near the amino (NH_2) terminus (amino acids 1 –112). This motif, identified in many proteins with DNA binding functions, is a zinc-dependent DNA binding domain, suggesting that BRCA1 may function as a transcription factor. The zinc finger motif requires zinc for folding and DNA binding activity, and is characterised by two conserved cysteine and histidine amino acid residues (Lovering et al., 1993). The BRCA1 protein also contains two putative nuclear localisation signals (at amino acid

position 500-508 and 609-615), a leucine zipper (at amino acid position 1209-1231) and an excess of negatively charged amino acids in the C-terminal region of the gene (Monteiro et al., 1996).

The number of conflicting reports on BRCA1 localisation has resulted in a variety of different suggested functions for the protein. However, Wilson et al. (1999) used immunocytochemistry to locate BRCA1. They confirmed that BRCA1 is a 200kDa molecule that is located in the nucleus of all cells, including malignant breast cells and argued against the fact that BRCA1 is secreted or membrane bound, as was previously found (Jensen et al., 1996). This study also found a correlation between the loss of BRCA1 expression and sporadic breast and ovarian cancers, which suggested that BRCA1 could be involved in the pathogenesis of sporadic breast and ovarian cancers.

When Thompson et al. (1995) compared BRCA1 gene expression in normal mammary epithelium and breast cancer tissue; they found the expression was five-to ten-fold higher in the normal tissue. The BRCA1 gene product was shown to be a negative regulator of mammary proliferation, whereby decreased BRCA1 expression increases the proliferation of breast epithelial cells. Mutations in BRCA1 are therefore thought to disrupt the function of BRCA1 and decrease its expression, leading to an increased growth of mammary epithelial cells.

Holt et al. (1996) analysed the function of BRCA1 in sporadic breast and ovarian cancer. Wild type BRCA1 protein was found to significantly inhibit growth of breast and ovarian cancer cells *in vitro*, while lung and colon cancer cell lines were not inhibited. Different mutations resulting in different lengths of the truncated protein were suggested to have different effects on the inhibition. BRCA1 mutations in the 5'end of the gene did not inhibit growth of any cell line, while mutations in the 3'end of the gene inhibited growth of ovarian cancer cells, but not breast cancer cells.

The carboxy terminal (COOH) of BRCA1 can act as a transcriptional transactivator when fused to the GAL4 DNA binding domain (Chapman and Verma 1996). A minimal amino acid region (1760-1863) is required for the transactivation, with additional sequences in the amino terminal required for maximum activity. If BRCA1 exhibited this transactivational function, it is expected that BRCA1 would bind to DNA and regulate some target genes, however this needed to be demonstrated. Similarly Monteiro et al. (1996) showed that the C-terminal portion of BRCA1, more specifically exons 21 to 24, could transactivate transcription. They also showed that missense and nonsense

mutations in this region would result in the loss of the minimal transactivation domain and not be able to carry out this function. This loss of function was suggested to result in a predisposition to tumour formation in mutation carriers, possibly by failure of BRCA1 to activate transcription of genes involved in suppressing transformation. Mutations that fell outside of this region were also shown to abolish function.

Koonin et al. (1996) examined the globular domains of the BRCA1 protein to find sequence similarities with other proteins. Other than the previously described RING finger, no similarity to other proteins in the database was found. However, a slight similarity between the C-terminus of BRCA1 and a region of the human 53BP1 protein that binds p53 was found. A similar motif was found in the yeast RAD9 protein, involved in G_1 and G_2 checkpoint associated cell cycle arrest in response to DNA damage. A new, conserved domain, consisting of the presence of two tandem domains (as a result of an internal duplication) was found and termed BRCT – BRCA1 C Terminus for each domain. This poorly conserved domain is found in proteins, which are involved in DNA repair such as RAD9 (Koonin et al., 1996).

Chai et al. (1999) investigated whether BRCA1 proteins do interact with p53 as was previously suggested. This was found to be the case, with the minimum transactivation domain sufficient for p53 interaction. This was the first report of a second p53 interaction domain in the second BRCT domain of BRCA1. The BRCT domain of BRCA1 (amino acids 1760-1863) was found to bind to the central region (corresponding to amino acids 79-307) of p53, and is sufficient to transactivate the $p21^{WAF1/CIP1}$ promoter. This binding suggests a BRCA1 function in p53 mediated tumour suppression, apoptosis and DNA repair.

In a report by Scully et al. (1996) the "nuclear dot" pattern of BRCA1 expression was found in the S phase of the cell cycle. The human RAD51 protein (hRAD51), homologous to the bacterial RecA protein, is characterised by a similar dot-like staining (Scully et al., 1997). hRAD51 is a member of a protein family that mediates double stranded DNA break repair and recombination. Scully et al. (1997) showed that BRCA1 and hRAD51 colocalise in S phase cells (and occupy the same region of the synaptonemal complex in prophase) and interact physically. The sequence of BRCA1 exon 11 (specifically amino acid position 758-1064) is indicated to be the binding site for RAD51. These associations suggest that BRCA1 is involved in cell cycle progression, DNA replication, chromosome recombination, and maintaining genome integrity. The interaction of p53 with RAD51, as well as the p53 interaction sequence in BRCA1

(Koonin et al., 1996) all strengthen the evidence for BRCA1 being involved in a DNA damage repair pathway.

Further support for a role of BRCA1 in DNA damage repair came from work by Gowen et al. (1998). Mouse embryonic stem cell lines with wild type and mutated Brca1 alleles were evaluated for their transcription coupled repair after exposure to ionizing radiation. BRCA1-deficient cells were found to be defective in repairing oxidative DNA damage, but not other types of damage, as a result of an incomplete BRCA1/RAD51 complex. A role for BRCA1 in DNA double strand break repair was investigated by Moynahan et al. (1999). Brca1-deficient mouse embryonic stem cells had a significantly reduced homologous repair mechanism; this was also the case when exon 11 (location of the RAD51 interacting domain) was deleted. These results support a role for BRCA1 in preserving genomic integrity by its function in homologous recombination to repair chromosomal DNA double strand breaks.

Wu et al. (1996) identified the BRCA1-associated RING domain protein – BARD1 that contains a RING motif on the amino terminus, and a sequence on the carboxy terminus that is significantly homologous to BRCT. BRCA1 missense mutations were shown to disrupt the BARD1/BRCA1 interaction.

The BRCA1 RING finger domain was studied by Jensen et al. (1998) as a potential protein interface, as it is found in proteins with proto-oncogene and transcription factor functions. They identified a novel protein – BAP1 - localized to the nucleus, having motifs and functions of an ubiquitin hydrolase enzyme. BAP1 was found to bind to the wild type BRCA1 RING finger, and enhance the growth suppression properties of BRCA1. BAP1 was mapped to chromosome 3p21.3 and suggested to be a possible tumour suppressor gene. The ubiquitin hydrolase function of BAP1 is a necessary posttranslational modification to modify protein function. BAP1 was also implicated in the BRCA1/RAD51 complex, and may therefore also play a role cell cycle control and DNA repair.

A study by Wang et al. (1998) isolated the c-Myc proto-oncogene as a BRCA1-binding protein. c-Myc has been shown to be involved in tumorigenesis, embryonic development and apoptosis. BRCA1 was demonstrated to negatively regulate c-Myc-mediated transcription, which lends further support to the hypothesis of BRCA1 as a component of a transcription factor complex. It was suggested that the mechanism of BRCA1 tumour suppressor action is by the down-regulation of the targets of the c-Myc transcription.

Loss of function BRCA1 mutations would therefore increase c-Myc activity and result in a susceptibility to transformation. However, other factors, including the effect of estrogen and progesterone, affect c-Myc expression.

More recently, work by Xu et al. (1999a) has assessed the role of BRCA1 in cell cycle checkpoints. Failure of normal cell cycle checkpoints can result in the accumulation of mutations, which may activate oncogenes and/or inactivate tumour suppressor genes, which would eventually lead to carcinogenesis. Mouse embryonic fibroblast cells with a deletion in exon 11 were shown to have an intact G_1-S checkpoint, but a completely defective G_2-M checkpoint. Therefore, the abnormal cells were allowed to proceed to M phase, and pass on the abnormal chromosomes to daughter cells. Approximately 25% of the cells were also shown to have amplification of their centrosomes, which lead to unequal chromosome segregation, abnormal nuclear division and aneuploidy. These results strengthen the evidence for BRCA1s role in maintaining genomic stability and inhibiting tumorigenesis, as well as a new role in regulating centrosome duplication.

Figure 2.1 shows the major functional domains in the BRCA1 protein.

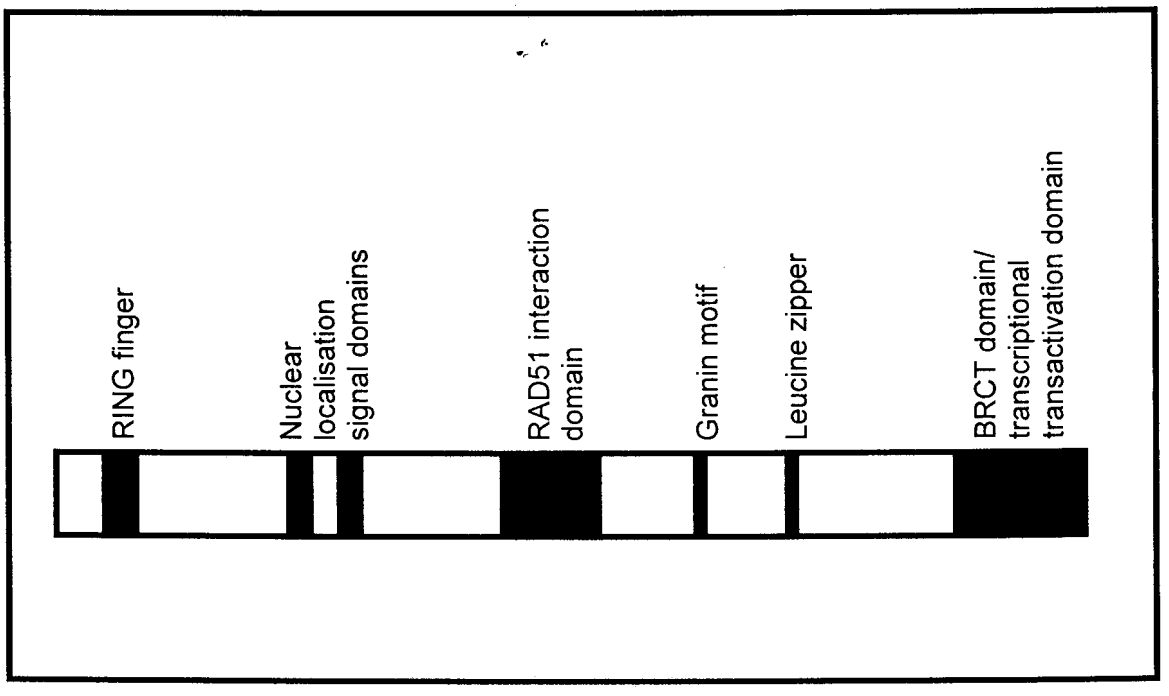


Figure 2.1 Schematic representation of the functional domains in the BRCA1 protein

Most recently, a number of BRCA1-associated proteins were identified by immunoprecipitation and mass spectrometry. The large multisubunit protein complex - BASC - the BRCA1-associated genome surveillance complex, consists of tumour suppressors, DNA damage sensors and signal transducers. (Wang et al., 2000). The BASC contains 15 subunits, including RAD50, and the MSH2, MSH6, MLH1 and PMS2 mismatch proteins, to name a few. Interestingly, BRCA2 and RAD51 were not found in this study, as in others. This result could not be explained. The complex is assumed to sense DNA damage by the ability to bind abnormal DNA structures including double-strand breaks and base-pair mismatches. The role of BRCA1 in this complex is suggested to be that of a scaffold protein that organises the different DNA damage sensors, and coordinates DNA repair in response to the damage. It is also suggested that BASC plays a role in post-replicational repair.

It has been shown that the mouse Brca1 protein shows approximately 58% homology to the human BRCA1 protein. In the mouse it is expressed in rapidly proliferating mammary epithelial cells during pregnancy and is downregulated during lactation. It has also been shown that Brca1 loss is associated with early embryonic lethality (reviewed in Xu et al., 1999b). This is in contrast to a human version, whereby a woman diagnosed with breast cancer at 32 years, with a strong family history of breast/ovarian cancer, was found to be homozygous for the 2800delAA BRCA1 mutation (Boyd et al., 1995). This represents the first and only report of human knockout for the BRCA1 gene. It is assumed that her risk of cancer is similar to that of heterozygotes.

By deleting exon 11 of Brca1 in mice mammary epithelial cells, Xu et al. (1999b) demonstrated that genetic instability and activation of Trp53 transcription led to the formation of mammary gland tumours, and incomplete mammary development during pregnancy. Increased apoptosis was found to have occurred, as well as a number of chromosomal changes and rearrangements (genomic instability). This work supported their previous work (Xu et al., 1999a) showing how BRCA1 is required for maintaining genomic integrity. Both results therefore suggest that Brca1 acts as a "caretaker" and when altered leads to an increased mutation rate in other genes including oncogenes and tumour suppressor genes.

In the mouse, Brca1s role as a "caretaker" is proposed under the current model (which may be applicable to humans) whereby Brca1, Brca2 and RAD51 interact as a complex to repair damaged DNA. Mutations in any of these genes would result in the accumulation of DNA damage and lead to the activation of a checkpoint mechanism

which would in turn activate p53. This would result in the upregulation of p21 which inhibits the cyclin-dependent kinases that allow for progression through the cell cycle, thereby initiating cell cycle arrest. The activation of other genes (some are as yet undiscovered) would result in apoptosis, to prevent the damaged DNA from being passed on to successive daughter cells (Reviewed in Brugarolas and Jacks, 1997).

in summary, there are a number of suggested putative functions for BRCA1. These include a role as a transcription factor for DNA binding, transcriptional transactivation of other genes, normal mammary cell proliferation during pregnancy and lactation, ubiquitin transfer to proteins targeted for degradation, maintaining the genomic integrity of a cell by cell cycle checkpoint signalling in response to DNA damage, transcription coupled and double stranded DNA break repair, homologous recombination, regulating centrosome duplication and apoptosis.

2.4 BRCA1 MUTATIONS

Many researchers began an intensive screen for BRCA1 mutations in breast/ovarian cancer families to establish the frequency and spectrum of mutations. To date, more than 500 unique germline BRCA1 mutations have been reported, that are distributed throughout the gene, leading to a range of truncated protein products. Most of the mutations occur in exon 11 (55%), but this is to be expected, as 62% of the gene is contained in this exon. Of all the mutations, 87% lead to premature termination of the protein product (BIC; Couch et al., 1996). Mutations include frameshift, nonsense, splice site as well as regulatory mutations. Missense mutations have also been reported, but their functional significance is difficult to ascertain. However, the C61G missense mutation in exon 5 removes the penultimate cysteine residue from the zinc finger domain, and is considered a pathogenic missense mutation (Friedman et al., 1994).

Amongst all the reported mutations, some unusual mutations with unexpected consequences have also been identified. One such mutation was identified in a breast and ovarian cancer family by Mazoyer et al. (1998). A nonsense mutation in exon 18 was found to result in the skipping of the in-frame exon 18, rather than lead to the truncation of 169 amino acids from the BRCA1 protein. The removal of exon 18 is expected to have a functional effect on the protein.

Thus far, no association has been found between the type and location of mutation with the age of onset of cancer. However, it has been suggested that mutations in the 3'end of the gene are associated with a relatively lower proportion of ovarian cancers (Gayther et al., 1995; Shattuck-Eidens et al., 1995). The "switch" point is hypothesized to be at the granin motif at codons 1214-1223 (Gayther et al., 1995).

The unexpected absence of somatic BRCA1 mutations seemed contradictory to the function of BRCA1 as a tumour suppressor gene. In normal progression to carcinogenesis, the loss of both BRCA1 alleles in the affected breast tissue is required for transformation. In the familial form of breast cancer, affected individuals are born with a germline BRCA1 mutation. The development of cancer is preceded by the functional loss of the remaining wild type allele in the breast or ovarian tissue. This second defect is usually a large deletion or rearrangement, which is seen as an apparent loss of heterozygosity (LOH) of an allele. In sporadic forms of the disease, two mutations are required in the same cell. However in the study by Futreal et al. (1994) no somatic mutations in breast or ovarian tumours were detected. In a study by Merajver et al. (1995) a BRCA1 mutation screen in sporadic cases of ovarian cancers identified four somatic mutations as well as LOH, lending support to the tumour suppressor function of the BRCA1 gene. New evidence shows that aberrant cytosine methylation of CpG island promoters results in decreased BRCA1 expression in sporadic human breast cancers (Rice et al., 2000).

Initially, Simard et al. (1994) found six novel mutations in 30 Canadian breast/ovarian cancer families. One of these mutations, 185delAG, was identified in four unrelated Ashkenazi Jewish families. Haplotype analysis revealed that these families shared a unique haplotype, even though they were not related. Four other unrelated families shared the 5382insC mutation, which was initially identified by Miki et al. (1994). These families also shared an identical, but unique haplotype. These initial results suggested that certain mutations were recurrent in some population groups.

2.4.1 BRCA1 Mutations In Different Populations

A number of mutations have been frequently reported in some population groups, and haplotype analyses have shown them to be representative of founder effects in the relevant populations. In Russia, BRCA1 mutations occur in 79% of high-risk breast/ovarian cancer families. Most families carrying one of two common mutations. Finnish breast/ovarian cancer families do not have any BRCA1 mutations. BRCA1

mutations can explain a small percentage of high-risk families in Britain (21%), France (24%), Germany (18%), Holland and Belgium(14%), Hungary (22%), Italy (29%), Sweden and Denmark (23%). BRCA1 mutations account for 40% of families in Canada, 47% in Israel and 39% in the United States (Szabo and King, 1997).

The 185delAG mutation has frequently been reported in high-risk Ashkenazi Jewish families (Friedman et al., 1995; Shattuck-Eidens et al., 1995; Simard et al., 1994; Struewing et al., 1995a; Tonin et al., 1995). Population based studies found this mutation to be present in one percent of the general Ashkenazi Jewish population (Struewing et al., 1995b). All these families share the same haplotype, suggesting they all share a common ancestor. Only two families with the 185delAG mutation have been reported to have a different haplotype. These are two families from Yorkshire, England, and are not Jewish. It is clear that there is a common ancestor for the "Ashkenazi Jewish" 185delAG mutation. This mutation was been estimated to have arisen 46 generations ago (Neuhausen et al., 1996a. When Bar-Sade et al. (1998) identified the 185delAG mutation in Ashkenazi and non-Ashkenazi Jewish patients, they found a difference in the associated haplotype. Non-Ashkenazi subjects were found to carry the mutation but a different haplotype, representing a different origin to the Ashkenazi patients. The common ancestor for the 185delAG mutation is now thought to have emerged prior to the divergence of these two population groups, earlier than the estimated 700 years ago.

Similarly, the 5382insC mutation has been reported often in families of European and Canadian origin, also sharing a unique haplotype (Friedman et al., 1995; Shattuck-Eidens et al., 1995; Simard et al., 1994). This mutation is estimated to have originated approximately 38 generations ago, and has been found in families of northern and eastern European origin (Neuhausen et al., 1996a).The 4184del4 mutation has been reported in families of European origin (Friedman et al., 1994; Friedman et al., 1995) as well as non-Caucasian families (Neuhausen et al., 1996a).

A study of Dutch and Belgian breast/ovarian cancer families identified 18 mutations that had not been reported outside the region (Peelen et al., 1997). Twelve mutations were recurrent and accounted for 80% of the families in which a BRCA1 mutation was identified. Haplotype analysis of the 2800delAA mutation (accounting for 24% of mutations detected) suggests that this mutation represents a founder effect in this population, which arose 38 generations ago. In Russia, BRCA1 mutations account for

75% of familial ovarian cancer, with two founder mutations accounting for 86% of all mutations (Gayther et al., 1997).

2.4.2 Risks Associated With BRCA1 Mutations

Once it was clear that mutations in BRCA1 predisposed to early-onset breast and ovarian cancer, the risks associated with carrying such mutations were estimated. Easton et al. (1995), used data on a collection of breast/ovarian cancer families and typed them with the polymorphic D17S579 marker that lies distal to BRCA1, to get maximum LOD scores. Mutations in BRCA1 were estimated to confer a breast cancer risk of 54% by 60 years and an ovarian cancer risk of 30% by 60 years. The overall lifetime penetrance of both breast and ovarian cancer was estimated to be 100%. Previous studies had estimated the risk of breast cancer in BRCA1 mutation carriers to be 85% by 70 years, higher than calculated by Easton et al. (1995). The discrepancy was suggested to be due to the fact that not all breast cancer cases are linked to BRCA1, and the presence of ovarian cancer is usually associated with linkage to BRCA1.

Ford et al. (1994) estimate the risk for breast cancer in BRCA1 carriers to be 73% by 50 years, and 87% by 70 years. The ovarian cancer risk was estimated to be 29% by 50 years and 44% by 70 years. The risk of prostate (8% by 70 years) and colon cancer (6% by 70 years) was also found associated with BRCA1 mutations. A patient carrying a BRCA1 mutation was also found to be at risk for the development of a second cancer.

New statistics suggest that the risk of developing breast cancer in women carrying a BRCA1 mutation differs significantly depending on the year they were born. For women born before 1940 their risk is 17% by 45 years and 38% by 55 years; while the risk increases to 47% by 45 years and 69% by 55 years in women born after 1940. This indicates that there is an environmental component to the risk (King et al., 2000)

The HRAS variable number of tandem repeats (VNTR) polymorphism is located downstream of the HRAS1 proto-oncogene on chromosome 11p15, and is suggested to modify the penetrance of some cancers. When BRCA1 carriers were typed for the VNTR, their ovarian cancer risk was shown to be greater when they carried one of two rare HRAS1 alleles (Phelan et al., 1996). There was no change in their breast cancer risk, however the precise mechanism of action of the HRAS1 alleles in modifying the effect of a BRCA1 mutation is unknown.

2.5 BRCA2 INTERACTIONS

Following on the discovery of BRCA1, a second gene involved in breast/ovarian cancer was mapped to chromosome 13q12-q13 by linkage analysis in 1994 (Stratton et al., 1994; Wooster et al., 1994). By 1995, Wooster et al. (1995) had identified BRCA2 by positional cloning. The BRCA2 gene has a similar structure to that of BRCA1. It consists of 27 exons, with a large exon 11. The 3418 amino acid protein does not show homology to other proteins, and has similar expression patterns to BRCA1 (Tavtigian et al., 1996). Mutations in BRCA2 predispose carriers to breast cancer, and ovarian cancer to a lesser extent than BRCA1. The risk of male breast cancer is significantly increased in BRCA2 mutations carriers, as is the risk to prostate and pancreas cancer.

The mutation spectrum of BRCA2 is similar to that of BRCA1 (Tavtigian et al., 1996; Wooster et al., 1995). Most mutations have only been reported once, but some have been frequently detected in certain population groups, as for BRCA1. The 6174delT BRCA2 mutation is the main BRCA2 mutation reported in Ashkenazi Jews (Neuhausen et al., 1996b), and the 999del5 mutation is the main BRCA2 mutation in Iceland (Thorlacius et al., 1996).

As the structure of BRCA2 is so similar to BRCA1, the functions of the two genes can be assumed to be of a similar nature. It was therefore suggested that BRCA2 also functions in DNA damage repair (Chen et al., 1998). These authors showed an interaction of BRCA1 and BRCA2 with similar responses to DNA damage. They were also found to coexist as nuclear dot structures, and found on synaptonemal complexes. It was apparent that the C-terminal segment of BRCA1 interacts with BRCA2. It is suggested that they participate in a DNA damage repair pathway and homologous recombination. Mutations in either of these genes would therefore disrupt the pathway and eventually lead to tumorigenesis.

2.6 OBJECTIVES

At the onset of this study, no information regarding the role of BRCA1 in inherited susceptibility to breast/ovarian cancer in South African families was known. Therefore the aim of this study was to determine the frequency and nature of *BRCA1* mutations in South African breast/ovarian cancer families. The information gained will allow the opportunity to apply appropriate counselling and clinical management of mutation positive families. It is clear that founder effects exist in some populations, and the Afrikaner population may be expected to exhibit some founder effects.

CHAPTER 3
SUBJECTS AND METHODS

All chemicals used were Analar grade from Merck or BDH, unless otherwise stated.

3.1 ETHICS

This study has been approved by the Ethics Committee of the University of Pretoria, Medical School (Protocol number 18/98). Informed consent was obtained from all patients selected for analysis, as stated in the approved protocol. See appendix for approval form.

