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Characterisation of *BRCA1* genomic rearrangements in South African breast and/or ovarian cancer families

BY

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ABSTRACT

Germ-line mutations within the breast cancer susceptibility genes, *BRCA1* and *BRCA2* are responsible for inherited susceptibility to breast and ovarian cancer. A wide spectrum of pathogenic mutations has been identified within both genes, but alterations within these genes occur far less frequently than originally believed. A large number of breast cancer families that showed linkage to *BRCA1* were not found to carry a pathogenic *BRCA1* mutation following the use of "classical" PCR based assays. In 1997, a large genomic rearrangement was reported within *BRCA1*, using Southern blotting. Numerous groups then employed semi-/quantitative methods to determine the presence and/or frequency of such alterations. This search extended the mutation spectrum of this gene, and to date at least 69 unique rearrangements have been reported. The contribution of these alterations to the burden of breast/ovarian cancer differs greatly between populations ranging from 0% to 36% of all *BRCA1* mutations in the Finnish and Dutch populations respectively.

Mutation screening has previously indicated that small mutations within the two BRCA genes are responsible for 59% of breast/ovarian cancer susceptibility in South Africa. To determine whether large rearrangements contribute to breast cancer susceptibility in South Africa, 74 *BRCA1/2* small mutation negative patients from 58 breast / ovarian cancer families were screened for large intragenic *BRCA1* rearrangements using Multiplex Ligation-dependent Probe Amplification (MLPA). In this first study of large genomic rearrangements within *BRCA1* in South Africa, three genomic aberrations were detected. A deletion of exon 22 (IVS21-36del510) was identified in a Dutch immigrant. This deletion represents one of the Dutch founder mutations. Both exons 23 and 24 were found deleted in a South African family of Greek ancestry. The breakpoints of this deletion were not characterized. Simultaneous deletions of these two exons (where the breakpoints could not be characterized) have been reported in the Italian and Spanish populations. One of the genomic aberrations detected by MLPA in the present study

erroneously appeared as a deletion of exon 18. Sequence analysis of this variant identified it as a single base pair substitution (c.5215G→A). This variant (R1699Q) has been reported previously, but its pathological significance is unconfirmed.

In total, two large genomic rearrangements were detected in two families, of which only one is a South African, of Greek ancestry. This indicates that such mutations play a small role (1.75%; 1/57) in familial breast / ovarian cancer in South Africa (Dutch immigrant excluded). No rearrangements were identified in the Afrikaner population, indicating that such mutations do not contribute to the burden of familial breast/ovarian cancer in this population (0/40). The remaining South African breast/ovarian cancer risk may to some extent be explained by large rearrangements within *BRCA2*, or by mutations in other low penetrance breast cancer susceptibility gene(s). *BRCA2* will now be screened by MLPA, followed by mutation screening of genes such as *p53* and *CHEK2* in high-risk families.

OPSOMMING

Oorgeërfde vatbaarheid vir bors- en ovariële kanker word veroorsaak deur kiemsellyn mutasies in twee gene, *BRCA1* en *BRCA2*. Deur gebruik te maak van klasieke PKR metodes is ‘n wye verskeidenheid mutasies geïdentifiseer in beide gene. Daar is egter ‘n aantal bors/ ovariële kanker families met genetiese koppeling tot *BRCA1* wat nie mutasies in dié geen blyk te dra nie. In 1997 is ‘n groot herrangskikking in *BRCA1* gevind met behulp van die Southern klad metode. Veskeie groepe het toe semi-/kwantitatiewe metodes gebruik om die teenwoordigheid en/of frekwensie van sulke veranderings te bepaal. Hierdie soektog het die mutasie spektrum van dié geen uitgebrei, en huidiglik is daar ten minste 69 unieke herrangskikkings al gerapporteer. Die bydrae wat hierdie herrangskikkings maak tot die las van bors/ovariële kanker verskil grootliks tussen populasies en strek van 0% tot 36% van alle *BRCA1* mutasies in die Finse- en Nederlandse populasies respektiewelik.