3.2 SELECTION OF PATIENTS

Patients referred by private practitioners, and those attending the familial cancer clinic, run by the Department of Human Genetics, University of Pretoria, were studied. Selection of families to be included was based on the number of affected persons (three or more cases), age at diagnosis (breast cancer cases < 50 years and/or ovarian cancer cases < 60 years), presence of bilateral disease and the occurrence of two or more primaries in an individual. Patients that were eligible for this study based on the above criteria included 51 families, six of whom are Ashkenazi Jewish. One family was of German origin, one South African Indian, and the remaining families were either Afrikaner or Afrikaner and British/Irish/Lebanese/ Portuguese/Scottish/Welsh. In total, 21 families are breast-ovarian cancer families, 27 families are breast cancer only families, and 2 families are ovarian cancer only families. Four families had at least one case of male breast cancer. Details of the families, as well as all other confirmed cases of cancer are listed in table 3.1. Generally only one affected individual from each family was subjected to the mutation analysis. In nine families (Families BRC 7, 9, 11, 13, 15, 26, 39, 41, 44) an additional affected member was also tested, as it was thought to increase the chance of finding a mutation, and it was not immediately obvious from the pedigree which member would be more likely. For example, if one member had bilateral breast cancer, but a sibling had unilateral cancer at a young age they were both screened.

Table 3.1 Phenotypes of Families Studied

Family	Number of breast cancer cases (Bilateral cases)	Age at onset		Age unknown	Number of ovarian cancer cases (Bilateral cases)	Other Cancers	Ethnic group
		≤ 50 years	> 50 years				
BRC 1	3 (1)	2	2				Ashkenazi Jewish
BRC 4	4 (2)	2	2		2		Afrikaner
BRC 5	11 (2)	9	4		1		Afrikaner
BRC 6	3	3				Lung, stomach, thyroid	Afrikaner
BRC 7	7 (2)	2	4	2		Kidney, liver, prostate, stomach, testis, uterus	Afrikaner
BRC 9	3 (1)	1	1	1	1	Stomach	Afrikaner
BRC 10	6 (2)	4	1	1		Bladder, lung, melanoma	Afrikaner
BRC 11	6 (2)	7	1		2	Stomach	Afrikaner
BRC 12	4	3		1			Afrikaner
BRC 13	4 (1)	5			1	Oesophagus	Ashkenazi Jewish
BRC 14	3	2	1				Afrikaner
BRC 15	19 (4)	10	3	6		Cervix, prostate	Afrikaner
BRC 17	11 (2)	4	4	3	1	Lung, pancreas	Afrikaner

Family	Number of breast cancer cases (Bilateral cases)	Age at onset		Age unknown	Number of ovarian cancer cases (Bilateral cases)	Other Cancers	Ethnic group
		≤ 50 years	> 50 years				
BRC 18	6 (1)	4		2		Prostate, skin	Afrikaner
BRC 19	3	2	1		1	Colon, skin, throat, thyroid	Afrikaner
BRC 20[a]	7			3		Prostate, skin, stomach, uterus	Afrikaner
BRC 21	1		1		2		Afrikaner
BRC 22	3 (1)	3					Afrikaner
BRC 23	2 (2)	1	1				Ashkenazi Jewish
BRC 24	4			4		Colon	English
BRC 26	1	1			1 (1)	Colon, prostate	Afrikaner
BRC 27	4	4			2	Colon	Afrikaner
BRC 28	3	2	1				Afrikaner & English
BRC 29	4	3	1			Liver, prostate, stomach	Afrikaner
BRC 30	2 (1)	1	1				Afrikaner & Lebanese

Family	Number of breast cancer cases (Bilateral cases)	Age at onset ≤ 50 years	Age at onset > 50 years	Age unknown	Number of ovarian cancer cases (Bilateral cases)	Other Cancers	Ethnic group
BRC 31	4	3		1		Colon, liver, lung, prostate, uterus	Afrikaner
BRC 32	3 (1)	2		1	1		Afrikaner
BRC 33[b]	7 (1)	1	1	5			Afrikaner
BRC 34[c]	4 (1)	2	3		1	Kidney	German
BRC 35	2	1		1	4 (1)		Ashkenazi Jewish
BRC 38	2	1	1		1		Afrikaner
BRC 39	6	4	1	1			Afrikaner & Irish
BRC 40	3	2	1				English
BRC 41	6	4	2				Afrikaner
BRC 42	2	1	1				Afrikaner
BRC 43	4(1)	2	2				Afrikaner & Irish
BRC 44	3	2	1				Afrikaner, English & Welsh
BRC 45	3 (1)	2	1				Afrikaner
BRC 46	4	4					Afrikaner

Family	Number of breast cancer cases (Bilateral cases)	Age at onset		Age unknown	Number of ovarian cancer cases (Bilateral cases)	Other Cancers	Ethnic group
		≤ 50 years	> 50 years				
BRC 47	1	1					Ashkenazi Jewish
BRC 48	7 (1)	5	3		1	Liver, prostate, throat	Indian
BRC 50	3	1	2		1		Portuguese
BRC 51[d]	1	1			1	Kidney, endometrial	Ashkenazi Jewish
BRC 52	3	1	2				English
BRC 53	2 (2)	1	1				Afrikaner
BRC 55	1			1	3		Scottish
OV 1	0				6	Leukemia, lung, stomach	Afrikaner
OV 2	0				2 (2)	Liver, stomach	Afrikaner
OV 3	1		1		3	Pancreas	Ashkenazi Jewish
FS 1	7 (2)	6	3		4	Testes	Afrikaner
FS 2	3			3	1		Afrikaner

[a] There are 4 cases of male breast cancer in this family.
[b] There are 2 cases of male breast cancer in this family.
[c] The index case has fallopian tube cancer. There are 2 cases of "gynaecologic" cancers and one case of male breast cancer in this family.
[d] The index case has breast, endometrial and ovarian cancer.

3.3 LABORATORY METHODS

3.3.1 DNA Extraction

Genomic DNA was extracted from peripheral blood of breast or ovarian cancer patients, using standard methods as described by Johns and Paulus-Thomas. (1989). This method involves the lysis of white blood cells from a 10ml EDTA blood sample by the addition of 30ml lysis buffer, pH 8.0 (0.32M sucrose / 10mM Tris-HCl / 5mM $MgCl_2$ / 1% Triton X-100), incubation on ice for ten minutes, followed by centrifugation at 9000rpm for 30 minutes at 4°C in a Beckman J-21 centrifuge, using a JA-17 rotor. The resulting pellet was resuspended in 9ml suspension buffer pH 8.0 (10mM Tris-HCl / 0.15mM NaCl / 5mM EDTA). The lysis was continued by the addition of 1ml 10% SDS (1% final concentration), 2.5ml freshly made 5M $NaClO_4$ (1M final concentration) and 12.5ml chloroform/isoamyl alcohol (24:1), and left on a Red Rotor™ Orbital Shaker for 30 minutes. After centrifugation at 1800rpm for 15 minutes at 20°C, the aqueous phase was removed to a new tube and a second chloroform/isoamyl extraction was performed (i.e. addition of 12.5ml 24:1 chloroform/isoamyl alcohol, shake for 30 minutes and centrifuged). After the second centrifugation, the aqueous phase was transferred to a beaker and the addition of two volumes (25ml) ice-cold 100% ethanol precipitated the DNA, which was then spooled onto a glass rod and dried for three minutes in a Savant Speedvac concentrator (model SVC100). The dried DNA pellet was resolubilized in 500µl TE buffer pH 8.0 (10mM Tris-HCl / 1mM EDTA). The concentrations of all dissolved DNA samples were determined by spectrophotometric analysis at 260 and 280nm on a Unicam 8625Series UV/Visible Spectrometer.

3.3.2 Mutation Analysis

The large size of the BRCA1 gene makes mutation analysis of the entire gene time consuming, costly and labour intensive. Therefore, three different approaches were used to screen the families. Initially all the families were screened for the three recurrent mutations: - 185delAG in exon 2, 4184del4 in exon11 and 5382insC in exon 20, using polymerase chain reaction (PCR) and allele specific oligonucleotide (ASO) hybridisation. It has been reported that 87% of all BRCA1 mutations result in truncated or absent proteins (Couch et al., 1996). As most disease-causing mutations lead to truncation of the protein product and

the large size of exon 11 (it comprises approximately 60% of the size of BRCA1 gene), the protein truncation test (PTT) was used to detect truncating mutations in exon 11. The remainder of the gene, as well as the exon/intron boundaries of exon 11 was screened by single-strand conformation polymorphism/heteroduplex analysis (SSCP/HA). Exon 11 was only screened for truncating mutations by the PTT and not analysed further by SSCP/HA.

3.3.2.1 Polymerase Chain Reaction (PCR) and Allele Specific Oligonucleotide (ASO) hybridisation for recurrent mutations

Polymerase Chain Reaction (PCR) and Allele Specific Oligonucleotide (ASO) hybridisation was used to identify the three commonly occurring mutations; 185delAG, 4184del4 and 5382insC in exon 2, 11 and 20 respectively. For each specific mutation the appropriate exon was amplified in a 20μl reaction including 50ng template genomic DNA, 10X buffer (200mM Tris-HCl pH8.4, 500mM KCl), 10X $MgCl_2$, 0.25μM each dNTP, 0.2μM each primer and 0.5 units *Taq* DNA Polymerase (GibcoBRL Life Technologies). The cycling conditions used were an initial three minute denaturation step at 94°C, followed by 35 cycles of 94°C - one minute, annealing temperature (T_{ann}) - one minute, 72°C - one minute, and a final extension step at 72°C for seven minutes, on a PTC-100 Programmable Thermal Controller (MJ Research, Inc). Following PCR, 5μl was loaded on a 1.6% agarose gel and visualised with ethidium bromide staining. Table 3.2 lists the primers, annealing temperatures (T_{ann}), $MgCl_2$ concentrations and product sizes for each exon amplified.

Table 3.2: PCR primers for recurrent BRCA1 mutations

Exon	Sequence	T_{ann} (°C)	$MgCl_2$ conc. (mM)	Product size (bp)
2	2F 5'- gaa aat gaa gtt gtc att t -3' 2R 5'- aca tac tag gga aga aaa gac -3'	55	3.0	264
11	11F 5'-aaa ggc atc tca gga aca tca – 3' 11R 5'-ttt atg ctt ttg ggg gag cac -3'	52	1.5	344
20	20F 5'-gct cca cca ctc cat tga ag -3' 20R 5'-ctg tgt aat ttg gat tcc c -3'	56	2.0	381

For the dot blotting procedure, 5µl PCR product was diluted with 15µl TE buffer pH 7.6 (10mM Tris-HCl / 1mM EDTA), and denatured at 99°C for ten minutes before blotting onto a dot-blot apparatus. This was done in duplicate to enable hybridisation to mutant as well as wild type ASOs. The DNA was vacuum-pulled through the apparatus and bound to a positively charged membrane (Hybond-N+, Amersham). After blotting, the membrane was air-dried and both sides of the membrane were cross-linked on a UV light box for five minutes. The ASOs, as listed in table 3.3, were end labelled with Digoxigenin-11-ddUTP as per the manufacturer's instructions (Boehringer Mannheim DIG Oligonucleotide 3' end labelling kit). The table also lists the appropriate melting temperatures (T_m) of each ASO.

Table 3.3: ASOs and hybridisation conditions for common mutations

Exon	ASO	T_m (°C)
2	23 delAG aaa tct tag tgt ccc	42
	23 WT aat ctt aga gtg tcc	42
11	1355del4 gaa aat aag aag agc a	42
	1355WT aaa taa tca aga aga g	40
20	InsC1755 aga atc ccc agg a	40
	WT1755 aga atc cca gga c	40

Each membrane was placed in a separate hybridisation bottle, with 1ml hybridisation solution/10cm^2 membrane. The salmon sperm DNA (20µg/1ml hybridisation solution) was first denatured at 99°C for ten minutes, placed on ice and then added to the hybridisation solution (5XSSPE / 5X Denhardts solution / 0.1% SDS). Prehybridisation was carried out at 5°C less than the T_m for two hours. The DIG-labelled ASO was used at a concentration of 5ng/ml hybridisation solution, and hybridisation was carried out overnight at 5°C less than the T_m. After hybridisation, the membranes were washed with 5XSSPE / 0.1% SDS for 15 minutes at room temperature, and then with prewarmed 2XSSPE / 0.1% SDS for 15 minutes at the T_m. Chemiluminescent detection with CDP-Star™ was performed as stated by the manufacturer's instructions (DIG Wash and Block Buffer Set, Anti-Digoxigenin-AP, CDP-Star, Boehringer Mannheim). The membranes were then exposed to Lumi-Film Chemiluminescent Detection Film (Boehringer Mannheim) for five minutes before developing. A positive hybridisation signal (seen as a black dot on the autoradiographic

film) seen on both blots (i.e. hybridisation to wild type and mutant ASO) indicated the presence of the specific BRCA1 mutation, as the BRCA1 mutations are heterozygous. Individuals found to harbour the common mutations were sequenced to confirm the presence of the specific mutation.

3.3.2.2 Protein Truncation Test (PTT)

Families in whom the recurrent mutations were not found to occur, were screened for truncating mutations in exon 11, using the Protein Truncation Test (PTT), as described by Hogervorst et al., 1995. This technique was used, as approximately 94% of exon 11 BRCA1 mutations are nonsense or frameshift mutations that lead to a truncated protein (Couch et al., 1996). The PTT involved amplification of exon 11 in three overlapping fragments (see table 3.4), followed by coupled transcription-translation reaction, and resolution of the resulting proteins on SDS-polyacrylamide gels. Possible mutations where seen as shifts in the banding pattern.

Each fragment was amplified in a 20µl reaction containing 100ng template genomic DNA, 10X Expand^{TM} High Fidelity buffer, 10X $MgCl_2$, 0.25µM each dNTP, 0.2µM each primer and 2.1 units Expand^{TM} High Fidelity PCR system mix (Boehringer Mannheim). This enzyme mix containing *Taq* DNA polymerase and *Pwo* DNA polymerase allows for the specific amplification of large DNA fragments with a high yield and high fidelity. TaKaRa ExTaq^{TM} was also used for amplification, using 100ng DNA, 10X buffer ($MgCl_2$ included), 0.25µM each dNTP, 0.2µM each primer, 1.25- and 2 units Taq for fragments A, B and C respectively. Each forward primer had been modified to include a T-7 RNA polymerase promoter, and a eukaryotic translation initiation sequence, to allow for an *in vitro* coupled transcription/translation reaction (Hogervorst et al., 1995). The PCR cycling conditions were 94°C for four minutes, followed by 30 cycles of; 94°C - one minute, annealing temperature (T_{ann}) - one minute, 68°C - two minutes, and a final 68°C extension step of seven minutes, on a PTC-100 Programmable Thermal Controller (MJ Research, Inc). After amplification, 5µl of the PCR product was electrophoresed on a 1% agarose gel.

Table 3.4: PTT primers and conditions

Fragment	Primer	T_{ann} (°C)	$MgCl_2$ conc. (mM)	Product size (kB)
11A PTT2F	5'-*ct tgt gaa ttt tct gag acg g-3'	58	1.5	1.3
PTT2R	5'-atg agt tgt agg ttt ctg ctg tg-3'			
11B PTT3F	5'- *ac aat tca aaa gca cct aaa aag-3'	52	1.5	1.4
PTT3R	5'-aac ccc taa tct aag cat agc att c-3'			
11C PTT4F	5'- *ca cca ctt ttt ccc atc aag tc-3'	52	2.5	1.1
PTT4R	5'-att att ttc ttc caa gcc cgt tcc-3'			

* - T7 promoter sequence - *cgc taa tac gac tca cta tag gaa cag acc acc atg g*

The PTT was performed using a slightly modified protocol of the TnT® T7 Quick Coupled Transcription/Translation System (Promega). A 15μl reaction consisting of 2μl PCR product, 10μCi L-[^{35}S] methionine (1000Ci/mmol, Amersham Life Science) and 12μl TnT® T7 Quick Master Mix was incubated at 30°C for 90 minutes and then placed on ice. Thereafter, 5μl PTT product was diluted with 20μl SDS sample buffer (0.0625M Tris-HCl pH 6.8, 20% glycerol, 2% SDS, 0.025% bromophenol blue, 5% 2 βMercaptoethanol). These diluted products were denatured at 99°C for two minutes and loaded on a 12% denaturing SDS-polyacrylamide gel (4% stacking gel - 37:1 acrylamide:bisacrylamide, 0.125M Tris-HCl pH 6.8, 0.1% SDS, 0.1% TEMED, 0.05% ammonium persulphate; 12% separating gel - 37:1 acrylamide:bisacrylamide, 0.375M Tris-HCl pH8.8, 0.1% SDS, 0.05% TEMED, 0.05% ammonium persuplhate). Electrophoresis was carried out at a constant current of 15mA in the stacking gel and 30mA in the separating gel, together with 1.5μg BenchMark™ Prestained Protein Ladder (Gibco Life Technologies). Gels were run at 18°C in the Protean®II xi Cell system (Biorad) in running buffer (25mM Tris-HCl, 192mM Glycine, 0.1% SDS) for two hours, or until the bromophenol blue reached the bottom of the gel. The gels were lifted onto blotting paper, covered with saran wrap, dried in a vacuum slab dryer (Hoefer Scientific Instruments) for two hours at 60°C, and then exposed to autoradiographic film (Cronex X-Ray Film). Possible mutations were seen as shifts in the banding pattern and sequenced to identify the mutation.

3.3.2.3 Polymerase Chain Reaction (PCR) Single Strand Conformation Polymorphism (SSCP) and Heteroduplex Analysis (HA)

Screening for *BRCA1* mutations in the remaining 21 exons as well as the 5' and 3' end of exon 11 (not covered by the PTT) was conducted by single-strand conformation polymorphism/heteroduplex analysis (SSCP/HA) (Breast Cancer Information Core 1997). Each exon was amplified in a 20µl reaction containing 50ng template genomic DNA, 10X buffer (200mM Tris-HCl pH8.4, 500mM KCl), 10X $MgCl_2$, 0.25µM each dNTP, 0.2µM each primer and 0.5 units *Taq* DNA Polymerase (GibcoBRL Life Technologies). The cycling conditions used were an initial three minute denaturation step at 94°C, followed by 35 cycles of 94°C - one minute, annealing temperature (T_{ann}) - one minute, 72°C - one minute, and a final extension step at 72°C for seven minutes, on a PTC-100 Programmable Thermal Controller (MJ Research, Inc). Following PCR, 5µl PCR product was loaded on a 1.6% agarose gel and visualised with ethidium bromide staining. The primers used were essentially as described by Friedman et al. (1994) with some exceptions (see table 3.5).

Table 3.5: SSCP/HA primers and PCR conditions

Exon	Primer		T_{ann} (°C)	$MgCl_2$ conc. (mM)	Product size (bp)
2	**2F**[a]	5'- aaaatgaagttgtcattttataaacc -3'	52	2.5	262
	2R	5'-gtcttttcttccctagtatgt-3'			
3	**3F**[b]	5'- aacgaacttgaggccttatg -3'	52	2.5	308
	3R[b*]	5'- ttggatttttcgttctcactt -3'			
5	**5F**	5'- ctcttaagggcagttgtgag-3'	55	1.5	278
	5R	5'-atggttttataggaacgctatg-3'			
6	**6F**	5'- cttattttagtgtccttaaaagg -3'	52	2	206
	6R*	5'-tttcatggacagcacttgagtg-3'			
7	**7F**[c]	5'- ggtttctcttggtttctttg -3'	55	2.5	326
	7R[d]	5'- aggactgcttctagcctg-3'			
8	**8F**	5'- tgttagctgactgatgatggt -3'	52	1.5	267
	8R*	5'- atccagcaattattattaaatac-3'			
9	**9F**	5'- ccacagtagatgctcagtaaata -3'	55	2	211
	9R	5'-taggaaaataccagcttcataga-3'			
10	**10F**[c]	5'- gatcttggtcatttgacagttc -3'	52	2	240
	10R[c]	5'-cccaaatggtcttcagaata -3'			
11-5'	**11-5F**	5'- ggaattaaatgaaagagtatgagc -3'	52	2.5	440
	11-5R[c]	5'-ctaagccaggctgtttgctt-3'			
11-3'	**11-3F**[c]	5'- aaaggcatctcaggaacatca -3'	52	2.5	344
	11-3R*	5'- gtgctccccaaaagcataaa -3'			
12	**12F**	5'- gcgtttatagtctgcttttaca -3'	52	2	227
	12R	5'-tgtcagcaaacctaagaatgt -3'			
13	**13F**	5'- aatggaaagcttctcaaagta -3'	52	2.5	320
	13R	5'-atgttggagctaggtccttac -3'			
14	**14F**	5'- ctaacctgaattatcactatca -3'	55	2.5	312
	14R	5'-gtgtataaatgcctgtatgca-3'			

Exon	Primer		T_{ann} (°C)	$MgCl_2$ conc. (mM)	Product size (bp)
15	**15F**[c]	5'- cagacttctaggctgtcttgc -3'	55	2.5	378
	15R[c]	5'-gtgtttgttccaatacagcag-3'			
16	**16F**	5'- aattcttaacagagaccagaac -3'	52	2	450
	16R	5'-aaaactctttccagaatgttgt-3'			
17	**17F**[c]	5'- agctgtgtgctagaggtaactc -3'	55	2	190
	17R[c]	5'-gtggttttatgcagcagatg -3'			
18	**18F**	5'- ggctctttagcttcttaggac -3'	55	2	258
	18R[c*]	5'-ctcagactcagcatcagc-3'			
19	**19F***	5'- ctgtcattcttcctgtgctc -3'	55	2.5	249
	19R	5'-cattgttaaggaaagtggtgc-3'			
20	**20F**	5'-atatgacgtgtctgctccac -3'	56	2	232
	20R	5'-tgcaaaggggagtggaatac -3'			
21	**21F**	5'- aagctcttccttttttgaaagtc -3'	52	2.5	299
	21R	5'-gtagagaaatagaatagcctct-3'			
22	**22F**	5'- tcccattgagaggtcttgct -3'	55	2.5	297
	22R	5'-gagaagacttctgaggctac -3'			
23	**23F**	5'- cagagcaagaccctgtctc -3'	60	3	255
	23R	5'- actgtgctactcaagcacca - 3'			
24	**24F**	5'- atgaattgacactaatctctgc -3'	55	2	280
	24R	5'-gtagccaggacagtagaagga-3'			

[a] Rohlfs et al., 1997; [b] Miki et al., 1994, [c] BIC, [d] created own primer.
*These primers have been modified, according to the correct intron sequence

Following PCR, 5µl of the amplified products were diluted with 2µl formamide dye (95% formamide, 0.125M EDTA, 0.0025% xylene cyanol, 0.0025% bromophenol blue). Before loading the samples, they were denatured at 95°C for five minutes and then placed on ice. The samples were run on 0.6X MDE®(Mutation Detection Enhancement, FMC Bioproducts) gels, in 0.6X TBE (0.892M Tris-HCl / 0.02M EDTA pH 8.0 / 0.890M Boric acid) on the Protean®II xi Cell system (Biorad), at 10°C overnight (between 19 and 24 hours, depending on the size of the exon). The gels were stained according to the silver staining protocol (Breast Cancer Information Core 1997. Gels were lifted onto blotting paper, covered with saran wrap and dried under a vacuum (Slab Gel Dryer, Hoefer Scientific Instruments) for 2

hours at 80°C. Possible mutations were seen as either SSCP and/or heteroduplex shifts on the gels, and sequenced to identify the mutations.

3.3.3 Sequencing

All shifts obtained by the PTT and SSCP/HA were sequenced to confirm the mutation. Before sequencing, the PCR products of the relevant exon were pretreated with 10 units Exonuclease I to remove any single stranded DNA (including primers) remaining from the PCR, and 1 unit Shrimp Alkaline Phosphatase to remove the excess dNTPS. Both these enzymes are activated at 37°C (for 15 minutes) and inactivated at 80°C (for 15 minutes). Sequencing was carried out by a modified protocol of the T7 Sequenase v2.0 PCR product kit (Amersham Life Science). An annealing mix consisting of 3μl pretreated PCR product, 20pmol primer and 10% DMSO was incubated at 99°C for three minutes, to denature the PCR product and allow the primer to anneal to its complementary sequence. The labelling mix consisted of 5X Sequenase buffer, 5X labelling mix, 5μCi (1000Ci/mmol) [α^{35}S] dATP (Amersham Pharmacia Biotech and ICN pharmaceuticals Inc) and 3.2 units Sequenase. 6.5μl of this labelling mix was added to the annealed DNA and incubated at 20°C for two minutes. 3.4μl of this labelled DNA was then added to 2.5μl of each ddNTP and incubated at 37°C for 30 minutes. The reaction was stopped by the addition of 4μl stop solution. Before these samples were loaded on a 6% denaturing polyacrylamide gel, they were heated to 80°C for three minutes. Gels were run for approximately two hours, lifted onto blotting paper, covered with saran wrap and dried under vacuum for two hours at 60°C.

All primers used in amplifying for the ASO and SSCP/HA, as well as the reverse PTT primers used to amplify exon 11, were used to sequence. However, the large size of the exon 11 overlapping fragments necessitated the need for other primers within the exon to enable the sequencing of smaller fragments. The PTT forward primers were not used to sequence, but another primer with a sequence complimentary to the T7 sequence was used. All primers used were as described by Castilla et al. (1994) (see table 3.6), except for the BRC 1-11:MF primer, which is described by Friedman et al. (1994).

Table 3.6: Exon 11 Sequencing primers

Primer	Sequence	cDNA position
BRC 1-11:2R	5'- ccccatcatgtgagtcatcaga - 3'	1323
BRC 1-11:4R	5'- ttgtgaggggacgctcttgta -3'	1617
BRC 1-11:5F	5'- gcatttgttactgagccacagata - 3'	1572
BRC 1-11:6F	5' – caaacggagcagaatggtca – 3'	1731
BRC 1-11:8R	5' – tttgcaaaacccttctccactta – 3'	2397
BRC 1-11:9R	5'- ttttgccttccctagagtgctaac -3'	2504
BRC 1-11:10F	5' - tatggcactcaggaaagtatctcg -3'	2448
BRC 1-11:11F	5' – acagtcgggaaacaagcatagaa –3'	2635
BRC 1-11:11R	5'- tttggcattatcaactggcttatc -3'	2948
BRC 1-11:12F	5'-aggctttcctgtggttggt -3'	2900
BRC 1-11:14R	5'-ggcccctcttcggtaacc -3'	3737
BRC 1-11:15F	5'-tcctagccctttcacccataca -3'	3686
BRC 1-11:15R	5' – agatgcctttgccaatattacctg – 3'	3959
BRC 1-11:MF	5'-ttgaatgctatgcttagattagggg-3'	3357
T7 sequence	5' – ctataggaacagaccaccatgg – 3'	

3.3.4 Genotyping

Patients with the same mutations underwent genotype analysis, involving the typing of polymorphic microsatellite markers within and flanking the BRCA1 gene. This was to determine whether these are independent events or whether they have arisen once. Unaffected members from families BRC1, 5 and 11 were also typed to deduce haplotypes. The following four polymorphic markers were used in the analysis; D17S1320, D17S855, D17S1322 and D17S1323, as used by Simard et al. (1994), and Neuhausen et al. (1996a). The D17S1320 marker flanks the BRCA1 gene, the remaining three markers are intragenic; D17S1323 in intron 12, D17S1322 in intron 19, and D17S855 in intron 20 (Smith et al., 1996). Figure 3.1 is a diagrammatic representation of the four markers used, in order from the centromere (cent) to the telomere (tel).

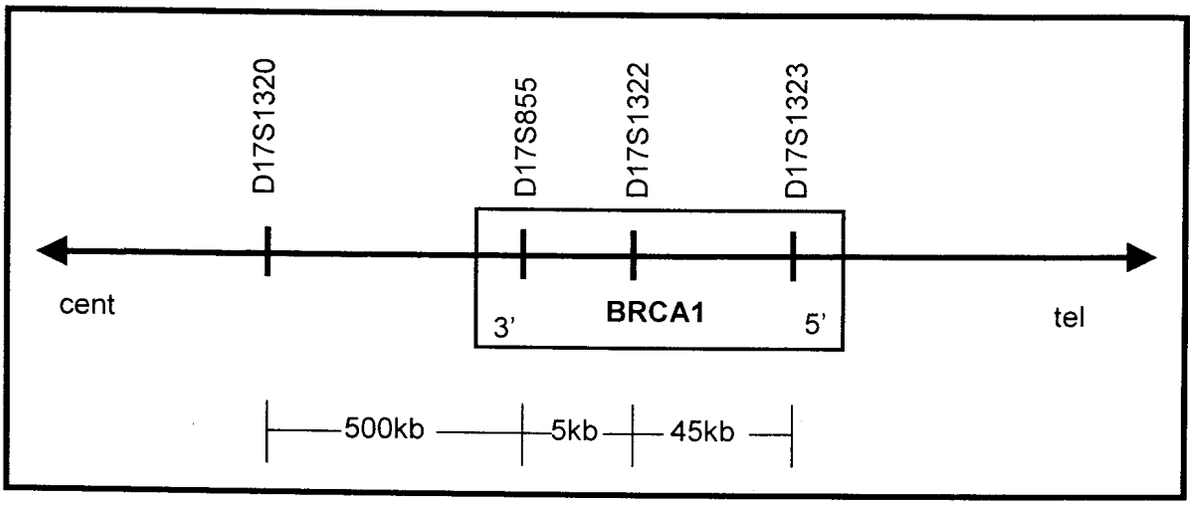


Figure 3.1 Schematic representation of BRCA1 genotype markers

One of each primer pairs was end-labelled in a 10μl reaction, by incubating the following mix at 37°C for one hour, followed by incubation at 95°C for five minutes, and then placing the mix on ice; 20pmol primer, 10X T4 polynucleotide kinase buffer (70mM Tris-HCl pH 7.6 / 10mM $MgCl_2$ / 5mM DTT), 5 units T4 polynucleotide kinase (Promega) and 41.675μCi (7000Ci/mmol) [γ^{32}P] dATP (Amersham Pharmacia Biotech and ICN pharmaceuticals Inc). Each marker was amplified in a 20μl reaction containing 50ng DNA, 10X buffer (200mM Tris-HCl pH8.4, 500mM KCl), 10X $MgCl_2$, 0.25μM each dNTP, 0.2μM each primer, 0.02μM end-labelled primer and 0.5 units *Taq* DNA Polymerase (GibcoBRL Life Technologies). The cycling conditions used were an initial three minute denaturation step at 94°C, followed by 35 cycles of 94°C - one minute, annealing temperature (T_{ann}) - one minute, 72°C - one minute, and a final extension step at 72°C for seven minutes, on a PTC-100 Programmable Thermal Controller (MJ Research, Inc).

After amplification, 10μl formamide dye (95% formamide, 0.125M EDTA, 0.0025% xylene cyanol, 0.0025% bromophenol blue) was added to all samples. These samples were then heated at 99°C for five minutes and placed on ice, before loading 3μl on a 6% denaturing polyacrylamide gel. A sequencing ladder was run at the same time to determine the sizes of the markers and hence the alleles. The gels were run for approximately three hours, until

the bromophenol blue had run off one hour. Table 3.7 shows the primers used for each marker, marker details, and PCR conditions (T_{ann}, $MgCl_2$ and product sizes).

Table 3.7: Primers for genotype analysis

Primer	Sequence	T_{ann} (°C)	$MgCl_2$ conc. (mm)	Product size (bp)
D17S1320-F D17S1320-R	5' - act ttc cag aaa atc tct gct c - 3' 5' - cca cgt ctt ttc tgt gtt cc - 3'	52	1.5	172-182
D17S855-F D17S855-R	5' - gga tgg cct ttt aga aag tgg - 3' 5' - aca cag act tgt cct act gcc - 3'	55	1.5	143-155
D17S1322-F D17S1322-R	5' - cta gcc tgg gca aca aac ga - 3' 5' - gca gga agc agg aat gga ac - 3'	55	1.5	121-139
D17S1323-F D17S1323-R	5' - tag gag atg gat tat tgg tg - 3' 5' - aag caa ctt tgc aat gag tg - 3'	52	1.5	151-161

The markers have been assigned allele letters according to Simard et al. 1994, and allele numbers according to Neuhausen et al 1996, as shown in the table below. Tables 3.8 – 3.11 show allele letters and numbers for haplotyping markers. The allele frequencies are those deposited in the Genome Database (GDB), and based on a European, Caucasian population.

Table 3.8: Allele frequencies of D17S1320 marker

Size (bp)	Frequency	Allele number
182	0.04	3
180	0.09	4
178	0.5	5
176	0.06	6
174	0.27	7
172	0.03	8

Table 3.9: Allele frequencies D17S855 marker

Size (bp)	Frequency	Allele number	Allele letter
155	0.05	2	B
153	0.19	3	C
151	0.18	4	D
149	0.15	5	E
147	0.12	6	F
145	0.26	7	G
143	0.05	8	H

Table 3.10: Allele frequencies of D17S1322 marker

Size (bp)	Frequency	Allele number	Allele letter
139	0.04	1	A
136	0.03	2	B
133	0.11	3	C
130	0.25	4	D
127	0.51	5	E
124	0.04	6	F
121	0.01	7	G

Table 3.11: Allele frequencies of D17S1323 marker

Size (bp)	Frequency	Allele number	Allele letter
161	0.02	1	A
159	0.04	2	B
157	0.19	3	C
155	0.03	4	D
153	0.04	5	E
151	0.68	6	F

CHAPTER 4

RESULTS AND DISCUSSION

4.1 BRCA1 GENE MUTATIONS

A total of seven different disease-causing mutations were detected in 15 of the 51 families screened (29.4%). Five (71.4%) of these mutations were frameshift mutations, and two (28.6%) were nonsense mutations. The PTT detected three different mutations in six families (11.8%), all of which are novel. The SSCP/HA technique identified two families with mutations (3.92%). A number of missense mutations, unclassified variants and polymorphisms were also detected. Interestingly, no splice-site mutations were detected, even though there have been reports of such mutations in other studies.

4.1.1 Recurrent Mutations

Three recurrent mutations were identified in the study, namely the commonly occurring 185delAG and 5382insC, and the novel E881X mutation (as shown in figures 4.1 – 4.5).

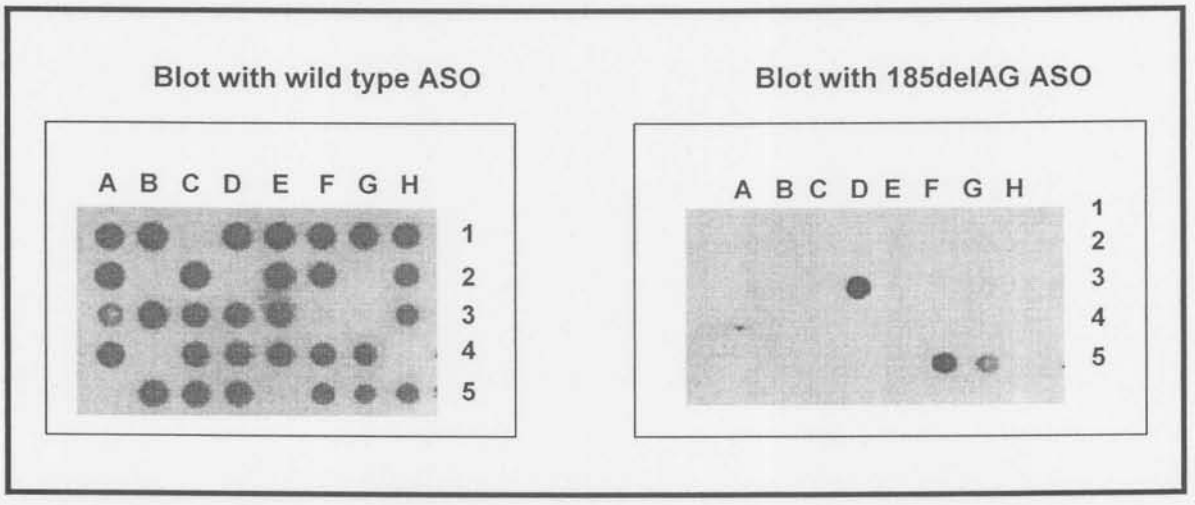


Figure 4.1: Dot blot analysis of 185delAG mutation
D3 is a positive control, patients at positions F5 and G5 carry the 185delAG mutation.

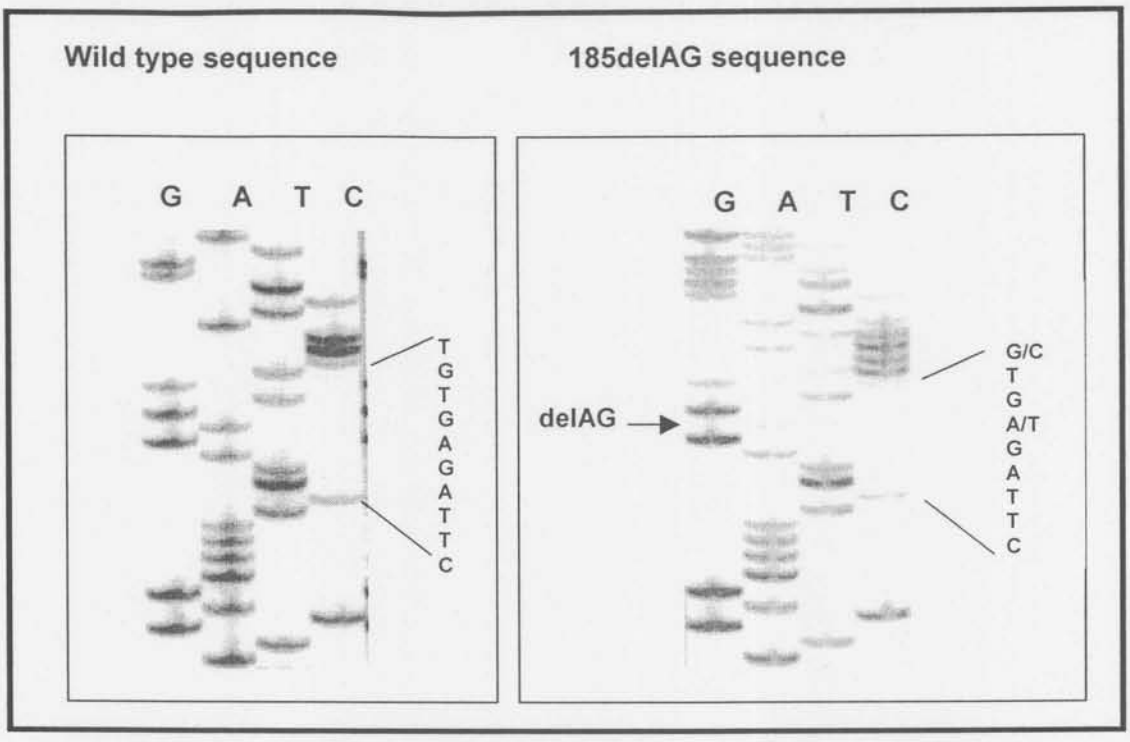


Figure 4.2: Sequence analysis of 185delAG mutation

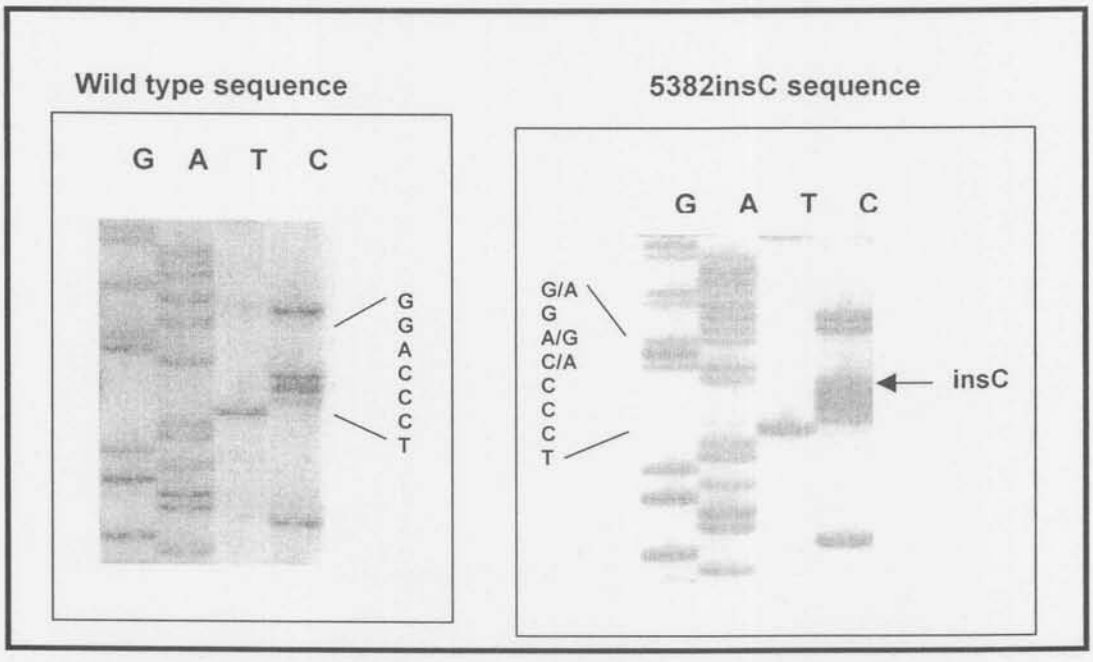


Figure 4.3: Sequence analysis of 5382insC mutation

It was not surprising that four Ashkenazi Jewish families in our study were found to harbour the 185delAG mutation. This mutation has been previously described in Ashkenazi Jewish families. Two Afrikaner families and one Ashkenazi Jewish family were found to carry the commonly reported 5382insC mutation.

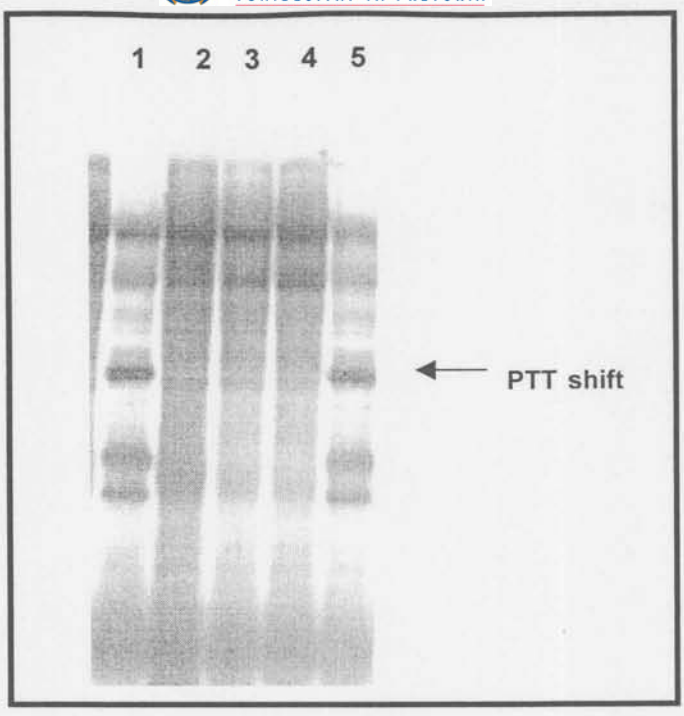


Figure 4.4: PTT of exon 11 (fragment B) showing truncated protein
SDS-polyacrylamide gel showing PTT, corresponding to the E881X mutation. Lanes 1 and 5 have an extra band/shift, lanes 2-4 have no shift.

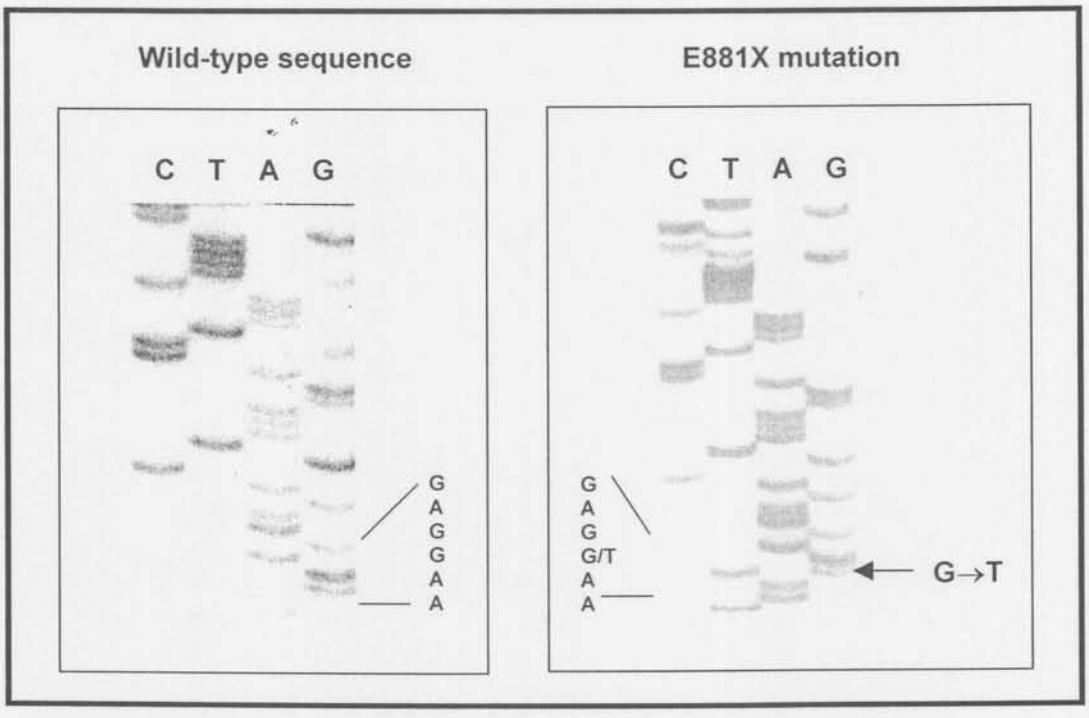


Figure 4.5: Sequence analysis of E881X mutation

The novel E881X nonsense mutation in exon 11 was identified in four Afrikaner families. The phenotypic details of these families can be seen in table 4.1, and the pedigrees of the families may be found in appendix 2.

Table 4.1: Phenotypic details of families identified with recurrent mutations

Family and Mutation	Ethnic origin	Average age at diagnosis of family	Breast cancer cases	Ovarian cancer cases	Other cancers
185delAG					
BRC 13	Ashkenazi Jewish	43.8 years	5	1	Oesophagus
BRC 35	Ashkenazi Jewish	39 years	2	5	
BRC 51	Ashkenazi Jewish	44.5 years	1	1	Endometrial
OV 3	Ashkenazi Jewish	53.5 years	1	3	Pancreas
		45.12 years			
E881X					
BRC 10	Afrikaner	41.8 years	9		
BRC 21	Afrikaner	54.7 years	1	2	Bladder, lung, melanoma
OV 1	Afrikaner	43.3 years		7	Lung, stomach,
FS1	Afrikaner	46.27 years	10	6	testis
		46 years			
5382insC					
BRC 1	Ashkenazi Jewish	40.25 years	4	-	Endometrial
BRC 5	Afrikaner	49.29 years	13	1	Lung, thyroid
BRC 11	Afrikaner	40.55 years	8	2	Stomach
		45.04 years			

It is clear from the above table that all of the families have a young average age at diagnosis, all less than 55 years (average 45.38 years). Although the families with the E881X mutation have a slightly higher average age at diagnosis (46 years) than the families with the 185delAG mutation (45.12 years) and the 5382insC mutation (45.04 years), there is no significant difference ($p=0.952$; ANOVA-test). Family BRC 35 has the lowest average age at diagnosis (39 years), family BRC 21 (54.7 years) the highest.

The families with the 185delAG mutation appear to have more cases of ovarian cancer, as compared to the 5382insC positive families with relatively more cases of breast cancer. This may reflect a similar observation by Gayther et al. (1995) who suggested a

genotype-phenotype correlation between the site of BRCA1 mutations and the occurrence of breast or ovarian cancer. It was suggested that mutations in the 5' end of the gene predispose to relatively more cases of ovarian cancer, while mutations in the 3' end give rise to more cases of breast cancer, with the location of the change point in exon 13 (between codons 1435 and 1443). This was also shown by Holt et al. (1996), where mutations at the 3'end of BRCA1 inhibited growth of ovarian cancer cells, but not breast cancer cells. The "switch" point is hypothesized to be at the granin motif at codons 1214-1223. This can hold true for these families, as the families with the 185delAG mutation in exon 2 have relatively more cases of ovarian cancer than the families with the 5382insC mutation in exon 20.

The average age at diagnosis of families with the E881X mutation is 46.6 years, which is slightly older than the average age of diagnosis for families with the 185delAG or 5382insC mutations. In the families with the E881X mutation, Family 10 has the youngest age at diagnosis (41.8 years) and family 21 has the highest age at diagnosis (54.7 years). The families with the E881X mutation have an interesting range of associated breast and ovarian cancers. Family 10 has no reported cases of ovarian cancer, which is slightly unexpected, in this area of the gene, as explained above by the genotype-phenotype correlations. But, their average age at diagnosis is quite young – 41.8 years. Family 21 has relatively more cases of ovarian cancer than breast cancer, with the highest average age at diagnosis of 54.7 years. Family OV1 has no cases of breast cancer, only ovarian cancer, with an average age at diagnosis of 43.6 years. It would therefore appear that the E881X mutation is associated with a higher penetrance of ovarian cancer than breast cancer, except for family FS1. Clearly there must be other factors modulating the effects of this mutation.