Mutasie-sifting van die twee BRCA gene het aangetoon dat klein mutasies in dié gene verantwoordelik is vir 59% van bors/ ovariële kanker vatbaarheid in Suid Afrika. Ten einde te bepaal of groot herrangskikkings bydra tot borskanker-vatbaarheid in Suid-Afrika, is 77 *BRCA1/2* negatiewe pasiënte van 61 bors/ovariële kanker families gesif vir groot intrageniese *BRCA1* herrangskikkings m.b.v “Multiplex Ligation-dependent Probe Amplification” (MLPA) metode. In hierdié eerste studie van groot genomiese herrangskikkings in *BRCA1* in Suid-Afrika is drie genomiese afwykings gevind. ‘n Delesie van ekson 22 (IVS21-36del510) is geidentifiseer in ‘n Hollandse immigrant. Hierdié delesie is ‘n Nederlandse stigters-mutasie. Beide eksons 23 en 24 is weggelaat in ‘n Suid-Afrikaanse familie van Griekse herkoms. Die breekpunte van hierdie delesie is nie gekarakteriseer nie. Gelyktydige delesies van hierdie twee eksons (waar die breekpunte nie gekarakteriseer is nie) is gerapporteer in die Italiaanse en Spaanse populasies. Een van die genomiese afwykings bepaal met MLPA in die huidige studie het verkeerdelik as ‘n delesie van ekson 18 voorgekom. Volgorde-analise van hierdie variant het dit as ‘n enkel

basispaar verandering (c.5215G→A) geidentifiseer. Hierdie variant (R1699Q) is voorheen al gerapporteer, maar die patogenisiteit daarvan is onbekend.

In totaal, is twee groot herrangskikkings in twee families opgespoor, waarvan slegs een ‘n Suid-Afrikaanse familie is (van Griekse afkoms). Dit dui dus aan dat sulke mutasies ‘n beperkte rol (1.75%; 1/57) in Suid-Afrikaanse familiële bors/ ovariële kanker speel. Geen herrangskikkings is in die Afrikaner populasie geïdentifiseer, wat daarop dui dat sulke mutasies geen rol in familiële bors/ovariële kanker in hierdie populasie groep speel nie (0/40). Die oorblywende bors/ovariële kanker risiko mag moontlik veroorsaak word deur groot herrangskikkings in *BRCA2*, of deur mutasies in ander lae-penetrasie borskanker-vatbaarheids gene. *BRCA2* sal nou gesif word met behulp van MLPA, gevolg deur mutasie-sifting van gene soos *p53* en *CHEK2* in hoë-risiko families.

TABLE OF CONTENTS

SECTION	Page
ACKNOWLEDGEMENTS	9
LIST OF TABLES	10
LIST OF FIGURES	11
LIST OF ABBREVIATIONS AND SYMBOLS	14
PREFACE	17
CHAPTER 1: BREAST CANCER SUSCEPTIBILITY AND *BRCA1*	**21**
1.1 INHERITANCE AND PENETRANCE	21
1.2 GENOMIC STRUCTURE AND ORGANIZATION	23
1.2.1 The *BRCA1* Promoter	24
1.2.2 Repetitive Elements	25
1.3 BRCA1 PROTEIN STRUCTURE AND FUNCTION	26
1.3.1 Structural motifs	26
1.3.2 BRCA1 function	27
1.3.2A DNA repair	28
1.3.2B Cell cycle checkpoints	31
1.3.2C Transcription control	32
1.4 *BRCA1* AND SPORADIC BREAST CANCER	34
1.5 CANCER PREDISPOSING MUTATIONS IN *BRCA1*	36
1.5.1 Small Mutations Within *BRCA1*	36
1.5.2 Large Rearrangements Within *BRCA1*	38
1.6 OBJECTIVES	44

CHAPTER 2: MATERIALS AND METHODS **45**

2.1 PATIENTS 45

2.2 METHODS 46

2.2.1 Extraction Of Genomic DNA 46

2.2.2 Southern Blot Analysis 48

2.2.2a) Probe Amplification And Labeling 48

2.2.2b) Restriction Digestion And Blotting 51

2.2.2c) Prehybridization / Hybridization And Stringency Washes 51

2.2.2d) DIG Detection 52

2.2.2e) Probe Stripping 54

2.2.3 MLPA ANALYSIS 55

2.2.4 MLPA ANALYSIS OF *BRCA1* 57

2.2.4a) Hybridization And Ligation Of Oligonucleotides 57

2.2.4b) Polymerase Chain Reaction 57

2.2.4c) Size Separation Of MLPA Fragments 58

2.2.4d) Data Analysis 58

2.2.5 FINE CHARACTERIZATION OF ABERRANT MLPA FRAGMENTS 60

2.2.5a) Amplification Of Putative Deleted Fragments 60

2.2.5b) Cycle Sequencing 61

CHAPTER 3: RESULTS AND DISCUSSION **63**

3.1 SOUTHERN BLOT ANALYSIS 63

3.1.1 Probe Amplification and Labeling 63

3.1.2 Southern Blot Results 64

3.2 MLPA DATA ANALYSIS 70

3.2.1 Determination Of Analysis Success 70

a) Reviewing the MLPA control mix fragments 70

b) Reviewing MLPA profiles 72

3.2.2 Determination Of The Presence Of A Mutation 74

a) Visual Inspection 74

b) Statistical analysis of MLPA results 76

3.3 MLPA RESULTS 83

3.3.1 Characterization Of The Suspected Exon 18 Deletion 83

3.3.2 Characterization Of The Exon 22 Deletion 90

3.3.3 Description Of The Exon 23-24 Deletion 94

3.4 SUMMARY OF RESULTS 99

CHAPTER 4: CONCLUSIONS 101

REFERENCES 104

APPENDIX A: Copy of Ethics Approval 124

APPENDIX B: Clinical details of patients 126

APPENDIX C: Sequence of BRCA1-MLPA probes, position of the ligation sites, and product sizes 131

APPENDIX D: Dosage quotients of MLPA analysis 134

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LIST OF TABLES

Table	***Name***	***Page***
Table 1.1	Breast and ovarian cancer risk for individuals carrying a *BRCA1* mutation.	22
Table 1.2	Reported rearrangements in *BRCA1*	40-42
Table 2.1	Ancestral links of families included in the study	46
Table 2.2	Primers and PCR conditions used to synthesize probes for Southern blot.	49-50
Table 2.3	Exons covered by the different probe mixes	52
Table 2.4	Size fragments generated with the different probe mixes	53
Table 2.5	Information for primers utilized to amplify and sequence.	61

LIST OF FIGURES

Figure	*Name*	*Page*
Figure 1.1	Schematic representation of the *BRCA1-NBR1* region.	23
Figure 1.2	Schematic representation of the structural and functional motifs of BRCA1 and interacting proteins.	26
Figure 1.3	Role of BRCA1 in DNA double strand break repair.	30
Figure 1.4	Contribution of small BRCA1 mutations to hereditary breast/ovarian cancer in different populations.	38
Figure 2.1	Basis of the MLPA technique.	55
Figure 2.2	Structure of short synthetic oligonucleotide	56
Figure 2.3	Structure of long M13 derived oligonucleotide	56
Figure 3.1	Agarose gel electrophoresis of labeled probes in probe mix 2	64
Figure 3.2	Southern Blot 1 (*Hind* III) and Southern Blot 2 (*Xba* I) hybridized with probe mix 2.	65
Figure 3.3	Southern Blot 3 (*Xba* I) hybridized with probes 6, 7 and 8.	66
Figure 3.4	Southern Blot 11(*Xba* I) hybridized with probes 6, 7 and 8.	67
Figure 3.5	Southern Blot 15 (*Hind* III) hybridized with probe mix 2.	69
Figure 3.6	Partial MLPA profiles to illustrate the effect of DNA concentration on the MLPA control mix fragments (64 – 94bp).	71
Figure 3.7	MLPA profile of wild-type control sample.	72

Figure	***Name***	***Page***
Figure 3.8	MLPA peak profiles of samples that displayed aberrant profiles	73
Figure 3.9	MLPA Peak Profiles of control samples	75
Figure 3.10	Superimposed electrophoresis profile of an unaffected (red) and mutation positive control (blue)	76
Figure 3.11	Layout of Wallace “RESULTS” spreadsheet	80
Figure 3.12	Histogram representation of Dosage Quotients	81
Figure 3.13	Histogram representation of MLPA fragment DQs for samples BRC119.1 and BRC119.2	83
Figure 3.14	Pedigree of family BRC119	84
Figure 3.15	Agarose gel electrophoresis of long-range PCR products of BRC119.1, 119.2 and unaffected control	85
Figure 3.16	Electropherograms of the anti-sense strand of exon 18 of samples BRC119.1, BRC119.2 and control	86
Figure 3.17	Genomic sequence of BRCA1 exon 18 and the flanking intron sequences	87
Figure 3.18	Human BRCA1 BRCT domains	89
Figure 3.19	Histogram representation of MLPA fragment DQs for sample BRC100.1	90
Figure 3.20	Pedigree of family BRC100	91
Figure 3.21	Agarose gel electrophoresis of long-range PCR product of BRC100.1	91

Figure	***Name***	***Page***
Figure 3.22	Electropherogram of the sense strand of the exon 22 deletion fragment	92
Figure 3.23	Genomic sequence of exon 22 and the flanking intron sequences	92
Figure 3.24	Genomic structure of intron 21-22	93
Figure 3.25	Histogram representation of MLPA fragment DQs for sample BRC158.1	94
Figure 3.26	Pedigree of family BRC158	95
Figure 3.27	Agarose gel electrophoresis of long-range PCR products of BRC158.1 and unaffected control	96
Figure 3.28	Genomic structure of the 3' region in which the DelEx23-24 mutation is located	97