The 185delAG mutation in exon 2 may be expected to have an effect on the DNA binding properties of the BRCA1 protein, as it falls within the RING finger domain. The E881X mutation falls within the putative domain of interaction of BRCA1 with RAD51 (Scully et al., 1997), and may impair the ability of this complex to recognise and repair damaged DNA. The 5382insC mutation in exon 20 falls within the transcriptional transactivation domain as described by Chapman and Verma (1996). This mutation was shown to abolish this activity, and therefore mutation carriers do not function correctly in activating transcription of other genes involved in suppressing transformation, and therefore lead to tumorigenesis (Monteiro et al., 1996).

4.1.1.1. Genotype Analysis with 17q Markers

The families with the same mutations were subjected to genotype analysis, as explained in the methods (section 3.3.4) to determine if these families share a common ancestor or if the mutation is an independent event in the families (figure 4.6).

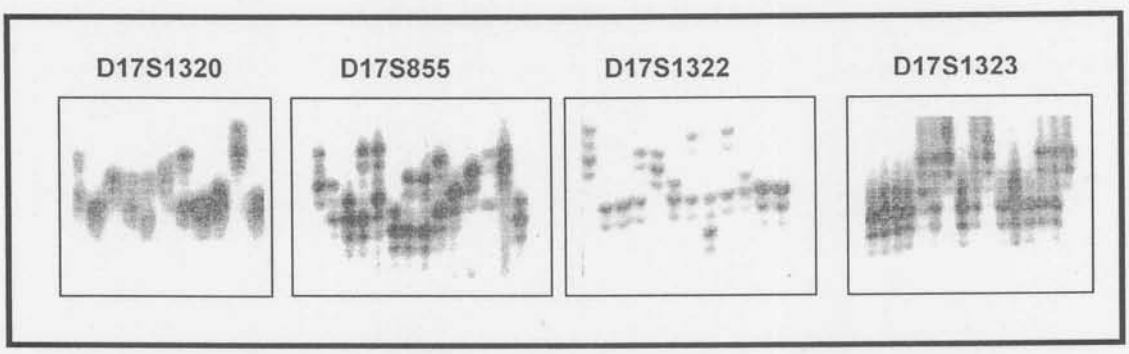


Figure 4.6: Autoradiograph of markers used for genotype analysis
6% Polyacrylamide gel of the microsatellite markers used in the genotype analysis.

In order to determine the allele frequencies of the markers in the Afrikaner population, fifty random Afrikaner control samples (100 chromosomes) were subjected to microsatellite genotyping of the same markers. This would give an idea of the allele frequencies of the markers in this population group as compared to those deposited in the GDB. The calculated allele frequencies in this population group are tabulated in tables 4.2-4.5.

Table 4.2: Allele frequencies of the D17S1320 marker

Allele	Afrikaner population frequency	Reported GDB frequency*
3	0.01	0.04
4	0.04	0.09
5	0.08	0.50
6	0.32	0.07
7[a]	0.10	0.27
8	0.42	0.03
9	0.02	

* Genome Database (http://www.gdbwww.gdb.org, GDB:249483)

[a]185delAG- and 5382insC associated allele

Table 4.3: Allele frequencies of the D17S855 marker

Allele	Afrikaner population frequency	Reported GDB frequency*	Reported Swedish frequency**	Reported Japanese frequency***
1	0.03		0.02	0.002
2	0.10	0.05	0.07	0.020
3	0.16	0.19	0.15	0.07
4[a]	0.20	0.18	0.22	0.30
5	0.15	0.15	0.15	0.19
6	0.22	0.12	0.13	0.17
7[b]	0.12	0.26	0.18	0.17
8	0.01	0.05	0.07	0.06

* Genome Database (http://www.gdb.org, GDB:249483)
** Johannsson et al., 1996.
*** Yokozaki and Tahara., 1999.
[a] 5382insC associated allele
[b] 185delAG associated allele

Table 4.4 : Allele frequencies of the D17S1322 marker

Allele	Afrikaner population frequency	Reported GDB frequency*
1		0.04
2	0.06	0.03
3[a]	0.01	0.11
4	0.16	0.25
5[b]	0.57	0.51
6	0.02	0.04
7		0.01

* Genome Database (http://www.gdb.org, GDB:249483)
[a] 185delAG associated allele
[b] 5382insC associated allele

Table 4.5: Allele frequencies of the D17S1323 marker

Allele	Calculated Afrikaner population frequency	Reported GDB frequency*
1	0.02	0.02
2	0.06	0.04
3[a]	0.22	0.19
4	0.02	0.03
5	0.04	0.04
6[b]	0.61	0.68
7		
8	0.02	

* Genome Database (http://www.gdb.org, GDB:249483)

[a] 185delAG associated allele
[b] 5382insC associated allele

The allele frequencies for marker D17S1320 obtained for the Afrikaner population differ significantly from those reported to the GDB ($p < 0.0001$; χ^2- test for contingency table). The presence of the 7 allele in the 185delAG and 5382insC haplotype, probably occurs purely by chance, as it is the second commonest allele in the general population (based on the GDB figures), it is associated with the mutation in the Ashkenazi Jewish families.

There is a significant difference ($p < 0.0001$; χ^2- test for contingency table) in the calculated allele frequencies of the D17S855 marker in the Afrikaner population compared to those of the GDB, Swedish and Japanese populations. It is not unexpected that the greatest difference observed is between the Afrikaner and Japanese populations, as one would expect the Afrikaner population to be more genetically similar to the Swedish and Europeans than the Japanese. The 7 allele is the most common allele reported in the GDB, and may be present in the Ashkenazi patients with the 185delAG, purely by chance. However, in the Afrikaners the 7 allele is not the most common and its presence in the 5382insC mutation may be mutation specific.

The calculated Afrikaner allele frequencies of the D17S1322 marker also differ significantly from those deposited in the GDB ($p = 0.033$; χ^2- test for contingency table). The presence of the 3 allele in the 185delAG mutation is probably specific to the mutation, however the 5 allele's presence in the 5382insC mutation may be purely due to chance, as it is at a high frequency in the Afrikaner and GDB population.

The Afrikaner and GDB allele frequencies of the D17S1323 marker are not significantly different (p= 1.000 ; χ^2- test for contingency table). The presence of both the 3 and 6 allele in the 185delAG and 5382insC mutations respectively may be a function of their high population frequency.

The results of the genotype analysis are displayed in table 4.6, with the markers listed in order from the centromere to the telomere, which is towards the 5' end of BRCA1. The typing of family FS 1 was unsuccessful.

Table 4.6: Genotype analysis of families with recurrent mutations

	Genotypes/ Inferred Haplotype by marker			
Family and mutation	**D17S1320**	**D17S855**	**D17S1322**	**D17S1323**
185delAG				
BRC 13	5/7	7/7	3/4	3/3
BRC 35	7/8	3/7	3/5	3/5
BRC 51		6/7	3/4	
OV 3	7/8	6/7	3/4	3/5
E881X				
BRC 10	5/7	4/5	3/5	5/6
BRC 21	7/7	4/5	5/5	6/6
OV 1	7/7	5/6	5/5	5/6
5382insC				
BRC 1	5/**7**	6/7	5/**5**	5/**6**
BRC 5	3/**7**	**4**/6	5/**5**	6/**6**
BRC 11	5/**7**	**4**/5	5/**5**	5/**6**

185delAG linked haplotype – 7/7/3/3
E881X linked haplotype – 7/5/5/6
5382insC linked haplotype – **7/4/5/6**

The families with the 185delAG mutation share a common haplotype - 7, 7, 3, 3. This is the common founding haplotype for all Ashkenazi Jewish families worldwide with this mutation (Neuhausen et al., 1996a). The mutation was originally estimated to have originate in ~1235A.D, 765 years ago in the Ashkenazi Jewish population (Neuhausen et al., 1996a). Other non-Ashkenazi Jewish patients have been identified with this mutation, but they have a different haplotype, suggesting an independent origin of the mutation in these carriers. However, it has been suggested that the common ancestor for 185delAG mutation carriers dates back to the time before the divergence of the different Jewish sub-populations. This is thought to be earlier than the early 1200s (Bar-Sade et al., 1998). It has been estimated that this mutation originated 2000 years ago. Our South African Ashkenazi Jewish families are therefore related to all other 185delAG patients worldwide. This ancient mutation is representative of a strong founder effect in this population group.

The Afrikaner families with the 5382insC mutation share a haplotype – 7, 4, 5, 6. This is the reported founding haplotype for families of northern European origin with this mutation. The Ashkenazi Jewish family with the 5382insC mutation has the linked haplotype, except for the D17S855 marker. The "6" allele is present at this locus in place of the linked "4" allele. The chance of mutation from the 4 to the 6 allele, in addition to the 5382insC mutation is probably unlikely. This is because it would have to involve a mutation at the dinucleotide locus, from a 151bp allele to a 147bp allele. This therefore could represent an independent mutation event for the 5382insC mutation in the Ashkenazi Jewish family, as it has occurred on a different haplotype background. Further studies may however indicate a recombination event, in which case the common mutation has occurred together with a subsequent recombination event, rather than an independent mutation. The 5382insC mutation is said to have arisen in the Baltic area ~800 years ago. It is believed to have originated in a Jewish person. It is interesting to note that genealogical studies have almost linked family BRC5 and 11, however the dates (date of birth of a key family member) that would link the two families differ by a year, which may be due to incorrect recording of the historic dates in the families. Haplotype analysis has confirmed that they are indeed related.

An interesting aspect arose with respect to the 5382insC mutation in family BRC 11. After identification of this mutation in the index patient (II1) other affected individuals were also tested for the mutation. Figure 4.7 is a simplified pedigree to understand the family members involved.

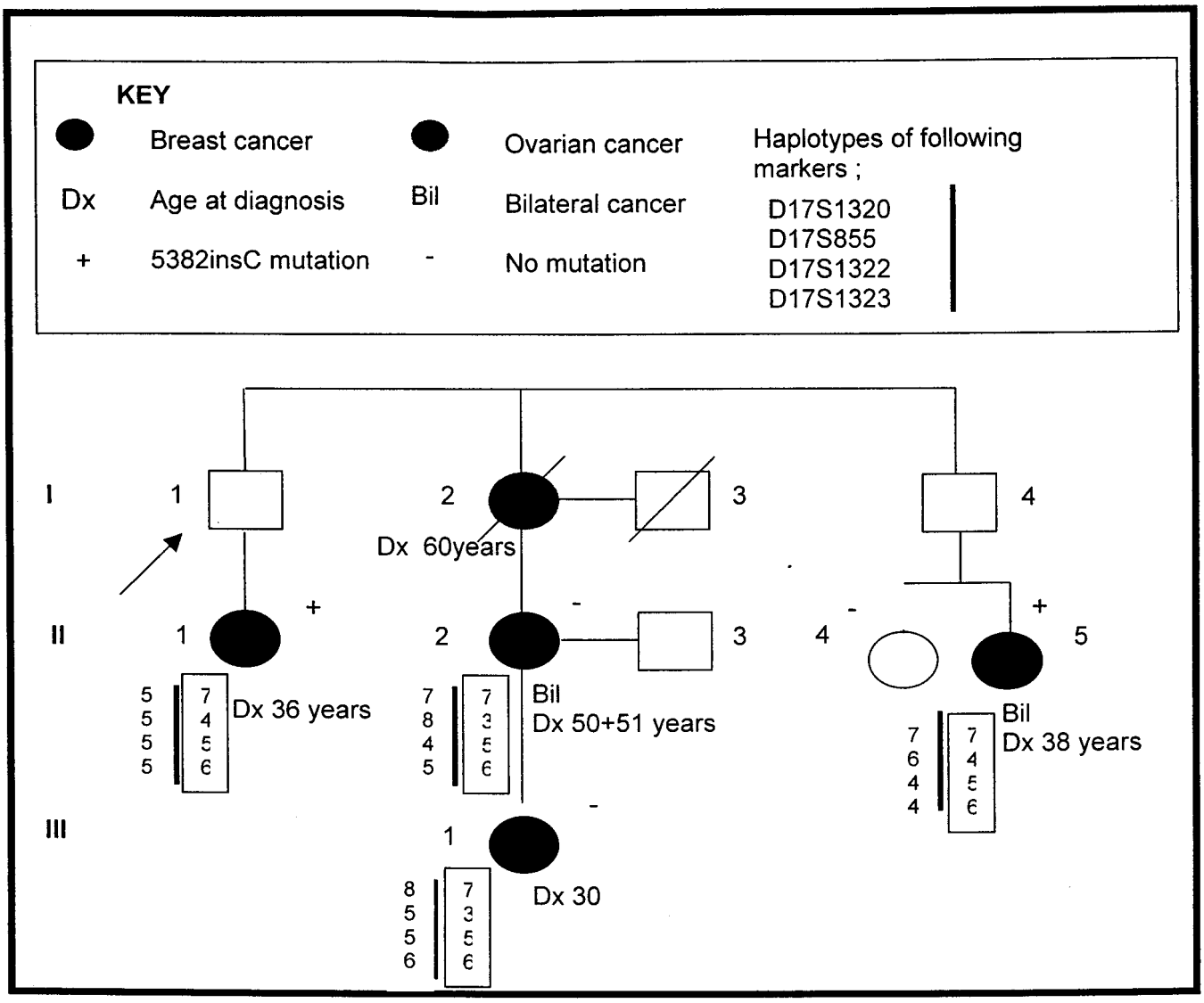


Figure 4.7 :Abbreviated Pedigree of family BRC11
The haplotype in the vertical rectangle segregates with the 5382insC BRCA1 mutation.

It was assumed that this is the mutation segregating in the family, as the unaffected II4 did not carry the mutation, and the affected individuals II1 and II5 tested positive. However, even though individuals II2 and III1 had breast cancer, they tested negative. Unfortunately, no DNA from I2 was available for testing. At the time, it was thought that a different mutation might have been inherited from the father (I3), who was Ashkenazi Jewish. However the 185delAG BRCA1 and 6174delT BRCA2 Ashkenazi Jewish specific mutations were not found. Individuals II2 and III1 were therefore subjected to a complete BRCA1 screen in an attempt to find the causative mutation, but none was found. BRCA1 haplotype analysis was also carried out on II2 and III1. They carried the haplotype associated with the 5382insC mutation, with the exception of marker D17S855. At this locus, the 3 allele was present in place of the 4 allele. This could be due to enzyme slippage during amplification, a naturally occurring mutation or possibly a recombination event. It is difficult to deduce the haplotype of individual I2 by looking at

other family members due to the absence of available DNA. The deletion and recombination of a fragment of the BRCA1 gene cannot be ruled out. The methods of mutation detection used in this study would not detect large rearrangements (including a deletion), therefore restriction enzyme and Southern blot analysis would be necessary to resolve this issue. Recently an in frame deletion of exon 20, of ~4Kb in size was reported in a woman with breast cancer (Carson et al., 1999). This deletion results in the loss of 28 amino acids from the carboxy terminus of the BRCA1 protein. The Southern blot analysis that was used to identify this deletion showed a reduced intensity of the exon 20 specific fragment. It is possible that we may have a similar deletion in our family 11.

It is possible that some of these individuals (I2, II2 and III1) may represent sporadic cases of cancer, i.e. phenocopies. However, the presence of two phenocopies is quite unlikely and it would be quite unusual to have three successive generations with a sporadic form of cancer. The presence of bilateral breast cancer as well as a young age at diagnosis leads one to believe that this should be an inherited form. The mutation may be inherited through the father, or be a de novo mutation in individual II2, that is subsequently inherited by her daughter. Only once a large BRCA1 rearrangement is excluded, should BRCA2 also be completely screened in these individuals. Clarification of these issues is important for correct counselling and risk assessment for this family.

The four Afrikaner families with the E881X mutation share a haplotype – 7,5,5,6, and may therefore share a common ancestor. The Afrikaner population originated from French, German and Dutch populations, and since this mutation has not been reported outside of South Africa, it could possibly be unique to South Africa, or more specifically an Afrikaner mutation. The mutation could have arisen some time after the arrival of Jan van Riebeeck at the Cape in 1652. Genealogic studies have indeed linked these four families to a common founding couple, Susanne Seugnet (arrived in the Cape from France in 1688) and Francois du Toit (arriving from France in 1686) who married in 1690 (Malherbe, 1959). They had nine children, three of whom were boys who can be linked in to the four E881X mutation families (figure 4.8). It appears that the mutation was mainly passed on through males for a number of generations. Susanne and Francois could therefore be the founding couple for this mutation. As this mutation has not been found in France, it is possible that it arose in either as a *de novo* germline mutation. However, it could also be that Susanne or Francois carried the mutation with them from France, where it might be extremely rare.

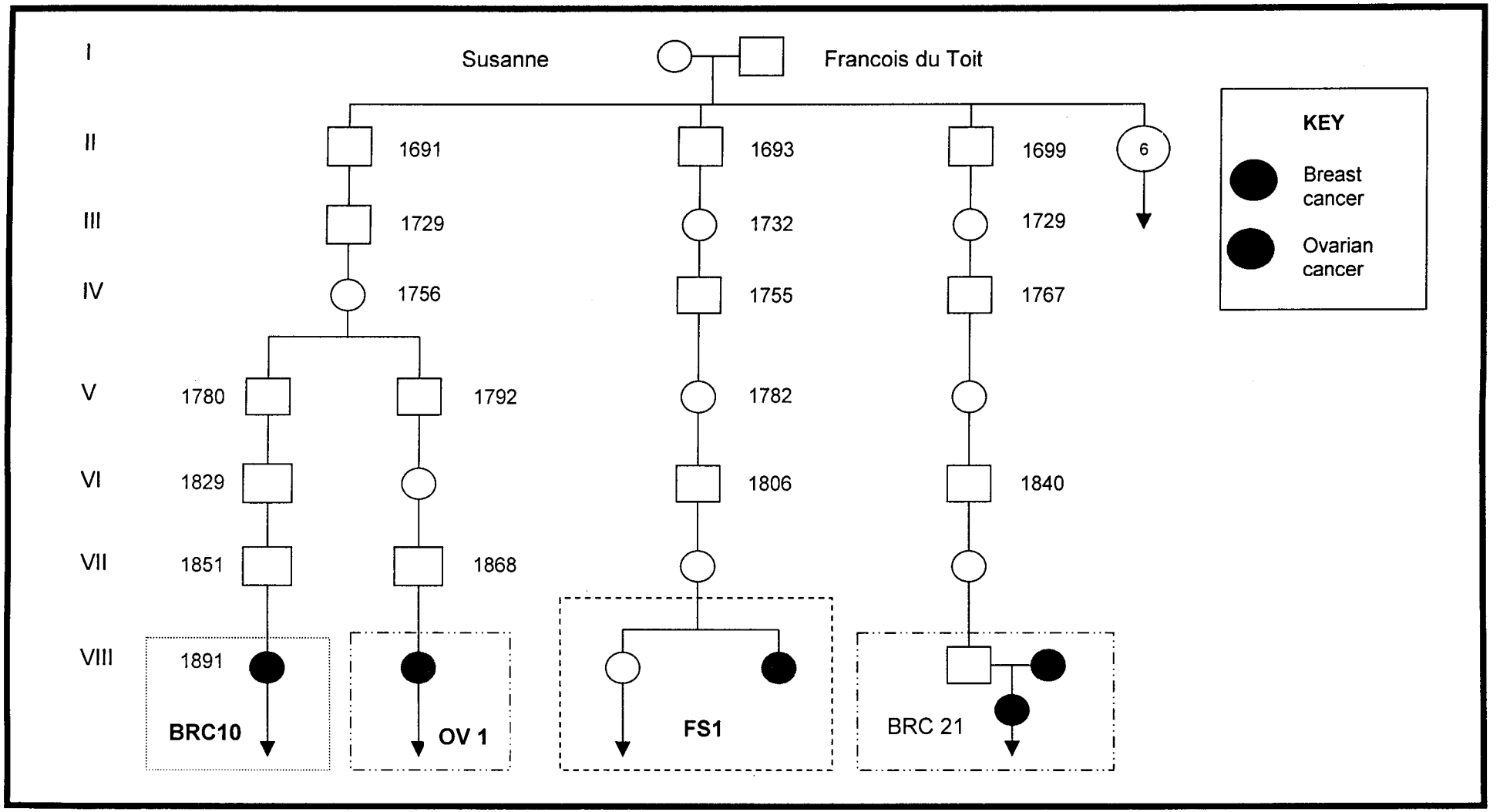


Figure 4.8: Simplified pedigree of the founding E881X mutation
The dates given with some individuals are the year of their birth/christening

4.1.2 Non-recurrent Novel Mutations

Family 55 was found to carry the S451X nonsense mutation, which has not been described (figure 4.9 and 4.10). The C to A transversion at codon 451 (nucleotide 1471), results in the introduction of a premature stop codon in the place of a serine amino acid.

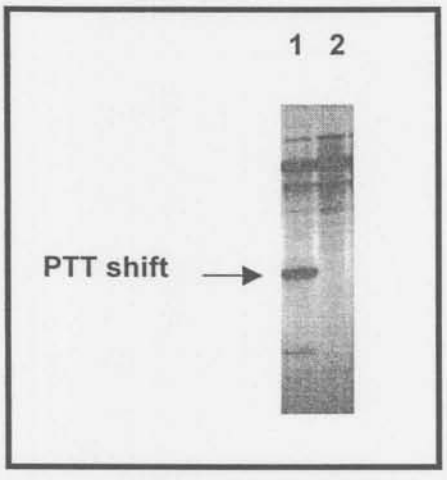


Figure 4.9: Autoradiograph of S451X PTT shift
SDS-polyacrylamide gel showing PTT shift in exon 11 fragment A, corresponding to the S451X mutation in family BRC55. Lane 1 has the shift, lane 2 has no shift.

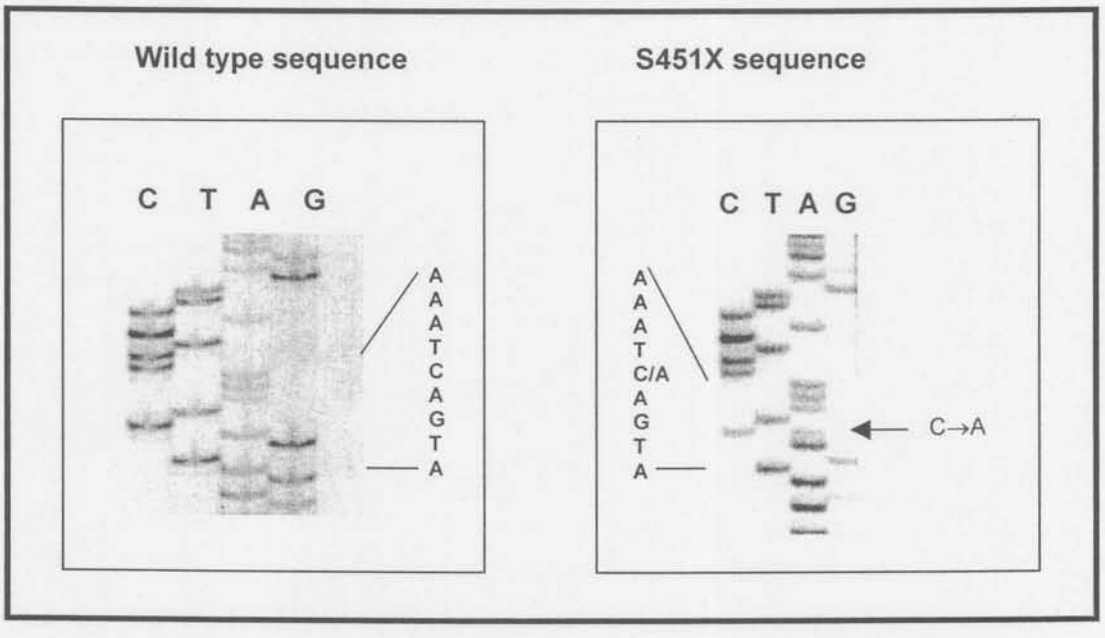


Figure 4.10: Sequence Analysis of S451X mutation
6% polyacrylamide gel showing the S451X mutation in family BRC 55.

An Afrikaner family was identified with the novel 1493delC frameshift mutation (figure 4.11 and 4.12). The C at nucleotide position 1493 (codon 458) is deleted, resulting in a premature stop codon at codon 474. This mutation has not been described.