LIST OF ABBREVIATIONS AND SYMBOLS

A	Alpha
A	Adenine
Ψ	Pseudo
AT	Ataxia telangiectasia
ATM	Ataxia telangiectasia mutated
ATR	ATM and Rad2 related protein
APH	Aqueous prehybridisation/hybridisation
BAP1	BRCA1 associated protein-1
BARD1	BRCA1-associated ring domain
BASC	BRCA1-associated genome surveillance complex
BC	Breast cancer
BIC	Breast Cancer Information Core
bp	Base pairs
BRCA1	Breast Cancer Susceptibility 1-gene
BRCA1	Breast Cancer Susceptibility 1 - protein
Brca1	Breast Cancer Susceptibility 1-gene: mouse homolog
Brca1	Breast Cancer Susceptibility 1-protein: mouse homolog
BRCA2	Breast Cancer Susceptibility 2-gene
BRCA2	Breast Cancer Susceptibility 2-protein
BRCT	BRCA1 C-terminal
C	Cytosine
cdk	Cyclin dependent kinase
CDP-Star	Disodium 2 – chloro – 5 - (4 – methoxyspiro {1,2 – dioxetane - 3, 2' - (5'-chloro) tricycle 3.3.1.$1^{3,7}$] decan} - 4-yl) – 1 - phenyl phosphate
CEN	Centromere
DIG	Digoxigenin
DIG-11-dUTP	Digoxigenin – 11- 2' – deoxy – uridine – 5' – triphosphate, alkali - labile
DNA	Deoxyribose nucleic acid
DNA-PK	DNA-dependent protein kinase
dNTP	Deoxy-nucleotide triphosphate
DQ	Dosage Quotient

DSB	Double strand break
dTTP	Deoxy – tyrosine -5' - triphosphate
ER	Estrogen receptor
fmol	Femtomole
FPG	1% Ficoll, 1% polyvinylpyrrolidone, 1% glycine
γ	Gamma
G	Guanine
HDR	Homology-directed repair
HRT	Hormone replacement therapy
IFN	Interferon
kb	Kilobases
KCl	Potassium Chloride
JNK/SAPK	c-Jun N-terminal kinase / stress-activated protein kinase
LFS	Li-Fraumeni syndrome
LR-PCR	Long range PCR
M	Molar
MAPH	Multiplex amplifiable probe hybridization
$MgCl_2$	Magnesium Chloride
µl	Microlitres
MLPA	Multiplex ligation-dependent probe amplification
mM	Millimolar
NHEJ	Non-homologous end joining
nmol	Nanomole
nt	Nucleotide
OC	Ovarian cancer
PAR	Population attributable risk
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PI	Phosphoinositide
pmol	Picomole
PRR	Positive regulatory region
Py-Pu	Polypyrimidine-polypurine
Q	Glycine
QMPSF	Quantitative multiplex PCR of short fluorescent fragments

R	Arginine
RT-PCR	Reverse Transcriptase PCR
SMP	Semi-quantitive Multiplex PCR
SSCP/HA	Single strand conformation polymorphism / heteroduplex analysis
T	Thymidine
TCR	Transcription coupled repair
TEL	Telomere
U	Units
UTR	Untranslated region
UV	Ultraviolet
VHL	Von Hippel Lindau
WT	Wild Type
ZBRK1	Zinc-finger and BRCA1-interacting protein with a KRAB domain

PREFACE

Breast cancer is the most common form of cancer to afflict women in South Africa, and overall the life-time risk for developing cancer of the breast is 1 in 36 for South African women, ranging from 1 in 13 for white women to 1 in 81 for black women (Sitas *et al*., 1998). In the development of breast cancer, both environmental and genetic factors play a role, where the most important factor for determining risk is a family history of the disease. This risk is a function of the number of affected individuals in the family, their age at diagnosis and degree of relatedness to affected individuals (reviewed in de Jong *et al*., 2002)

Well established environmental factors that lead to an increase in risk include age, geographical location (the USA and western countries show elevated risk), childhood exposure to ionizing radiation, reproductive factors, use of exogenous hormones, diet and obesity (reviewed in Dumitrescu and Cotarla., 2005). The large differences in breast cancer incidence in different regions of the world may be attributed to genetic differences together with different lifestyles and environmental exposures. An interesting study by Ziegler *et al.* (1993) on Asians who migrated to the US (from a region with low breast cancer incidence to a region of high breast cancer incidence), revealed that once they spent as little as ten years in the new country, their breast cancer incidence increased. McPherson *et al*. (2000) found that the migrant's descendants take up the risk of the native population within one or two generations, indicating that life-style and environmental factors play an enormous role. Reproductive factors shown to cause a moderate to high increase in the risk of breast cancer include late age of menopause (older than fifty four), early age of menarche (younger than twelve), nulliparity and late age at first birth (reviewed in Dumitrescu and Cotarla., 2005). Hormonal factors that result in a low to moderate increase in risk include use of hormonal replacement therapy (HRT) and use of oral contraceptives. Obesity in postmenopausal women and high levels of alcohol consumption are well-established factors that result in a moderate increase in risk. Alcohol intake (one or more drinks per day) is directly associated with an increased risk for breast cancer in both pre- and postmenopausal women and risk increases by 9% with every 10g increase in daily intake (Smith-Warner *et al*., 1998).

The most important risk factor is however a family history of the disease. Hereditary breast cancer is responsible for 5-9% of all breast cancer cases (Ford and Easton, 1995), where the disease results from the inheritance of a defective gene or allele. In 1990 linkage analysis provided evidence that breast cancer susceptibility in some families was linked to chromosome 17q21.3 (Hall *et al*., 1990). The gene involved, **br**east **ca**ncer susceptibility gene **1** (*BRCA1*) was

cloned shortly afterwards by Miki *et al.* (1994). Researches however soon discovered that *BRCA1* was responsible for only 45% of multiple case families with site-specific breast cancer. Furthermore, families that had a high incidence of male breast cancer did not carry mutant *BRCA1* alleles. Researchers then sought to find a second breast cancer susceptibility gene, and *BRCA2* was subsequently identified on chromosome 13 (Wooster *et al*., 1994; Wooster *et al*., 1995). Mutant forms of these two breast cancer susceptibility genes, and in particular *BRCA1,* have been found to increase an individual's susceptibility to ovarian cancer (Miki *et al*., 1994). The lifetime risks for *BRCA1*- and *BRCA2*-associated breast cancers are almost equal at approximately 80%, while the age of onset is generally later for *BRCA2*-mutation carriers (Narod and Foulkes., 2004; Schubert *et al*., 1997). The life-time risk for ovarian cancer is 40-65% for *BRCA1* and 20% for *BRCA2* mutation carriers (Ford *et al*., 1998). Apart from increasing breast and ovarian cancer risk, *BRCA1* has been found mutated in cases of fallopian tube carcinoma (Aziz *et al*., 2001; Hartley *et al*., 2000; Reeves *et al*., 2004; Zweemer *et al.,* 2000) and increases risk for prostate cancer. The penetrance and risk associated with germline *BRCA1* mutations is discussed in more detail in Section 1.1. *BRCA2* mutations are strongly associated with an increased breast cancer risk for males (reviewed in Rahman and Stratton, 1998).

Not all cases of hereditary breast carcinoma are attributed to the two breast cancer susceptibility genes, *BRCA1* and *BRCA2.* A detailed study by Ford *et al.* (1998) revealed that while virtually all (84%) kindreds with autosomal dominant inheritance of both breast and ovarian cancer together, presented with mutations in one of the two BRCA genes, a large proportion (67%) of families with site-specific, early onset breast cancer (four or more cases), did not show linkage to either of these genes. A number of other studies also found that the majority of site-specific breast cancer kindreds are not explained by either *BRCA1* or *BRCA2* mutations (Rebbeck *et al*., 1996; Serova *et al*., 1997; Vehmanen *et al*., 1997). Following the discovery that a proportion of familial breast cancer was unaccounted for, researchers sought to locate *BRCA3*, the third high penetrance breast cancer susceptibility gene. In spite of the development of high-throughput analysis of DNA samples, the availability of a complete genetic map and the sequencing of the entire human genome, the gene could still not be found, although two groups believed they had located the gene at 8p11-21 and 13q21-31 (Kainu *et al*., 2000; Seitz *et al*., 1997). These loci were however quickly disqualified, based on the lack of mutations within these regions in families with a strong history of breast cancer (Rahman *et al*., 2000; Thompson *et al*., 2002).