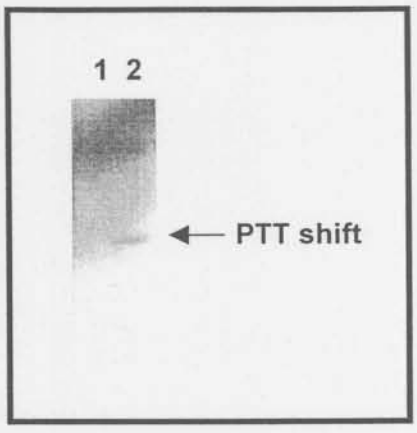


Figure 4.11 Autoradiograph of 1493delC PTT shift
SDS-polyacrylamide gel showing PTT shift in exon 11 fragment A, corresponding to the 1493delC mutation in family BRC27. Lane 1 has no shift, lane 2 has the shift.

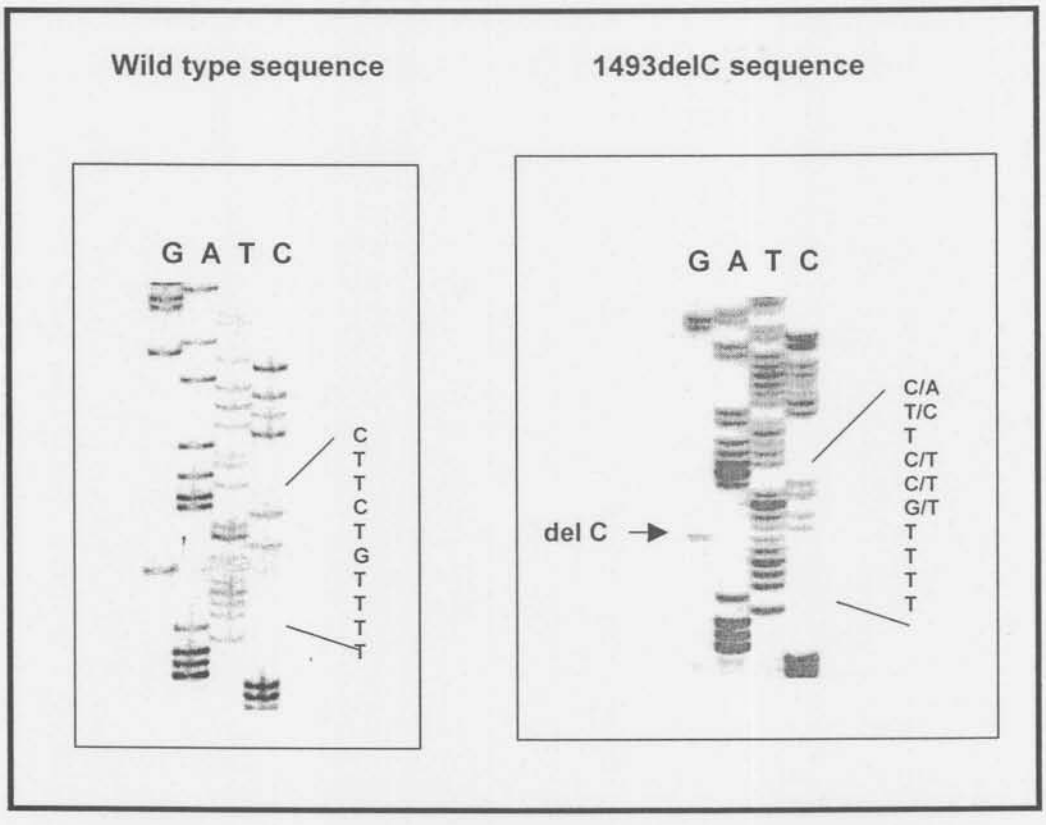


Figure 4.12: Sequence Analysis of 1493delC mutation
Antisense sequence of the 1493delC mutation in family BRC27.

A South African Indian family was identified with the novel 4957insC frameshift mutation in exon 16. The C inserted at nucleotide 4957 (codon 1613) results in the creation of a premature stop codon at codon 1621. Interestingly enough, this patient also has a G to C substitution at the same nucleotide position on the same allele as the insertion, resulting in an amino acid substitution. However, the patient also has an additional nucleotide substitution in the same codon on the other allele. It is an A to G transition at nucleotide 4956, changing the amino acid. The resulting S1613A missense mutation results in the substitution of a serine to an alanine amino acid at codon 1613, and has not been reported. This is explained in diagrammatic form in figure 4.14.

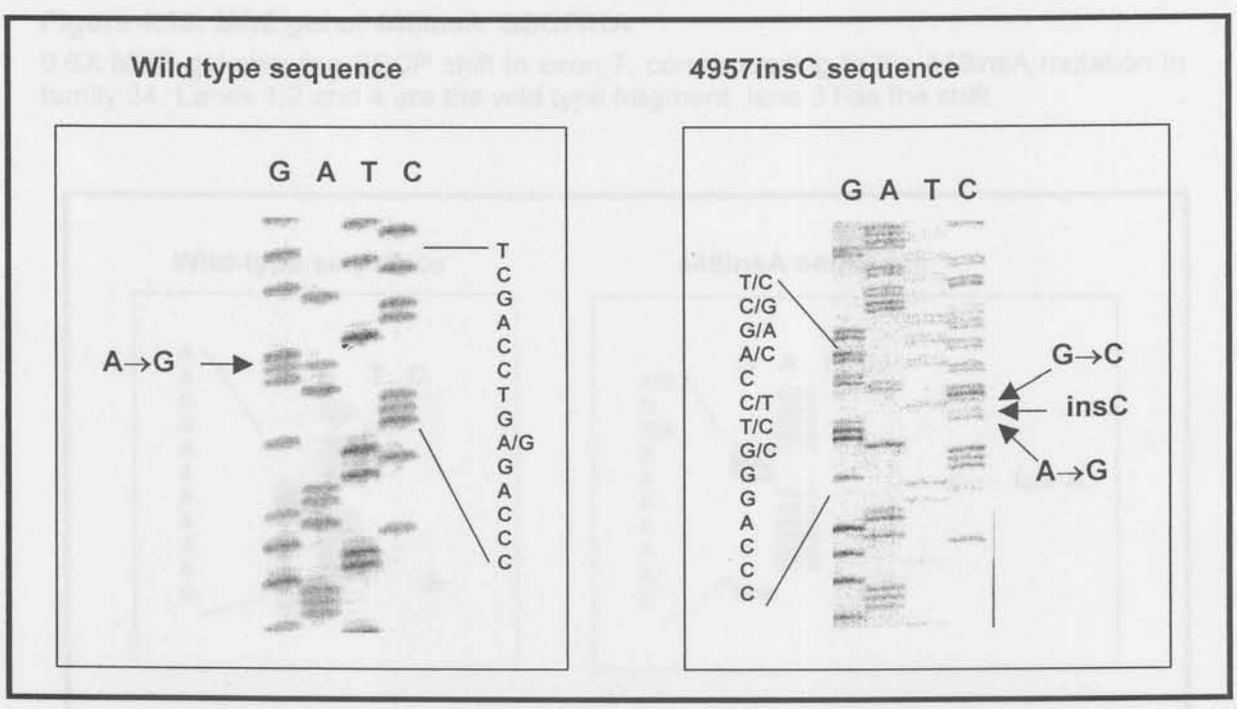


Figure 4.13: Sequence Analysis of 4957insC mutation
6% polyacrylamide gel showing the wild type sequence, including the presence of the S1613G polymorphism (4956A→G), and the 4957insC mutation in family 48, in addition to the novel S1613A variant (homozygous 4956A→G change and heterozygous 4957G→C change).

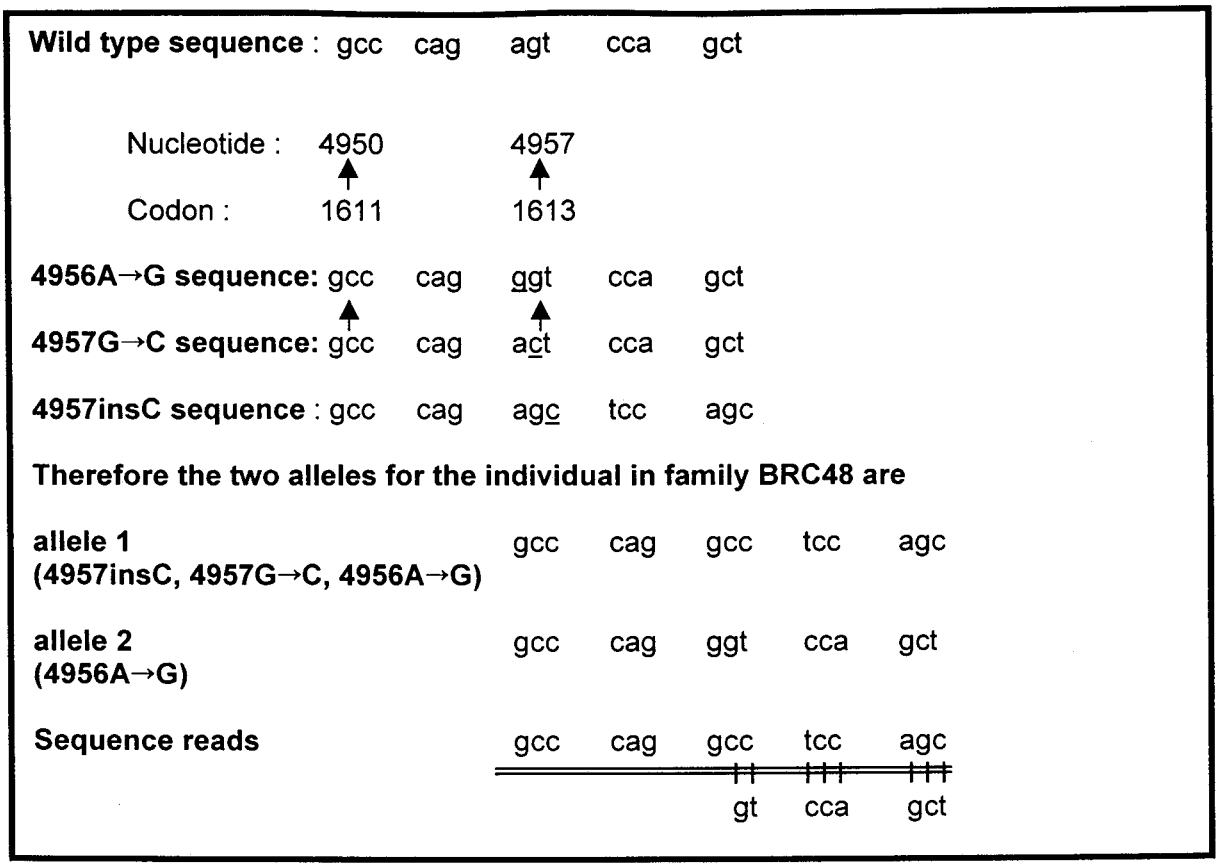


Figure 4.14: Schematic representation of the mutations in Family BRC48.

Phenotypic details of the families with novel mutations are seen in table 4.7. The pedigrees of these families can be found in the appendix. Additional phenotypic details can be found in table 3.1.

Table 4.7: Details of families with novel mutations

Family and Mutation	Ethnic origin	Average age at diagnosis of family	Breast cancer cases	Ovarian cancer cases	Other cancers
S451X BRC 55	Scottish	unknown	1	3	Stomach
1493delC BRC 27	Afrikaner	49.5 years	4	2	Colon
4957insC BRC 48	Indian	46.8 years	8	1	Liver, prostate, throat

The average age at diagnosis of families with mutations is younger than 50 years. Family 48, with the 4957insC mutation has the lowest average age at diagnosis of 46.8 years. Family 55, with the S451X mutation, has relatively more cases of ovarian cancer than breast cancer. This may once again be a genotype-phenotype effect, with the 5' end of the gene associated with more cases of ovarian cancer than breast cancer. However, Family 27 with the 1493delC mutation also in exon 11 has relatively more cases of breast cancer as compared to ovarian cancer. Family 48 has many more cases of breast cancer as compared to ovarian cancer. This fits in with the genotype-phenotype correlation of more cases of breast cancer associated with mutations in the 3'end of the gene – the mutation occurs in exon 16.

The S451X nonsense mutation in exon 11 falls within the putative RAD51/BRCA1 interaction domain (Scully et al., 1997), and the mutation may impair the ability of this complex to recognise and repair damaged DNA. The 4957insC mutation in exon 16 falls within the transcriptional transactivation domain as described by Chapman and Verma (1996). It is possible that this mutation impairs the transcription transactivating ability of other genes involved in suppressing transformation, and may lead to tumorigenesis (Monteiro et al., 1996). It is possible that this change, as well as the additional substitutions in this region have led to the presence of liver, prostate and throat cancers in this family, by the above-mentioned process.

4.1.3 Previously Reported Mutations

One previously reported mutation, the 448insA frameshift, was identified in a family of German ancestry (BRC 34). The proband had cancer of the fallopian tube (figure 4.15 and 4.16). This mutation in exon 7 arises from an insertion of an A at nucleotide 448 (codon110). The reading frame is shifted, and a premature stop codon is introduced at codon 113. Other cases of fallopian tube cancer have been reported with BRCA1 mutations (Simard et al., 1994; Tonin et al., 1996). The pedigree of this family can be seen appendix 2, and the phenotypic details in table 3.1.

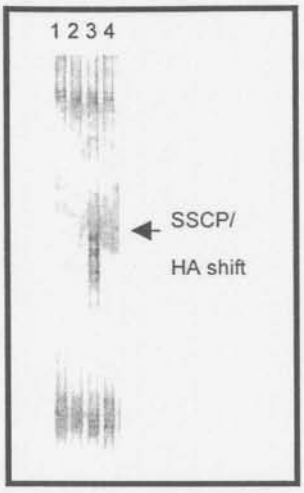


Figure 4.15: MDE gel of 448insA SSCP/HA
0.6X MDE gel showing SSCP shift in exon 7, corresponding to the 448insA mutation in family 34. Lanes 1,2 and 4 are the wild type fragment, lane 3 has the shift.

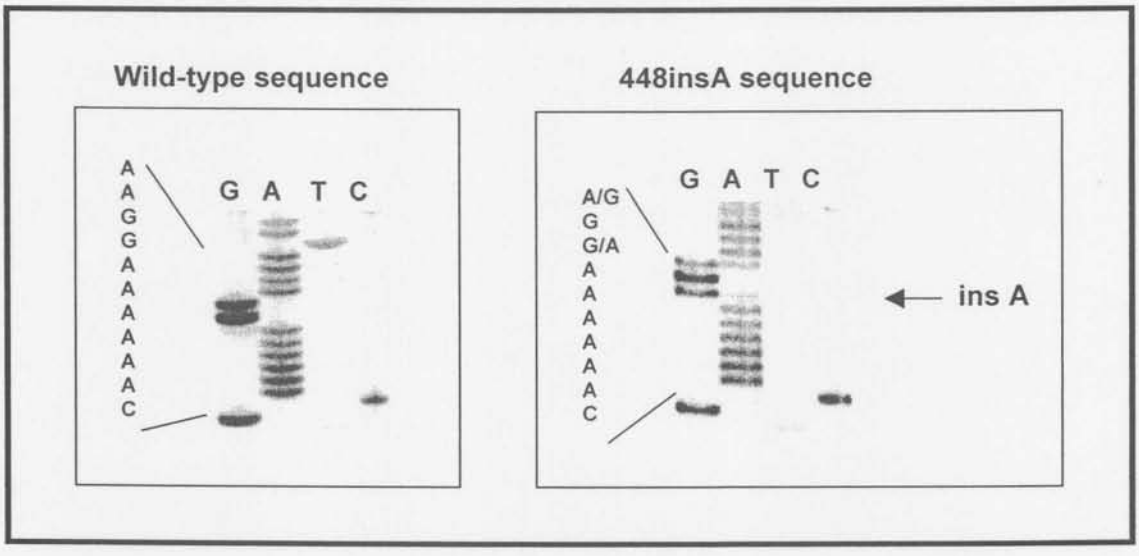


Figure 4.16: Sequence analysis of 448insA mutation
6% polyacrylamide gel showing 448insA mutation in family 34.

In addition to the fallopian tube cancer case there are also two cases of "gynaecologic" cancer, an ovarian cancer, and three breast cancer cases reported for family 34. There is also a case of male breast cancer, which is usually associated with a BRCA2 mutation. This family has a relatively young age at diagnosis of 53.5 years (51.38 years if the "gynaecologic" cancers are also taken into account). The mutation falls in the 5'

region of BRCA1 within the zinc finger domain. The mutation may have affected the DNA binding properties of the gene, and led to the observed phenotypes in the family.

4.1.4 BRCA1 Germline Mutations of Unknown Functional Significance and Polymorphisms

A number of different mutations were identified using the SSCP/HA. However, a number of criteria need to be fulfilled in order to classify these as pathogenic mutations or polymorphisms. These include the type of change (i.e. frameshift, nonsense), which would have a functional effect on the protein. The location of the change is equally important, it is expected that disease-causing mutations would be more likely to occur within the coding region or an intron/exon boundary of a gene. If a mutation segregates with the cancer in a family, and is not found in unaffected members, it is likely to be a disease-causing mutation. If this mutation does not occur in the general population, one would consider it a pathogenic mutation, as one would not expect a disease-causing mutation to be present in many unaffected individuals. The amino acid affected will also give an indication of the functional significance of a sequence variant. Changes to a highly conserved amino acid are likely to affect the function of the polypeptide, and would therefore be considered pathogenic. Finally, functional analysis is required to determine the exact consequence of a mutation, and its phenotypic effect (Cotton and Scriver, 1998).

4.1.4.1 Missense Mutations of Unknown Functional Significance

A number of missense mutations were detected in the 38 families that were screened by SSCP/HA (table 4.8). These mutations are characterised as such because they involve a change in the amino acid within the protein sequence. At this time, the mutations are unclassified with respect to their role, but they could be polymorphic or pathogenic.

Table 4.8: Missense Mutations Detected

Family	Exon	Mutation	Nucleotide Change	Amino Acid Change	Described
BRC 51	2	V14L	159 G→C	valine→leucine	No
BRC 27	11	D458E	1493 C→G	aspartic acid→glutamic acid	No
BRC 10	11	D693N	2196 G→A	aspartic acid → asparagine	Yes
BRC 10	11	D749V	2365 A→T	aspartic acid → valine	No
BRC 10	11	P871L	2731 C→T	proline→leucine	Yes
BRC 26	11	P871L	2731 C→T	proline→leucine	Yes
OV1	11	P871L	2731 C→T	proline→leucine	Yes
FS1	11	P871L	2731 C→T	proline→leucine	Yes
BRC 21	11	S1040N	3238 G→A	serine→asparagine	Yes
BRC 6	11	R1347G	4158 A→G	arginine→glycine	Yes
BRC 18	15	S1512I	4654 G→T	serine→isoleucine	Yes
BRC 48	16	S1613A	4956A→G 4957G→C	serine→alanine	No

Family BRC 51 was identified with the disease-associated 185delAG mutation, in addition to the V14L variant. This missense mutation is probably not pathogenic, but may have a modulating influence on the mutation. Family BRC 27 was found to carry the D458E variant, in addition to the 1493delC frameshift mutation. It is interesting that the mutation and the variant occur at the same nucleotide position (1493), as the C that is deleted occurs on its own, and is not part of a run of nucleotides. This may represent some sort of hot spot for nucleotide change. The change is unlikely to be a pathogenic mutation, as both aspartic acid and glutamic acid have acidic side chains, so the change is conservative (Alberts et al., 1989).

The D693N missense mutation in family BRC10 is a non-conservative change, and has been described previously as a polymorphism (Couch et al., 1996; Greenman et al., 1998; Shattuck-Eidens et al., 1997). Family BRC 10, with the E881X nonsense mutation was also identified with an additional variant – D749V. The amino acid change is non-conservative as aspartic acid has an acidic side chain and valine has a nonpolar side chain (Alberts et al., 1989). This may render the change a pathogenic missense mutation, if a number of other criteria are also met (Cotton and Scriver, 1998). Once

again, the presence of the nonsense mutation would be sufficient to give rise to the cancer and the missense change may modify the phenotype. However it would be difficult to ascertain this.

The P871L mutation was identified in family BRC 10, 26, OV1 and FS1, and has been described previously in ovarian cancer patients and shown to be polymorphic in the general population (Janezic et al., 1999; Durocher et al., 1996). The significance of this in family BRC 26 is challenging, as there was no mutation identified in this family. Families BRC10, OV1 and FS1 have the E881X mutation. The significance of this polymorphism may be correlated with the fact that both family BRC 10 and FS1 with the E881X mutation had relatively more cases of breast cancer than ovarian cancer, compared to the other two families with the same mutation. This was unexpected for mutations in this area of the gene, and it was suggested that the E881X mutation might be an ovarian cancer specific mutation. However, the presence of the P871L polymorphism may modify the expression of the gene, and give rise to an increased susceptibility to breast cancer, in conjunction with the E881X mutation. Janezic et al. (1999) reported that the polymorphism lies within a functional domain of BRCA1, but the significance of the change will only be fully understood after functional studies have been carried out. This mutation is a conservative change, and has been shown to be conserved in the mouse BRCA1 protein (Durocher et al., 1996). Therefore, it is clear that the E881X is the overriding cause of the cancer in the families. However, how this phenotype or expression is modified by all the additional polymorphisms and variants is at this stage unknown.

Family BRC 21 has a missense mutation in addition to the novel nonsense mutation E881X identified. The change in amino acid is conservative, as both amino acids have uncharged polar side chains (Alberts et al., 1989). The nonsense mutation on its own would be expected to be sufficient to cause the cancer phenotype. But, this mutation has been reported previously as a pathogenic missense mutation (Newman et al., 1998) and a probable polymorphism (Couch et al., 1996; Newman et al., 1998; Shattuck-Eidens et al., 1997). Durocher et al. (1996) reported this mutation as a rare sequence variant, due to its presence in controls and lack of segregation with the disease in certain families. This variant must have different population frequencies, resulting in its different classifications in different studies. Keeping in mind the interaction of BRCA1 with Rad51 in exon 11, to repair DNA damage (Scully et al., 1997), it is possible that the location of the S1040N missense mutation may interfere with this interaction and allow

for an incorrect sequence to be replicated. The effects of this would at this stage, be difficult to determine.

Family BRC 6 was found to carry a missense mutation (R1347G) that has been described as a polymorphism (Berchuck et al., 1998), pathogenic missense mutation (Newman et al., 1998), and unclassified variant. The arginine (with a basic side chain) to glycine (nonpolar side chain) amino acid change is non-conservative, and may affect the function of the polypeptide. However, 50 random Afrikaner samples were tested for the presence of this variant, and it was not detected. This may suggest that it is in fact a pathogenic missense mutation. Unfortunately segregation studies of the mutation with breast cancer could not be conducted in this family, as there is only one surviving member affected with breast cancer. In a study by Durocher et al, 1996, it is reported that this amino acid is not conserved in the mouse BRCA1 protein.

The S1512I missense mutation in family BRC 18 has been described as a polymorphism in ovarian cancer patients (Janezic et al., 1999; Berchuck et al., 1998), and an unclassified variant in a Finnish family (Huusko et al., 1998). It is a non-conservative change, with serine having an uncharged polar side chain, and isoleucine having a nonpolar side chain (Alberts et al., 1989). It may be a considered a pathogenic missense mutation if it is not found in the general population, and it segregates with the cancer in this family. Functional studies will be the ultimate test to see the significance of the change.

The S1613A missense mutation in family BRC 48 was found in addition to the 4957insC frameshift mutation detected by SSCP/HA, as explained on page 57. An S1613G polymorphism has been described in breast and ovarian cancer patients and controls (Durocher et al., 1996; Janezic et al., 1999; Osorio et al., 2000; Shattuck-Eidens et al., 1997). This is a non-conservative change, but the frameshift mutation is clearly the cause of the cancer in this family. How the missense change affects the phenotype will be difficult to determine.

In order to classify the missense mutations as pathogenic, rare variants or polymorphisms it is necessary to determine their frequency in the general population. Some of them may be common in the Afrikaner population, and will therefore represent polymorphisms. The location of the mutation can also have an effect on the mutated protein product. Mutations in the Ring finger, with its zinc binding properties will affect amino acids 1 to 112. This area of the gene is involved in the degradation of proteins by

ubiquitination. Therefore, loss of this RING finger could lead to protein levels that would stimulate proliferation. Mutations in the carboxy terminus (affecting amino acids 1528 to 1863) would abolish the transcriptional activation functions, and result in tumour suppression. The BRCT domain of BRCA1, which is located between the RING finger and the carboxy terminus, is required for DNA damage repair. Therefore, mutations in this region could lead to failed repair of damaged genes, which could lead to cell cycle arrest or cell proliferation. Therefore for all mutations and variations, especially those where the functional significance is unknown, the site of mutation may play an important role in the effect on the protein. Similarly, functional analysis is needed to determine the exact functional effect on the protein, however, there is as yet, no functional assay for BRCA1.