Another high penentrant gene associated with breast cancer susceptibility is the p53 tumor suppressor gene, that when mutated in the germline causes Li-Fraumeni syndrome. This syndrome is characterized by increased susceptibility to soft tissue and osteosarcomas,

premenopausal breast cancers, brain and adrenocorticocal carcinomas and leukemias (Garber *et al*., 1991). Mutated alleles have a low population frequency and account for less than 1% of familial breast cancer cases (Borreson *et al*., 1992; Prosser *et al*., 1991). This gene is therefore not considered to be the third high penetrance breast cancer susceptibility gene. Many scientists now believe that a third high penetrance breast cancer susceptibility gene does not exist. Peto (2002) is of the opinion that BRCA negative families with a history of breast cancer may harbor mutations that affect susceptibly in a more complex way, by gene-gene or gene-environment interactions for example. Other scientists have suggested that there is no other single gene responsible for the remaining families (Narod and Foulkes, 2004). The remaining proportion of familial cases could possibly be due to low penetrance gene mutations, in which case environmental factors might play a significant role in determining the risk for breast cancer development in individuals harboring these changes.

It has however become increasingly evident that the contribution of BRCA mutations to breast/ovarian cancer susceptibility may be underestimated. This is due to the fact that classical qualitative PCR-based techniques were utilized that cannot detect large genomic rearrangements. Since rearrangements may occur in genes that are clinically important, methods have recently been altered to include quantitative or semi-quantitative tests that can determine gene dosage or copy number. The use of such techniques has led to the identification of at least sixty-seven different large rearrangements in *BRCA1* (see Table 1.2) and 13 in *BRCA2* (Agata *et al*., 2005; Bunyan *et al*., 2004; Nordling *et al*., 1998; Tournier *et al*., 2004; Walsh *et al*., 2006; Wang *et al*., 2001; Woodward *et al*., 2005). These rearrangements are often mediated by *Alu* sequences, which make up a large portion of *BRCA1* (discussed in section 1.2.3). *BRCA2* contains far fewer *Alu* repeats than *BRCA1*, which may to some extent explain why fewer rearrangements have been reported in this gene. Varying proportions of *BRCA1* rearrangements have been detected in different population groups, some of which represent founder effects. This is discussed in detail in section 1.5.2.

A large number of techniques are currently available for the detection of heterozygous deletions and duplications. The different techniques available to date can be grouped into three categories, based on the approach used: 1) Cytogenetics, 2) Southern blotting and 3) PCR (Armour *et al*., 2002). Currently, Multiplex Ligation-dependent Probe Amplification (MLPA) is the most commonly used technique for detecting such mutations in *BRCA1*. This method can detect the relative quantity of as many as 40 different DNA sequences in a single reaction. The basis of this technique is that probes, only once annealed to target sequences are amplified and

quantified as opposed to the direct amplification of target sequences themselves. A more detailed explanation of this technique is given in Section 2.2.3.

Previous studies on the white South African population have indicated that small mutations in *BRCA1* and *BRCA2* account for 20% and 39% of familial breast/ovarian cancer susceptibility respectively (Reeves *et al*., 2004; Schlebush, 2004). In the South African Afrikaner population small *BRCA1* and *BRCA2* mutations are responsible for 15% and 43% of familial breast cancer respectively, where founder effects were observed in both genes in this population (Reeves *et al*., 2004; Schlebush, 2004). The contribution of these genes to the burden of breast cancer in South Africa may however be an underestimation, due to the fact that quantitative techniques were never utilized. In the current study, Southern blotting and MLPA were employed in an effort to determine whether large genomic rearrangements in *BRCA1* play a role in South African breast/ovarian cancer etiology.

CHAPTER 1

BREAST CANCER SUSCEPTIBILITY AND *BRCA1*

1.1 INHERITANCE AND PENETRANCE

BRCA1 (MIM 113705) is a tumor suppressor gene, and therefore mutation of both alleles is required within the normal cell for neoplastic transformation to occur (Knudson *et al*., 1975). It is therefore often observed that the wild-type allele of the gene is lost in tumors of heterozygous carriers. Consequently, such cancer predisposition is inherited in a dominant fashion, while the predisposing allele behaves as a recessive allele in somatic cells. The mutation inherited through the germline is generally small and restricted to the gene, while the second 'hit' usually occurs somatically and involves large stretches of DNA. Knudson (1993) predicted that genes, which confer a risk of cancer due to germline mutations, are expected to be somatically mutated in sporadic cancers of the same type. This theory has been found to hold true for a number of cancers, such as retinoblastoma, colon cancer and Von Hippel Lindau syndrome, but not for breast cancer. Given that *BRCA1* plays an important role in inherited susceptibility to breast cancer, it is rather surprising that it is very infrequently mutated in sporadic breast cancer. It has however been shown that *BRCA1* or its resultant product may be inactivated by other mechanisms besides somatic mutation (Rice *et al*., 2000).

Germline mutations within *BRCA1* have been detected in 15-20% of affected women with a family history of breast cancer, but in 60-80% of affected individuals with a family history of both breast and ovarian cancer (Couch *et al*., 1997; Peto *et al*., 1999). There have been many conflicting reports as to the penetrance of mutations within *BRCA1*. This may be largely due to the selection processes involved in the studies. Penetrance estimates based on multiple-case families with breast and or ovarian cancer will be higher than population based studies. In practice, the majority of genetic testing is performed on individuals who have a strong family history of the disease. As a result of the discrepancies surrounding penetrance, Antoniou *et al*., 2003 completed a meta-analysis of 22 independent studies to determine risk for individuals unselected for family history. The average cumulative risks in *BRCA1* mutation carriers either selected (Easton *et al*., 1995) or unselected (Antoniou *et al*., 2003) for family history are indicated in table 1.1.

Table 1.1: **Breast and ovarian cancer risk for individuals carrying a *BRCA1* mutation.**

	Breast Cancer Risk (%)		***Ovarian Cancer Risk (%)***	
	Age 50	**Age 70**	**Age 50**	**Age 70**
Multiple case families	51	85	23	63
Unselected for family history	37	65	13	39

Values for unselected family history are derived from a graph supplied by Antoniou *et al.*, 2003.

Clearly the penetrance of *BRCA1* mutations is much higher for individuals that have a strong family history of the disease as opposed to individuals without, and risk is higher for individuals with a strong family history. Such risk estimates are extremely valuable for effective genetic counseling.

1.2 GENOMIC STRUCTURE AND ORGANIZATION

BRCA1 is an exceptionally large gene, which stretches over 81kb of genomic DNA and consists of 5 592 bases (Miki *et al*., 1994). The gene comprises 24 exons, ranging between 40 and 3 425bp, of which only 22 are coding (exons 1 and 4 are non-coding). An *Alu* element was originally misidentified as exon four. Introns represent 90.9% of the sequence (Smith *et al*., 1996). There is a large central exon (exon 11) of 3.4kb, which encodes approximately 60% of the protein that consists of 1 863 amino acids. Miki *et al.* (1994) revealed that the transcript is 7.8kb in size and is most abundant in the testis and thymus, although present in the breast and ovaries. They also found that an alternatively spliced *BRCA1* transcript deficient of exon 11 occurs in many tissues at relatively high levels. Various smaller alternatively spliced transcripts of 7, 4.6, and 1.5 - 2.2 kb have been identified, all with distinctively different patterns of expression (reviewed in Orban and Olah, 2003).

BRCA1 lies upstream of the *NBR1* (**N**ext to ***BR**CA**1***) gene (also known as 1A1-3B), and the 5' ends of both *BRCA1* and *NBR1* have been duplicated (Brown *et al*., 1996). A partial copy of the *BRCA1* gene (Ψ-*BRCA1*) lies next to a partial copy of the *NBR1* gene (*NBR2*), illustrated in figure 1.1. It is in fact exons 1a, 1b and 2 of *BRCA1* that are duplicated, and make up the Ψ-*BRCA1* gene. While exons 1a, 1b and 3 of *NBR1* are duplicated, together with 295bp of the intergenic region, and so make a portion of the *NBR2* gene (Brown *et al*., 1996). *NBR2* consists of five exons, with the last exon alternatively used, and spans approximately 30kb between *BRCA1* and Ψ-*BRCA1*. *NBR1* was originally cloned as a candidate gene for CA125, the ovarian cancer antigen (Campbell *et al*., 1994). No link between this gene and breast/ovarian cancer has however been demonstrated.