4.1.4.2 Unclassified Variants and Polymorphisms

Three unclassified variants and six polymorphisms were detected in the 38 families that were screened by SSCP/HA (Table 4.9). Same sense mutations (silent/synonymous mutations) are assumed to be polymorphic as they should have no functional significance, these are single nucleotide polymorphisms (SNPs).

Table 4.9 Unclassified Variants and Polymorphisms

Family	Exon	Mutation	Nucleotide Change	Described	Designation
[a]	IVS7	IVS7+33delCTT	IVS7+33delCTT	No	Polymorphism ?
[b]	IVS8	IVS8-58delT	IVS8-58delT	Yes	Polymorphism
BRC 10	11	S694S	2201C→T	Yes	Polymorphism
BRC 21	11	A870A	2729 T→A	No	Polymorphism
BRC 50	12	S1370S	4229T→G	No	Polymorphism
BRC 48	IVS14	IVS14-63C→G	IVS14-63C→G	No	Unclassified variant
[c]	IVS18	IVS18+66G→A	IVS18+66G→A	Yes	Polymorphism
BRC 19	IVS18	IVS18+64A→G	IVS18+64A→G	No	Unclassified variant
BRC 24	IVS18	IVS18+64A→G		No	Unclassified variant

[a] 27 families were identified with this variant, which could indicate that it is polymorphic.
[b] This variant was detected in 18 families.
c This variant was detected in 22 families.

The IVS7+33delCTT mutation was detected in 27 families (52.9%), the IVS8-58delT in 18 families (35.29%), the IVS 18+64A→G in two families (3.92%) and the IVS18+66G→A in 22 families (43.14%). The large number of families found with the IVS7delCTT mutation and the fact that the position of the change is not likely to influence the splice donor site, being at position +33 (Strachan and Read, 1996), suggests that this is a polymorphism, like the previously described IVS8-58delT ((Osorio et al., 2000; Shattuck-Eidens et al., 1997) and IVS18+66G→A (Osorio et al., 2000) mutations. The IVS14-63C→G and IVS18+64A→G novel variants are also unlikely to affect splicing, as they occur too far away from the splice site.

The S694S silent mutation clearly has no affect on the polypeptide. It has been described in breast and ovarian cancer patients and controls (Couch et al., 1996; Durocher et al., 1996; Janezic et al., 1999; Shattuck-Eidens et al., 1997). Two other novel silent mutations were detected in this study, i.e. A870A and S1370S, both of which are unlikely to be pathogenic.

Unfortunately from the data alone, it is difficult to draw any conclusive correlations between the presence of the uncharacterised variants and/or polymorphisms and phenotype with respect to breast and/or ovarian cancer and the age at diagnosis. It could be possible that a haplotype of all or a combination of the polymorphisms may offer a protective effect, and result in a later age at diagnosis or a milder phenotype, or it could be less protective. Obviously, more work needs to be carried out to determine if there is a significant association. This should include work to determine whether the variants are in cis or trans and whether they segregate with the cancer phenotype in the family. More importantly, families where no BRCA1 mutations have been identified must first be screened for mutations in BRCA2, and possibly the other breast cancer susceptibility genes.

Shattuck-Eidens et al. (1997) have described a haplotype of variants, including the polymorphisms and unclassified variants we have detected. Certain haplotypes are more common than others and represent common chromosomes on which mutations and other variations occur. It has also been shown by other studies, that many of the polymorphisms are in linkage disequilibrium (Durocher et al., 1996).

The uncharacterised variants, polymorphisms, missense and even silent mutations cannot be passed off as benign changes in line with recent studies. It has been suggested that discrete sequences within exons known as exonic splicing enhancers (ESEs) are important in promoting splicing (Blencowe, 2000). These elements are often targets for mutations in some human diseases, such as Spinal muscular atrophy and Becker muscular dystrophy. It is therefore not unlikely that a seemingly silent mutation or other variation may affect an ESE, and have implications for splicing. If similar elements are found to be present in the introns as well, the theory may also be applied to the uncharacterised variants detected in the introns. This may be used to explain how a seemingly simple variant could possibly be disease causing, by means of interfering with normal splicing mechanisms.

4.2 SUMMARY OF DISEASE CAUSING MUTATIONS

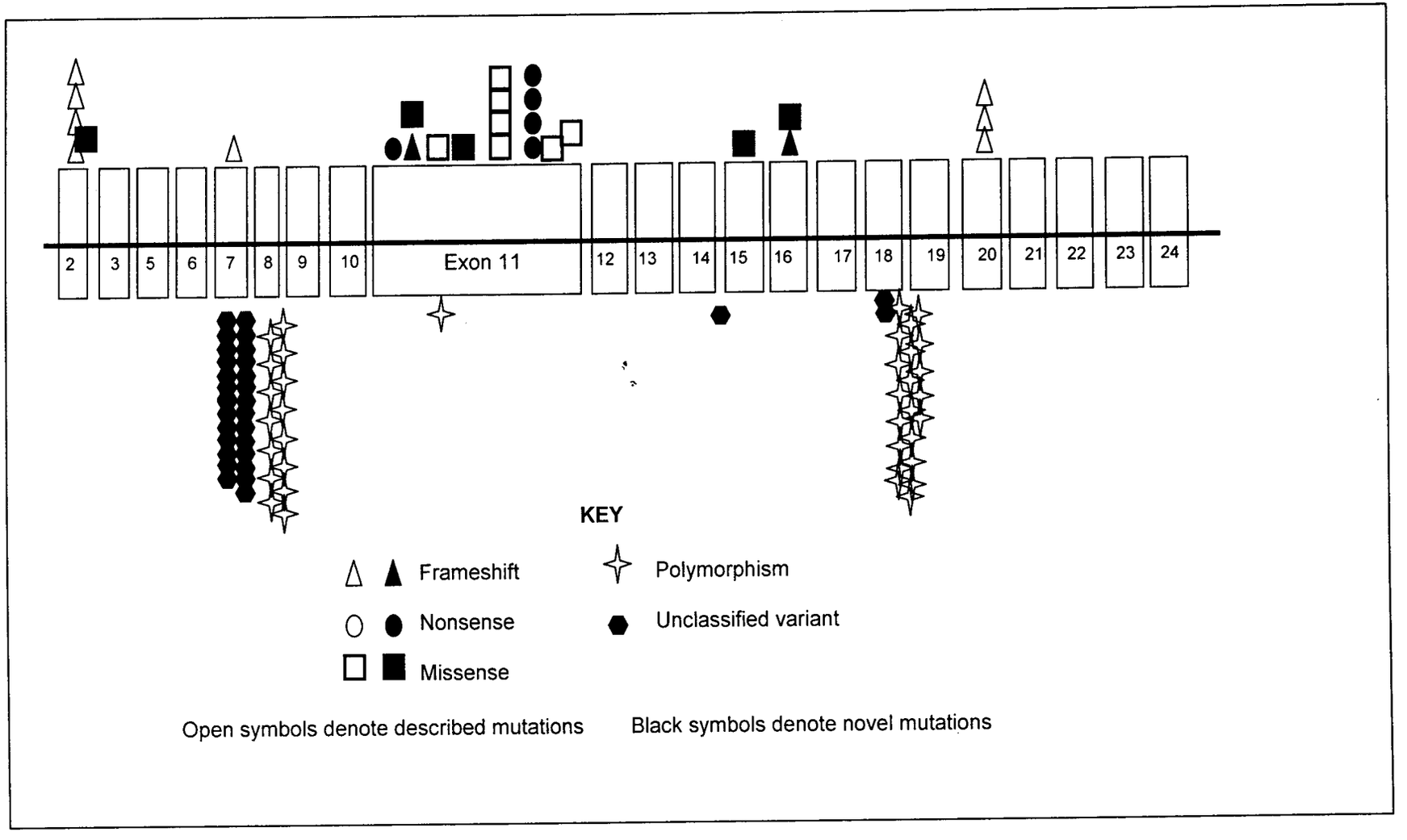
The recurrent 185delAG and 5382insC mutations were detected in seven families (13.7%), representing 7.8% and 5.9% respectively, of all mutations detected. The S451X nonsense mutation was identified in one family (2%); the E881X nonsense mutation was identified in four families (7.8%), and the 1493delC mutation was identified in one family (2%). The 448insA mutation has been previously described, and the

4957insC mutation is novel. A summary of these mutations is seen in table 4.11, the distribution of mutations, unclassified variants and polymorphisms can be seen visually on a schematic diagram of the BRCA1 gene in figure 4.17, mutations that are pathogenic or potentially pathogenic are shown above the gene, those below the gene are non-pathogenic mutations that are often polymorphic.

Table 4.10: BRCA1Pathogenic Germline Mutations Identified in South African Families.

Family	Exon	Nucleotide Change	Mutation	Method of Detection	Comment
BRC 13	2	185delAG	185delAG	ASO	Described
BRC 35	2	185delAG	185delAG	ASO	Described
BRC 51	2	185delAG	185delAG	ASO	Described
OV 3	2	185delAG	185delAG	ASO	Described
BRC 34	7	448insA	448insA	SSCP/HA	Described
BRC 55	11	1471C→A	S451X	PTT	Novel
BRC 10	11	2760G→T	E881X	PTT	Novel
BRC 21	11	2760G→T	E881X	PTT	Novel
OV 1	11	2760G→T	E881X	PTT	Novel
FS 1	11	2760G→T	E881X	PTT	Novel
BRC 27	11	1493delC	1493delC	PTT	Novel
BRC 48	16	4957insC	4957insC	SSCP	Novel
BRC 1	20	5382insC	5382insC	ASO	Described
BRC 5	20	5382insC	5382insC	ASO	Described
BRC 11	20	5382insC	5382insC	ASO	Described

Figure 4.17: Schematic Representation of the Location of All Mutations Detected



A large proportion of the breast/ovarian cancer families (57.1%) were found to harbour a disease-causing BRCA1 mutation. Similarly, 50% of the ovarian cancer only families had a BRCA1 mutation, while 7.4% of the breast cancer only families harboured a BRCA1 mutation. Mutations in exon 11 were found in 40% of the families with a detectable BRCA1 mutation. This is less than expected for exon 11 (as exon 11 accounts for 60% of the gene), but all the mutations are expected to result in a truncated protein product. There are a number of factors that could account for the low proportion of mutations identified in exon 11. These include the fact that the PTT does not detect missense mutations, the possibility of large chromosomal aberrations, regulatory mutations or mutations that fall outside the regions covered by the PTT.

4.3 COMPARISONS TO OTHER STUDIES

A significant proportion of our South African families (29.4%) were found to have a BRCA1 mutation. These results are shown in context with results of studies from the rest of the world in table 4.12. The extent to which BRCA1 mutations are involved in South African families is much higher than most other countries, but lower than in America, Russia, Israel and Sweden. However, there were novel mutations obtained, which had not been described elsewhere. The South African results are also similar to Canada and Sweden, but this is probably purely coincidental, as the origins of the populations are very different.

In order to compare our results with those of others, we need to break down the South African families into an Afrikaner and an Ashkenazi Jewish component. This is used to compare the proportion of mutations found with similar countries of origins. In this study, 72.5% of the patients studied were of Afrikaner descent, and 13.7% were Ashkenazi Jewish. Therefore we can compare the results from Holland and Belgium (24% and 23.8% respectively), to the proportion of Afrikaner families with mutations (18.9%). One would have expected these results to be similar, as the Afrikaner population originated from Dutch, German and French populations. Although, on the whole the results in this study (29.4%) are quite similar to those of Belgium and Holland (23.8% and 24% respectively).

Table 4.11: Comparison of our study with others

Population	Percentage BRCA1 mutations found	Reference
Iceland	1%	Thorlacius et al., 1996
Finland	7%	Huusko et al., 1998
Spain	9.38%	Osorio et al., 2000
United Kingdom	10.2%	Xu et al., 1997b
Taiwan	11.1%	Li et al., 1999
America	16%	Couch et al., 1997
United Kingdom	18.2%	Greenman et al., 1998
Scandinavian	22.6%	Håkansson et al., 1997
Belgium	23.8%	Goelen et al., 1999
Holland	24%	Ligtenberg et al., 1999
Canada	24.7%	Tonin et al., 1998
South Africa	**29.4%**	**Present study**
Sweden	33%	Zelada-Hedman et al., 1997
Israel	47%	Levy-Lahad et al., 1997
America	67.6%	Friedman et al., 1995
Russia	75%	Gayther et al., 1997
America	80%	Serova et al., 1996

The mutations detected in the present study were novel, and have not been detected outside South Africa. The E881X mutation is one such mutation, which originated in a French couple and has a founder effect in the Afrikaner population. The Afrikaner patients in our study are expected to mostly have a similar genetic makeup to the Dutch population, and it was surprising to find that our results did not resemble those of the Dutch studies. None of the mutations unique to the Dutch population were detected in the South African families, even though the methods used in this study were similar to those used by the Dutch. However, our mutation detection techniques would not detect large rearrangements, which have been shown to make up 36% of all mutations in Dutch breast/ovarian cancer families (Petrij-Bosch et al., 1997). Thus a founder effect may be seen in the South African families of Dutch origin, if the BRCA1 gene is analysed for large genomic deletions in these families.

Similarly the South African Ashkenazi Jewish families can be compared to the Israeli population, where the 185delAG founder mutation was found in 71.4% of families. This is much higher than the proportion of families in Israel (47%), but there were only seven families used in this study. The 185delAG mutation identified in these families is the same ancient mutation that has been identified in other Ashkenazi Jewish families around the world.

Only 1% of breast/ovarian cancer families in Iceland can be explained by mutations in BRCA1 (Thorlacius et al., 1996). In this population, BRCA2 mutations are far more common than BRCA1 mutations. In fact, the 999del5 BRCA2 mutation explains most of the breast/ovarian cancer, representing a strong founder effect. Mutations in Finnish breast/ovarian cancer families are seen in a very low percentage (7%) of families (Huusko et al., 1998). The frequency of BRCA2 mutations is equally low, but the mutations that are described represent a founder effect, and are unique to the Finnish population.

In Spain, a low proportion of families were found with BRCA1 mutations. A surprising result from this study by Osorio et al. (2000) was the absence of any mutations in exon 11. A large percentage of mutations are expected to occur here, due to the large size of this exon (60% of the gene). It has also been reported that 94% of all exon 11 mutations would result in premature chain termination (Couch et al., 1996).

In Russia, 75% of familial ovarian cancer families can be explained by mutations in BRCA1 (Gayther et al., 1997). This, together with the fact that two mutations (5382insC and 4153del A) account for 86% of these mutations, represents a founder effect in this population.

The high frequency of mutations in American families, as explained by Serova et al. (1996) may be due to the fact that the study only investigated 20 families, which may be considered a small size, and the mutation detection involved complete sequencing of the entire coding region of BRCA1. The study also involved cDNA analysis to reveal abnormal BRCA1 transcripts as well as looking for regulatory mutations which would identify decreased RNA levels. This is a comprehensive screen of the entire coding region as well as the promoter areas, but is rather labour-intensive and costly.

It is interesting to note the differences in frequencies of BRCA1 mutations reported in the same country, in different studies, most notably in the United Kingdom and America .

This may represent a difference in the selection criteria of patients between studies, as well as different mutation detection techniques used (Xu et al., 1997b; Greenman et al., 1998).

4.4 EFFICIENCY OF MUTATION DETECTION

In this study, 29.4% of families were found to carry a BRCA1 mutation. This may represent the true number of families with mutations, or it may reflect the sensitivities of the mutation detection methods used. DNA sequencing of the entire BRCA1 gene would fully elucidate the mutation status of the patients, but this technique is time consuming, labour intensive and costly. Due to the differences in sensitivities of techniques and their ability to introduce artifacts, it is important to sequence any possible mutation to confirm its presence. We are confident that our screening methods, together with the confirmation by sequencing are sufficient evidence for the presence of a mutation. In addition to this we have chosen the methods we used specifically to detect disease-causing mutations. The PCR-ASO technique can only be used to detect specific mutations, however the respective exons were also completely screened by the SSCP/HA to screen for other mutations. The PTT of exon 11 detected truncating mutations (frameshift or nonsense mutations), but would miss any missense mutations. It may be necessary to screen exon 11 by SSCP/HA for these mutations, but this would be time-consuming. In addition, it is difficult to prove that a missense mutation is disease causing. It is also difficult to comment on the significance of the intronic variants and polymorphisms detected. It has been estimated that more than 80% of mutations will be detected using SSCP/HA (Hayashi and Yandell, 1993). Therefore if the 15 families identified with mutations represent this sensitivity of detection, then we would have missed a further three families, out of the 51 studied. When SSCP is used in conjunction with heteroduplex analysis it may increase the detection rate, due to the effect of one technique resolving subtle or ambiguous shifts generated by the other technique (Axton and Hanson, 1998). This effect can be seen by the fact that some mutations show only SSCP or heteroduplex shifts only, and some have both shifts. The methods used in this study are the accepted methods by most other researchers today.

A number of recurrent mutations have been detected in other populations groups, but the South African population is probably most comparable to the Dutch population. In this population group a large number of families were found to carry the 2804d4elAA mutation that has not been reported elsewhere (Peelen et al., 1997). This mutation was

not detected in the South African families even though it is detectable using the PTT technique. In addition to this, 36% of all mutations in Dutch breast/ovarian cancer families are one of three large Alu-mediated deletions (Petrij-Bosch et al., 1997). It is possible that the South African Afrikaner families could have these founder mutations, but a method to detect the large rearrangements is required.

In this study, only the coding sequence of BRCA1 was screened for mutations, therefore mutations in the regulatory regions would have been missed. Germline mutations that affect the promoter, gene expression or gene silencing would therefore also be excluded. Similarly, any large deletions or rearrangements would be missed too by the PCR-based techniques used in this study. Thus, the frequency of BRCA1 mutations in the families studied may be higher than reported here, due to the limitations of the mutation detection techniques used in this study. Complex rearrangements and large deletions of the BRCA1 gene may occur as a result of Alu-mediated recombination, which may explain the majority of families with no detectable BRCA1 mutations. Also, most times only one individual from each family was analysed. It is possible that a mutation was not detected because that individual was a phenocopy, and the mutation may be found by analysing a different individual .

Clearly the next step in assessing these South African breast/ovarian cancer families would be to identify BRCA2 mutations in addition to large deletions or rearrangements, more specifically those that are common to the Dutch population (Petrij-Bosch et al., 1997), in the 70% of families with no detectable BRCA1 mutations. It would also be useful to do linked marker analysis in the larger families to give an indication if BRCA1 is indeed involved or not, by virtue of co-segregation of the alleles with the phenotype.

CHAPTER 5
CONCLUSION

This study represents the first study of BRCA1 mutations in South African breast/ovarian cancer families, and it has been shown that 29.4% of the families studied have a disease causing BRCA1 mutation. Many of these mutations are novel and represent a founder effect in the Afrikaner population. This founder E881X mutation can be traced back to a French couple who arrived in South Africa in the late 1600s. As the mutation has not been found in France, it is an Afrikaner specific one. It appears to confer a higher penetrance of ovarian cancer.

Recurrent mutations were also detected in the families studied. It was shown that our South African families with the same mutations are genetically related to each other and other patients worldwide with the same mutations. Missense mutations were also identified, but more research needs to be carried out to determine if these are pathogenic. Many unclassified variants of unknown significance, polymorphisms and silent mutations were also identified. There may prove to be an interesting link in the presence of these polymorphisms and the presence of cancer, due to the appearance of a haplotype of polymorphisms, in patients with and without a detectable mutation. More work needs to be conducted to determine the significance of this.

It is thought that BRCA1 accounts for the majority of breast/ovarian cancer and ovarian cancer only families, as well as a lesser proportion of breast cancer only families. This study confirmed that this is also the case in South African families, with the majority of breast/ovarian cancer families (57.1%), and a large proportion (50%) of ovarian cancer only families carrying mutations. A much smaller proportion (7.4%) of breast cancer only families were identified with mutations in BRCA1. The frequency of mutations found in these families may increase, as the possibility of large rearrangements in the BRCA1 gene will be addressed.

These results have already allowed for accurate counselling and risk assessment in the mutation positive families. Patients are therefore able to manage their cancer risk by way of regular screening, chemoprevention or prophylactic surgery. Screening of new families is now simplified by screening for the founder mutations first. There is also the prospect for predictive testing, which requires psychological assessment, taking into account the patients' wishes and well being of the whole patient. However, a word of

caution is given here, with respect to the experience we have had with family BRC11. It is important to conduct this type of testing in controlled laboratory conditions, and be completely sure of the mutation identified before proceeding with counselling. Even then, one must remain aware of the limitations, and until the risks associated with certain mutations is fully understood, presymptomatic diagnosis, should only be used with caution and sensitivity to the patients' needs.

The involvement of BRCA2 mutations needs to be addressed in the remaining families where no BRCA1 mutation was identified. The presence of large Alu-mediated deletions, rearrangements or regulatory mutations in these families may also explain the absence of detectable BRCA1 mutations. Until both these aspects have been completely analysed, one may assume that other genes are involved (BRCA2 or BRCA3), or the appearance of the cancer in the families is not due to a familial component.

Note added in proof: Of the remaining 36 families with no detectable BRCA1 mutation, 13 have subsequently been found to carry BRCA2 mutations.

CHAPTER 6
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APPENDIX 1

PROVINSIALE ADMINISTRASIE

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GAUTENG

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Enquires: Dr R Sommers

Reference:

Tel: (012) 354 1560

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Address: Ethics Eittee
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Private Bag x 169
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0001

Date: 25-02-98

Nommer : 18/98

TITEL : Molecular Genetic Analysis of Inherited Predisposition to Breast/ Ovarian Cancer: The Frequency and Nature of BRCA1 and BTCA3 Mutations in South African Breast / Ovarian Cancer Families.

AANSOEKER : Prof E J van Rensburg; Dept Human Genetics Pretoria Academic Hospitals;PRETORIA.

This Protokol and Informd Consent has been considered by the Ethics Committee,Faculty of Medicine,Univ.of Pretoria and Pretoria Academic Hospitals on 25-02-98 and found to be acceptable.

Prof A.L. Coetzee	MA(ClinPsych);DSocSc (Leiden);MPA(Pret): Psychologist
Dr J.E.Davel (female)	MBChB:Hospital Superintendent
Prof A.P.du Toit	BA;DiplTheo(Pret) BA (Hons) (Rhodes);MA;DPhil(Pret): Philosopher
Prof C.I. Falkson (female)	MBChB;M.Med(Int);MD;Med. Oncologist
Prof G. Falkson	MBChB;M.Med(Int);MD;OSG: Medical Oncologist
Prof S.V. Grey (female)	BSc(Hons)(Stell);MSc(PU vir CHO) DSc(Pret): Deputy Dean
Dr S.W. Johnson	MBChB:Hospital Superintendent
Dr V.O.L. Karusseit	MBChB;MFGP(SA);M.Med(Chir); FCS (SA): Surgeon
Ms B.C.F. Magardie(female)	BCur:Matron/Senior Nursing Sister
Senior Sr J. Moerane(female)	BCur(EetAl)SeniorNursing Sister
Prof T.R. Mokoena	MBChB;FRCS(Glasgow);DPhil (Oxford): Surgeon
Prof H.W. Pretorius	MBChB;M.Med (Psych)MD: Psychiatrist
Dr P. Rheeder	MBChB;MMed(Int);LKI(SA);MSc (KLIN.EPI):Specialist Physician
Prof J.R. Snyman	MBChB,MPharmMed: Pharmacologist
Prof De K Sommers	BChB; HDD; MBChB; MD: Pharmacologist
Advokaat L.G.Thomas (female)	B.Iuris(University of the North); LLB(University of the Western Cape)
Prof F.W. van Oosten	BA;LLB;LLD(Pret);LLD (Unisa):Prof in Criminaland Medical Law

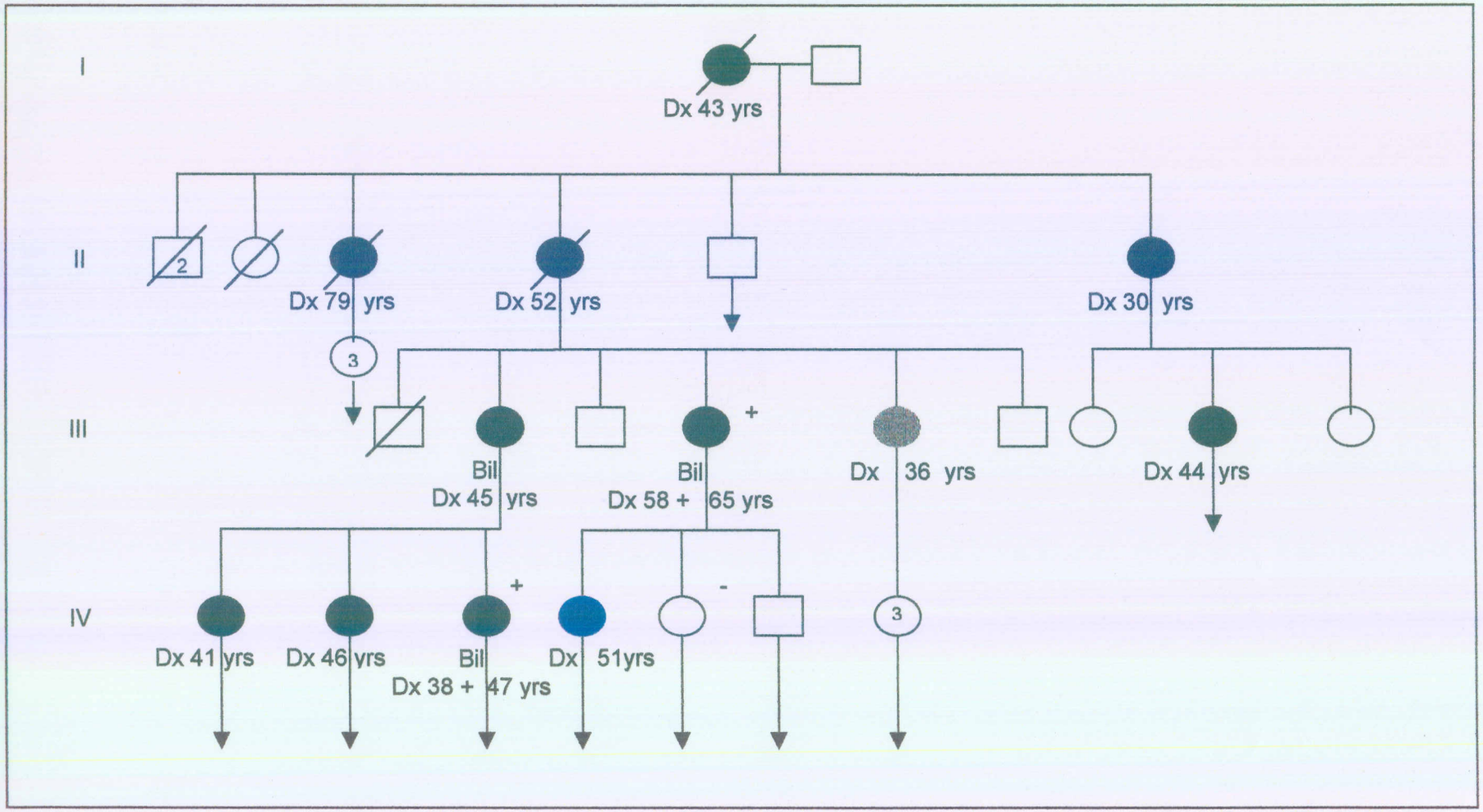
PROF G FALKSON; MBChB; M.Med (Int);MD;
VOORSITTER

APPENDIX 2

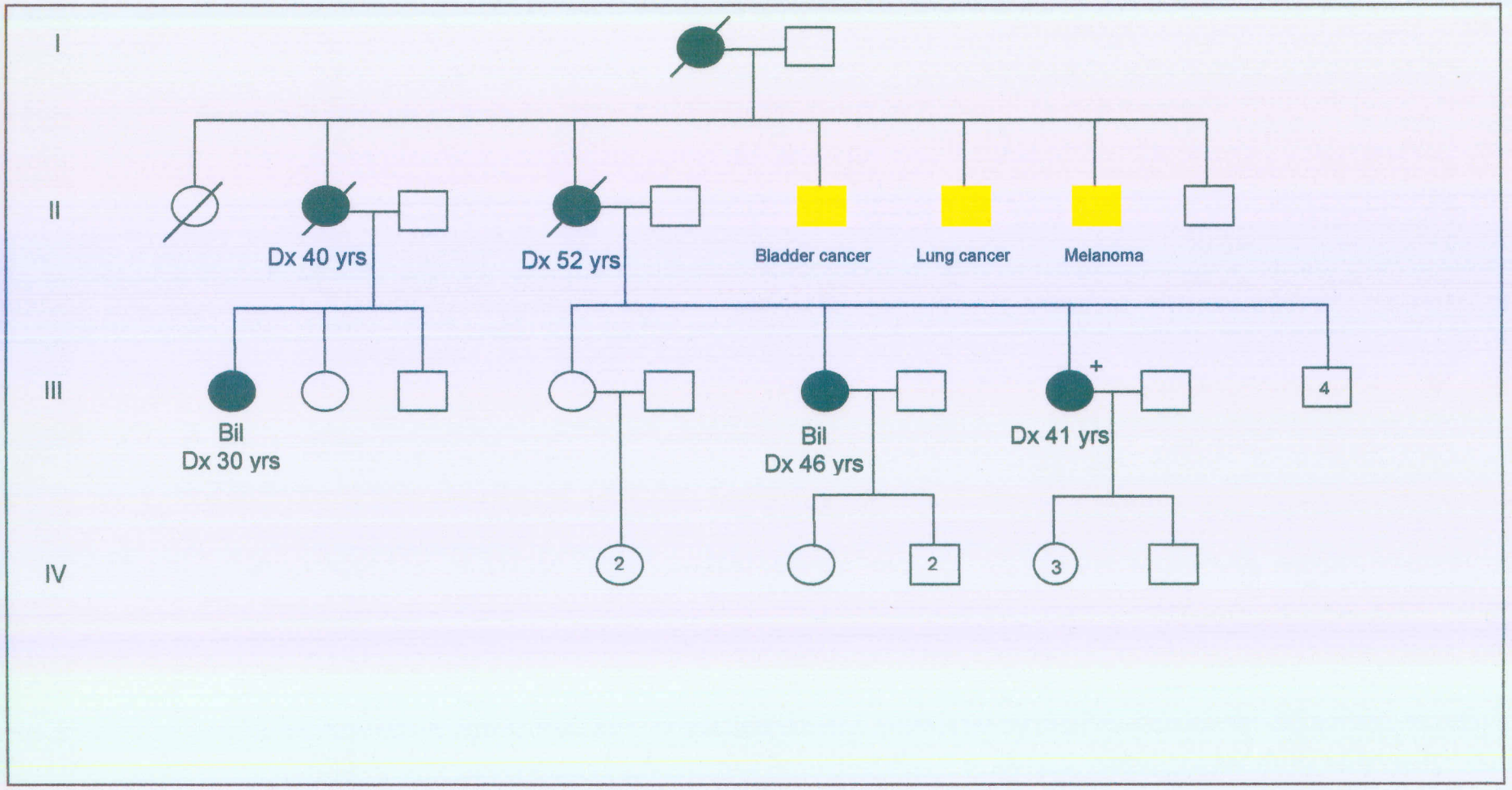
Pedigrees of families with mutations listed in table 4:11 are shown in the appendix.
The key, as shown below, applies to all pedigrees. Those individuals that have tested positive for the specific mutation are denoted with +, those that have tested negative are shown as -.
Spouses have not always been included in the pedigree.
Where there are a number of unaffected children in successive generations, a ↓ symbol is shown.

KEY					
●	Breast cancer	●	Ovarian cancer	●	Breast and ovarian cancer
Dx	Age at diagnosis	Bil	Bilateral cancer	●	Other cancer
+	Mutation present	-	No mutation	yrs	years

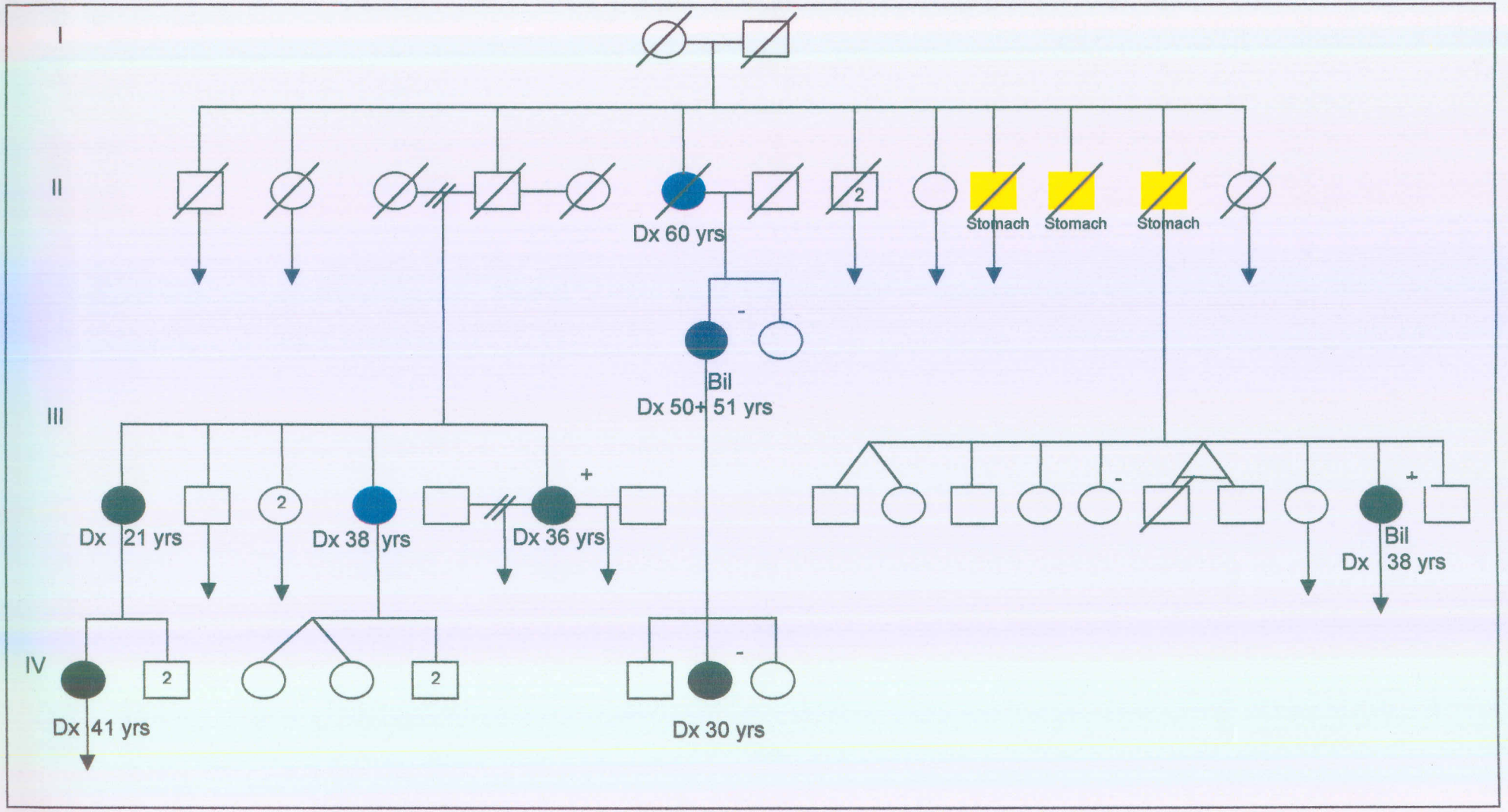
BRC 5 – 5382insC



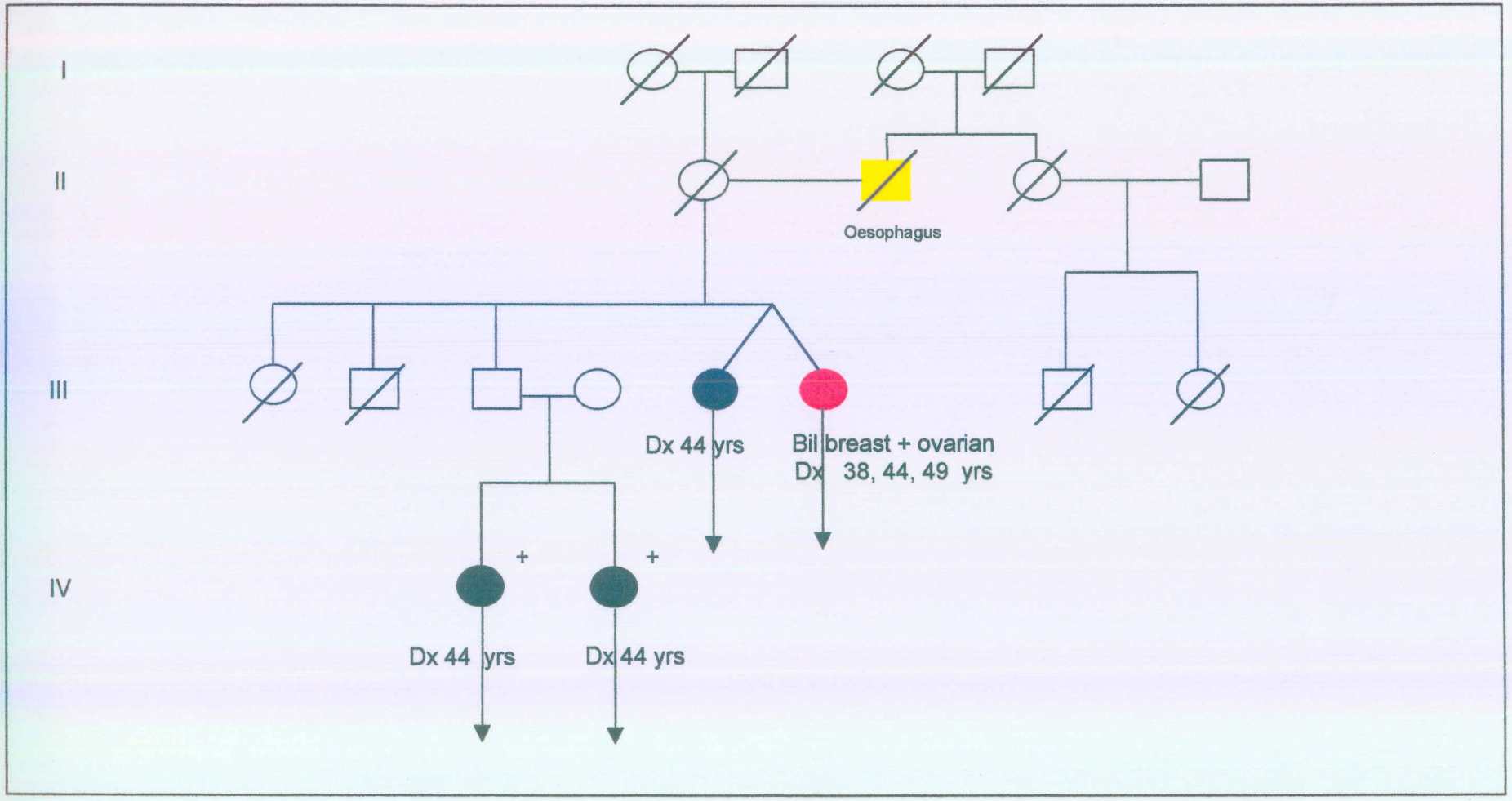
BRC 10 - E881X mutation



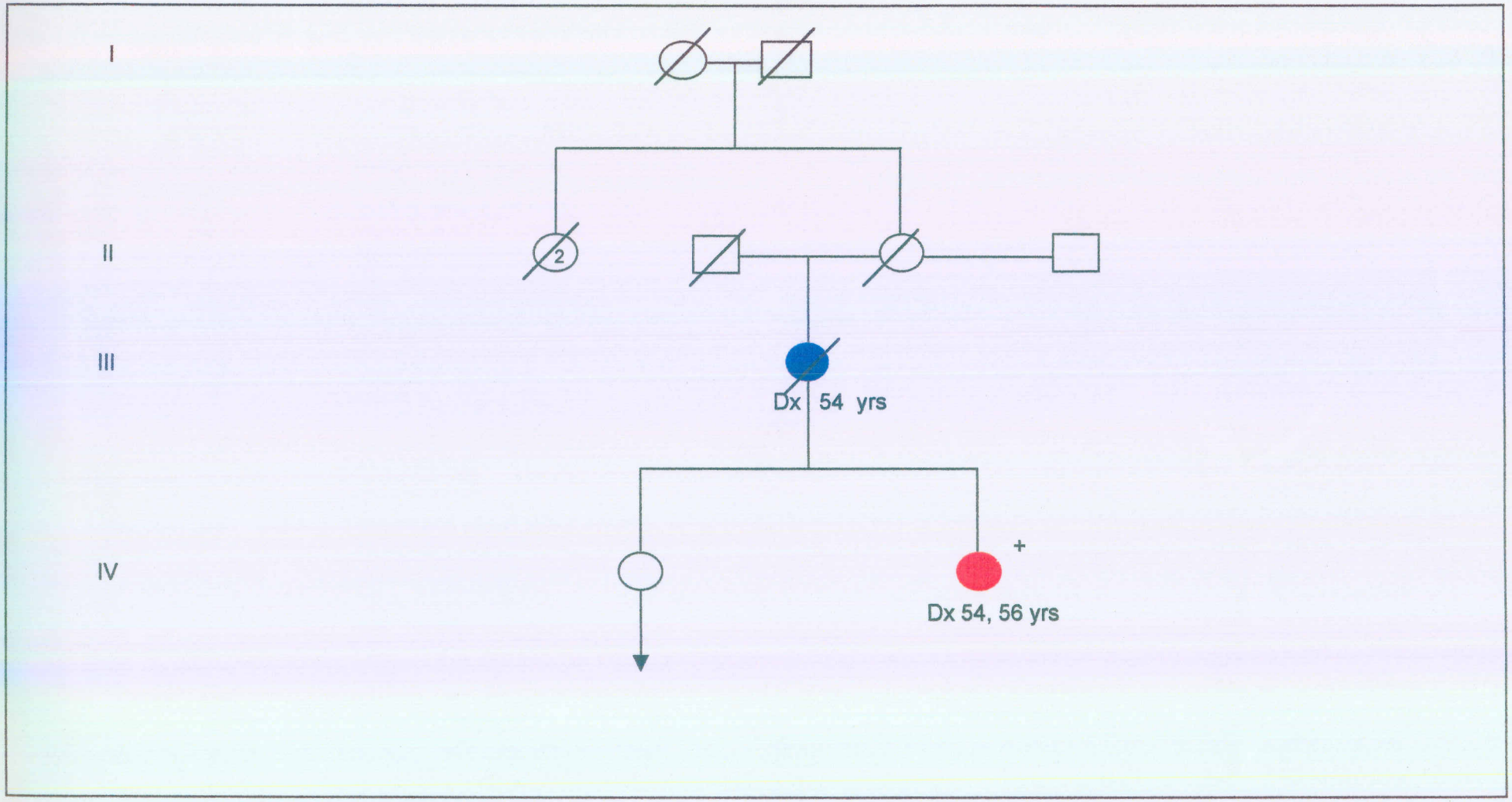
BRC 11 - 5382insC mutation



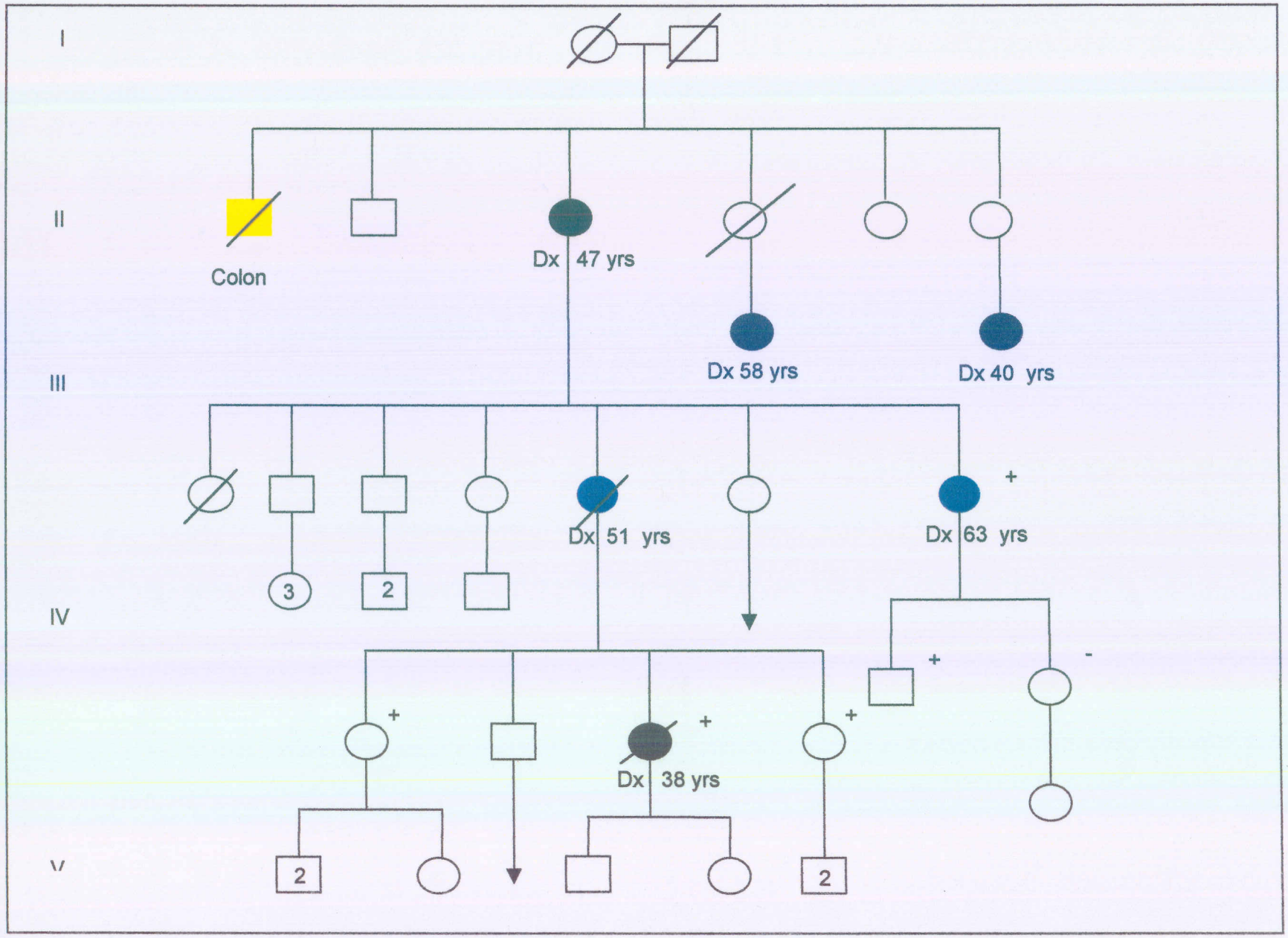
BRC 13 – 185delAG



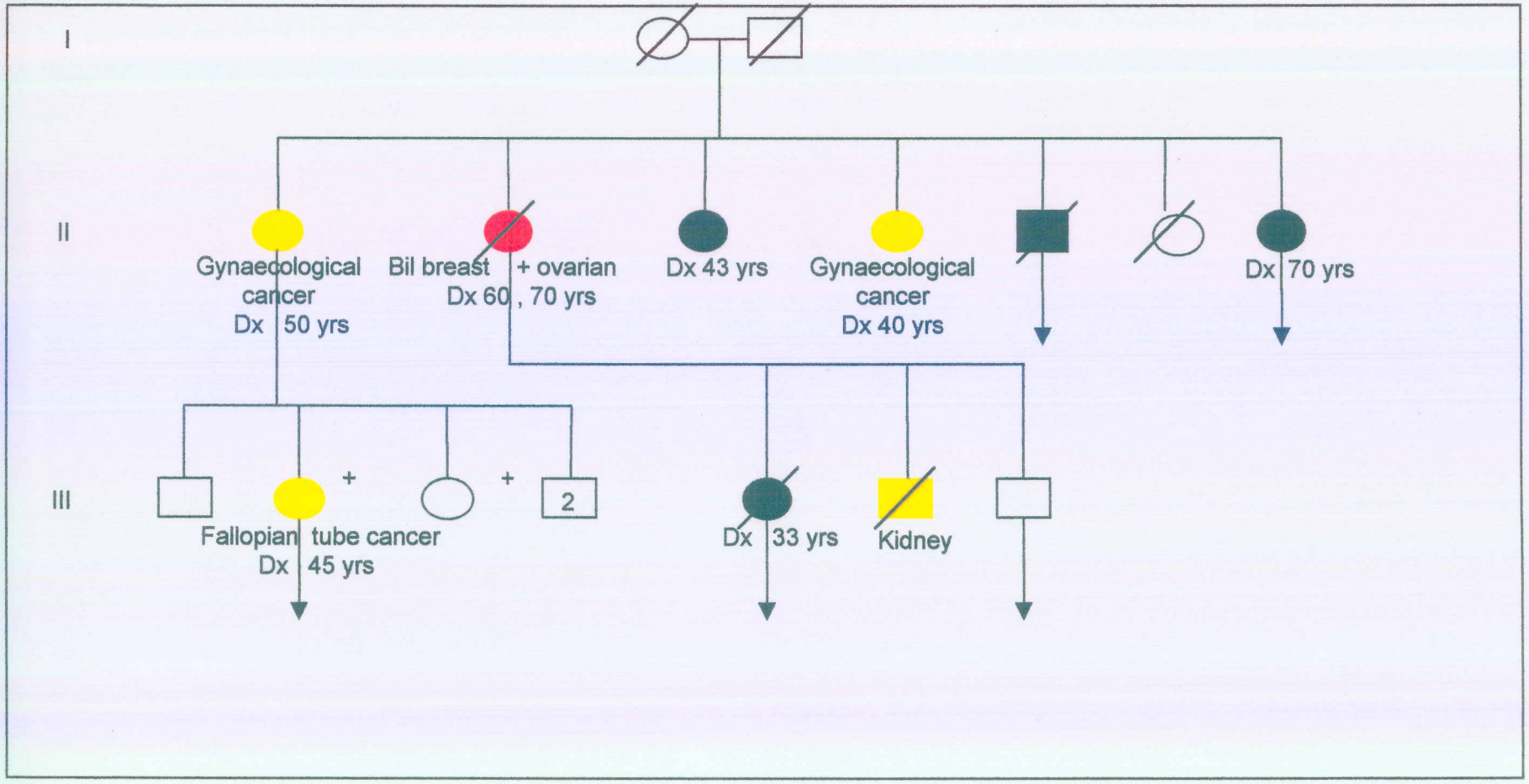
BRC 21 – E881X



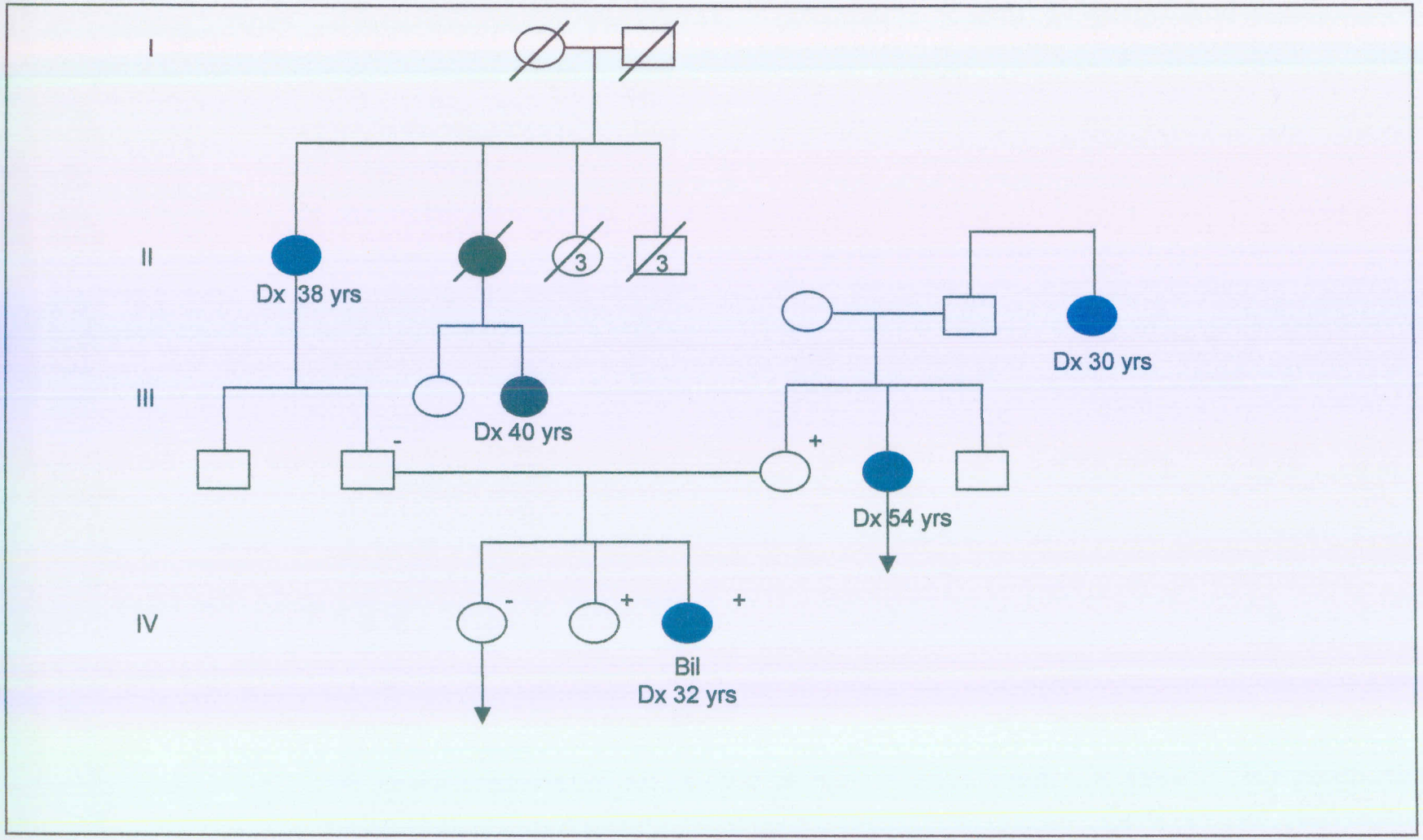
BRC 27 – 1493delC



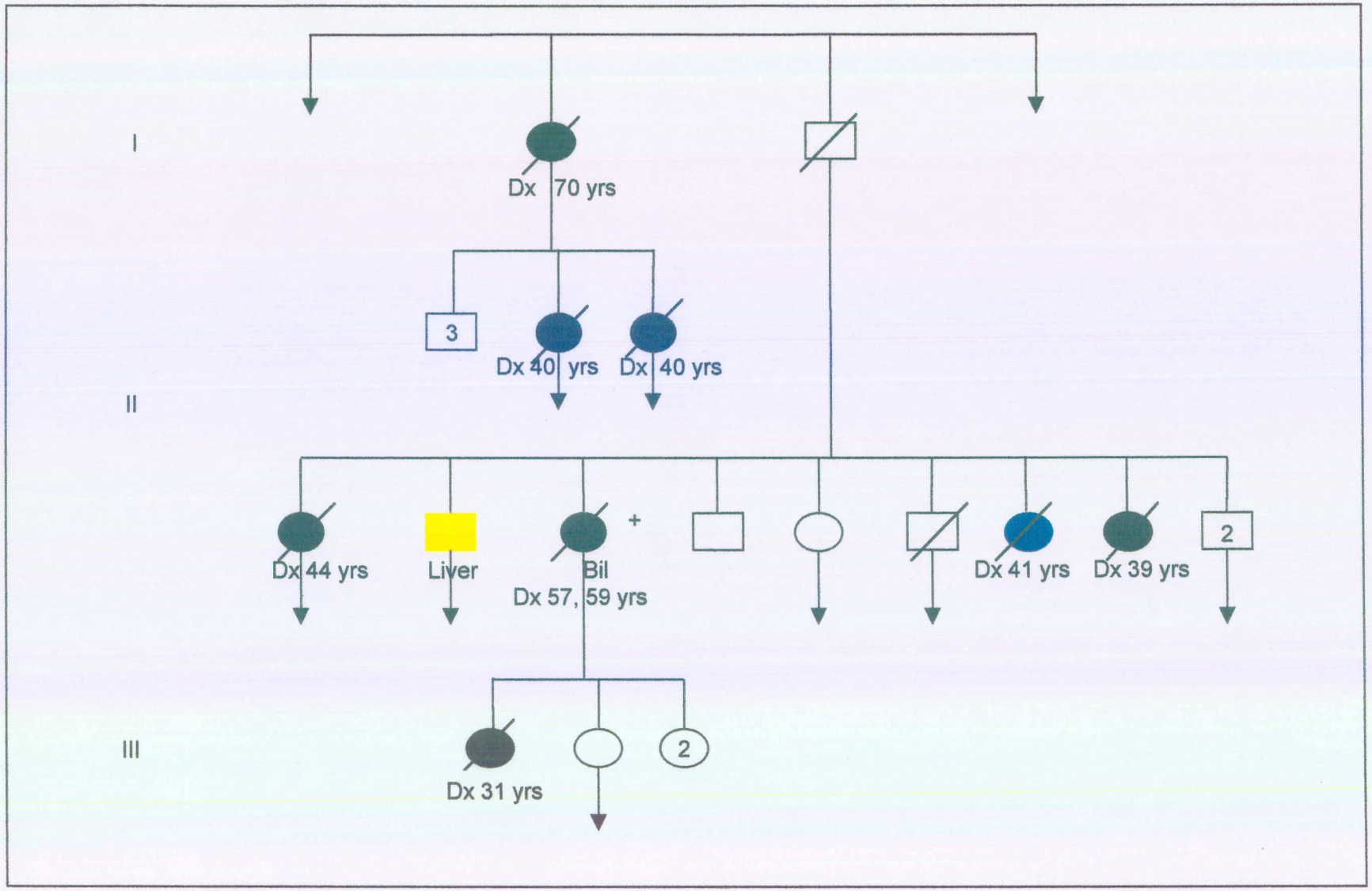
BRC34 – 448insA



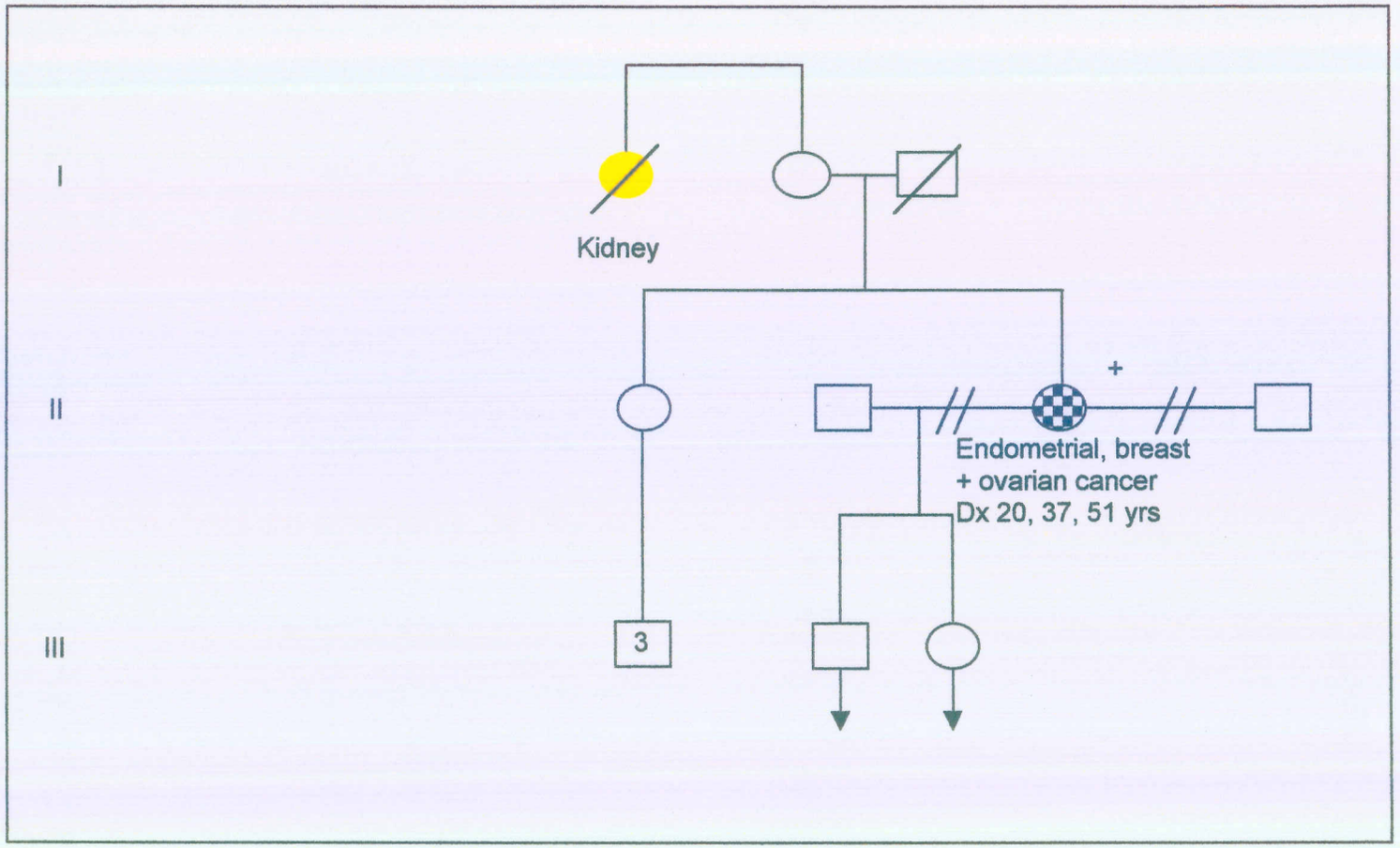
BRC 35 – 185delAG



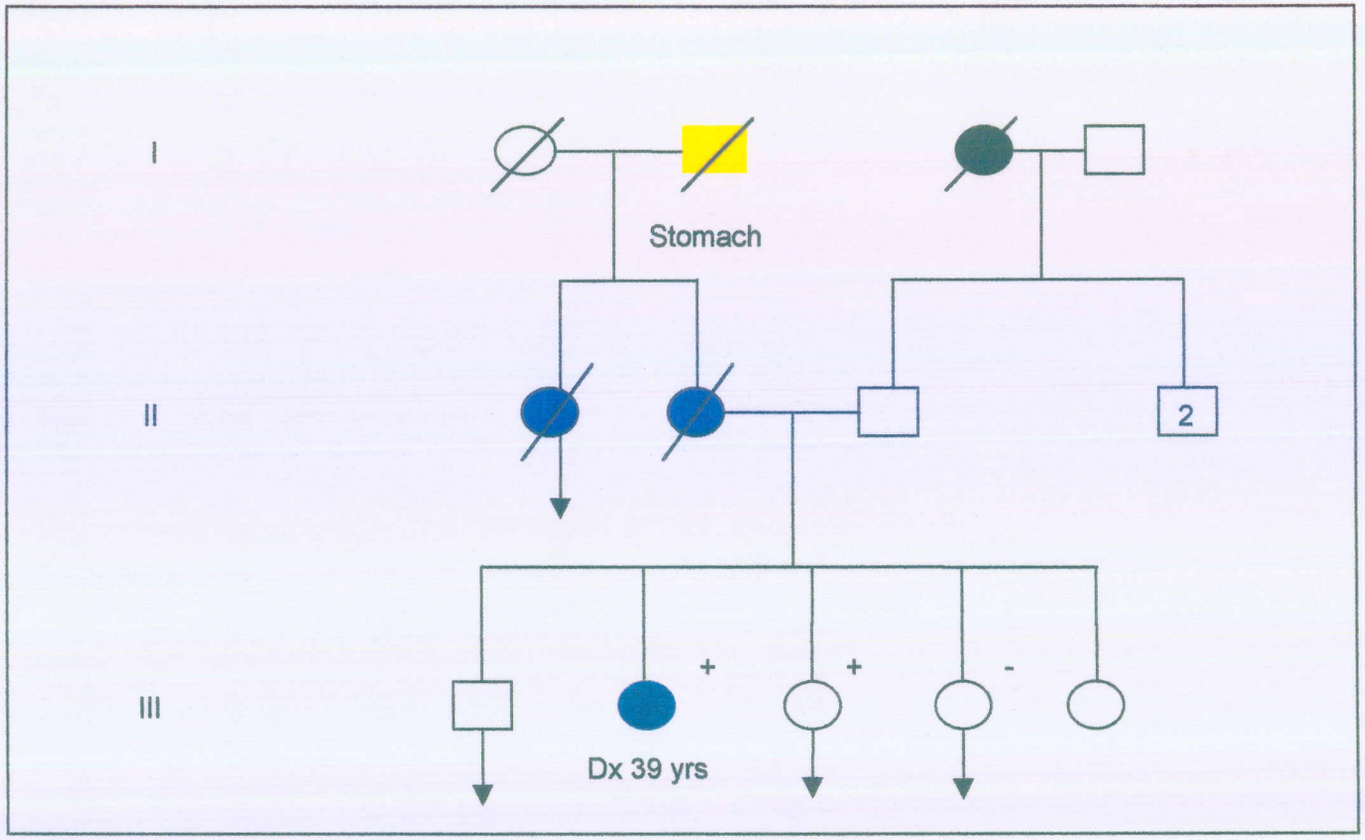
BRC 48 – 4957insC



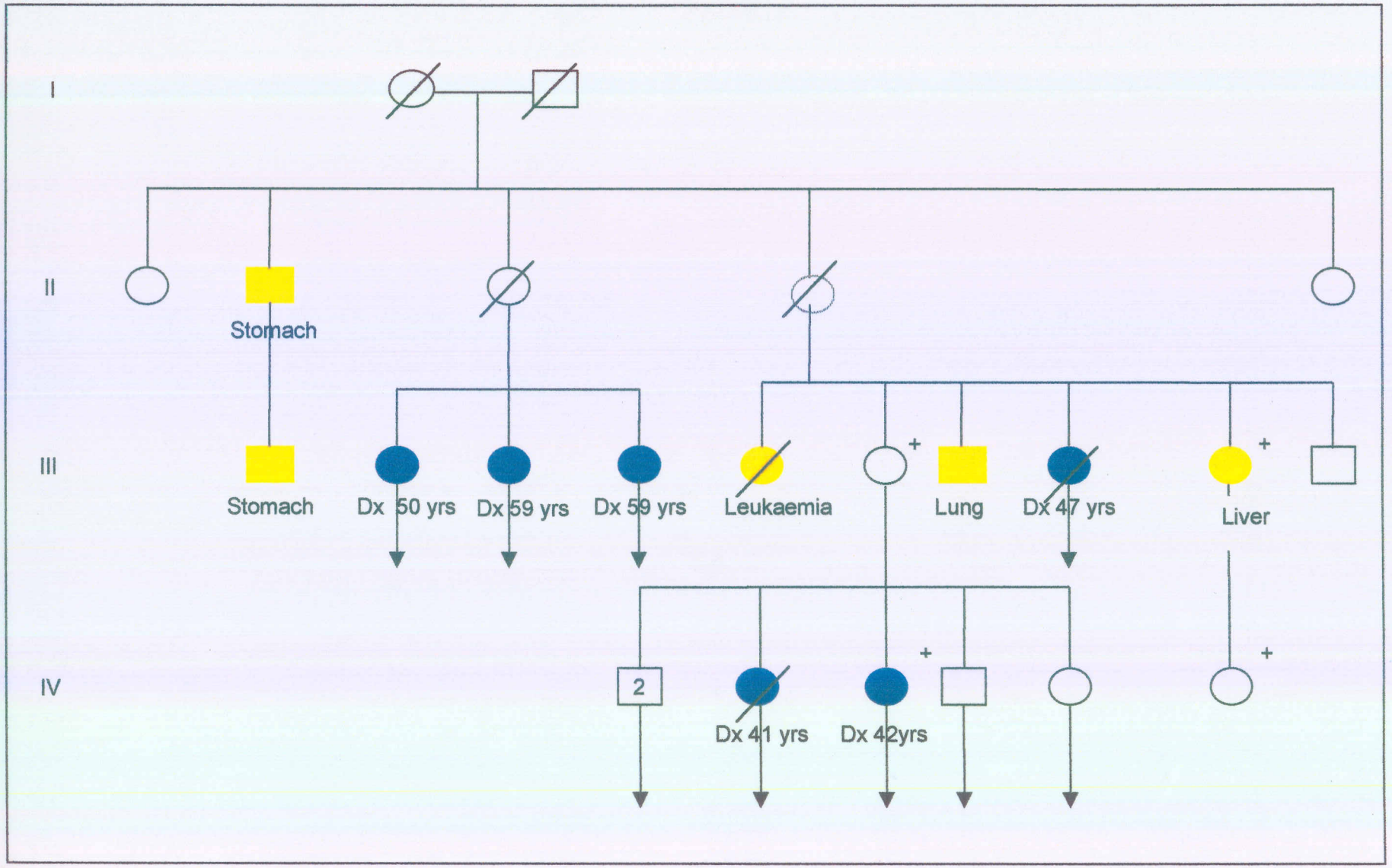
BRC 51 – 185delAG



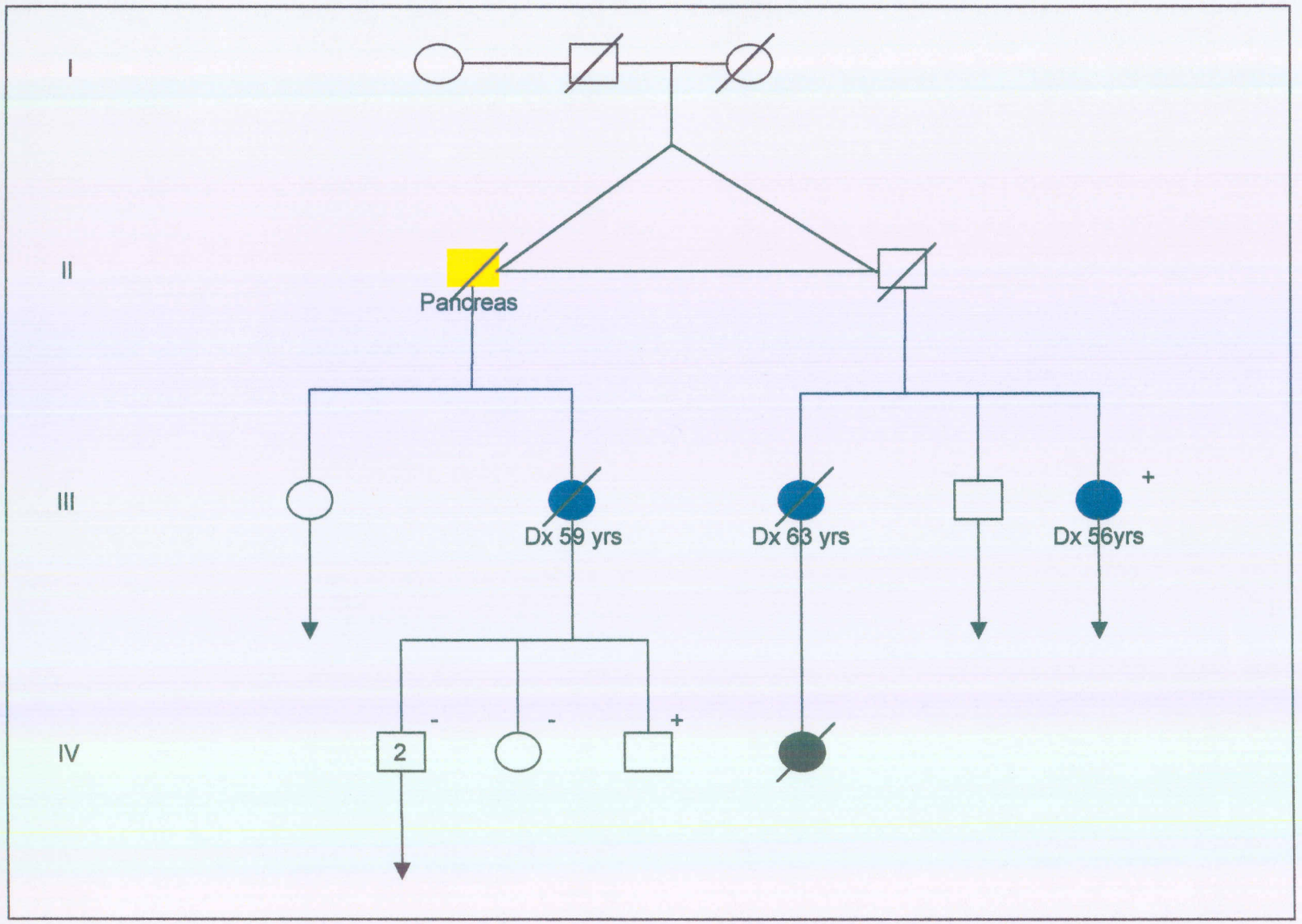
BRC 55 – S451X



OV 1 – E881X mutation



OV 3 - 185delAG



FS1 – E881X