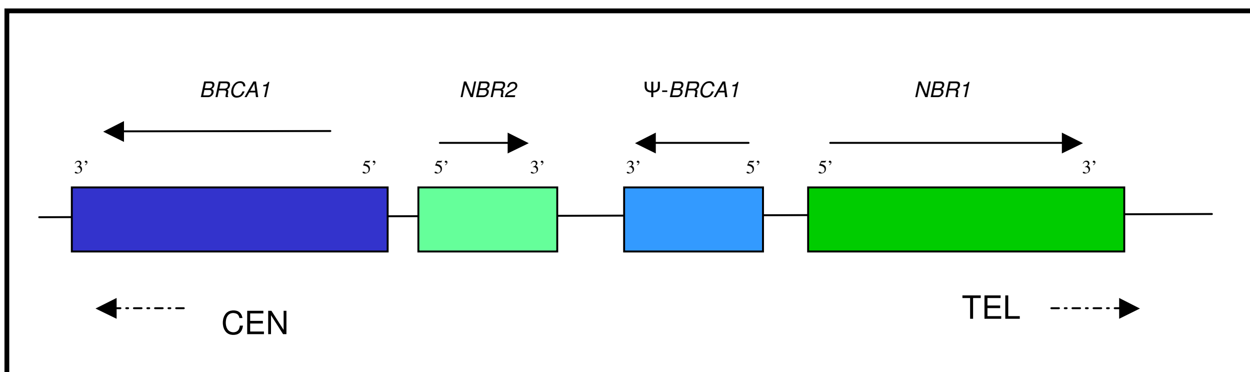

Figure 1.1: Schematic representation of the *BRCA1-NBR1* region.
Solid arrows indicate transcription direction. Dashed arrows indicate the position of the centromere and telomere on chromosome 17 (adapted from Xu *et al*., 1997a).

The intron-exon structure is maintained in both Ψ-*BRCA1* and *NBR2* and there exists a high degree of nucleotide sequence identity between the respective genes. These facts suggest that these genes are non-processed pseudogenes (arose from integration of genomic material and not cDNA copies) and that the duplication is a recent event in evolutionary terms (Brown *et al*., 1996). In support of this notion, Xu *et al*. (1997a) found that *NBR2* is absent in species other than primates. SSCP analysis of *NBR2* in 50 breast and 50 ovarian tumors did not reveal a single mutation, indicating that mutational inactivation of this gene is uncommon in the development of these tumors (Xu *et al*., 1997a).

1.2.1 THE *BRCA1* PROMOTER

BRCA1 expression is complexly regulated (Xu *et al.,* 1997b). There are two distinct promoters separated by 277bp, α and β, which produce two discrete transcripts. Both transcripts are present in the majority of tissues, but higher expression of the exon 1a (α-) transcript occurs in the mammary gland, while higher expression of the exon 1b (β-) transcript occurs in the placenta (Xu *et al*., 1995). Interestingly, promoter α is bidirectional and is also responsible for transcription of *NBR2* (Xu *et al*., 1997b). The two *BRCA1* promotors are both TATA-less, but reside in GC-rich regions, common in TATA-less promoters (Azizkhan *et al*., 1993, as cited by Smith *et al*., 1996). Also GC boxes are located 5' to exons 1a and 1b, and overlap with exon 1a. These boxes bind Sp1 and mediate interaction of TATA-less promoters and TFIID. Other *cis* elements are present that can bind other transcription factors and function uni- or bi-directionally (Xu *et al*., 1997b).

Although an alternative ERE (estrogen receptor element) is only found in the β promoter, both *BRCA1* promoters respond to estrogen stimulation. This implies that estrogen regulation of the α promoter occurs by means of a more complicated mechanism, and not by the classical ER pathway (Xu *et al*., 1997b). The human exon 1b is not conserved in mice, and therefore no β transcript occurs. It is thus unlikely that two promoters regulate *BRCA1* expression in the mouse. Only an SP1 binding site and CCAAT box are conserved within the promoter regions of human *BRCA1* and mouse *brca1* genes (Xu *et al*., 1997b).

Thakur and Croce (1999) identified 222 bases (-202 to +20) that house essential regulatory elements of the *BRCA1* promoter. This segment of DNA is believed to contain several weak regulatory sites. A positive regulatory region or PRR, which has a short polypyrimidine-

polypurine (Py-Pu) tract proceeded by a CREB site was identified in this region. The presence of a complete and functional PRR is critical for normal transcription of this particular gene (Thakur and Croce 1999), and to confirm this, methylation of a CpG dinucleotide in the CREB site of the PRR was reported in a sporadic breast cancer case (Mancini *et al*., 1998). This positively acting site has been identified as a GABP*χíβ* binding site, known as the RIBS element (Atlas *et al*., 2000). Although this CREB site is present, the proximal promoter is not responsive to cAMP induction. It is suggested that CREB acts as a constitutive positive element for *BRCA1* expression, and that inactivation of this site would have a serious effect on expression of *BRCA1* (Atlas *et al*., 2001).

1.2.2 REPETITIVE ELEMENTS

Genomic regions of *BRCA1* include very high densities (46.3%) of repetitive DNA elements (Smith *et al*., 1996). A total of 138 individual *Alu* repeats were identified, which make up 41.5% of the total 81kb sequence. Of these 94 are complete, while 44 are partial elements (between 69-231 nucleotides with 70-100% sequence homology to the consensus sequence of *Alu* subfamilies). A further 4.8% of the gene consists of other repetitive elements, where fragments from the L1 family are most frequent. Simple sequence repeats are also abundant, with 54 identified intragenically (Smith *et al*., 1996). Such high levels of *Alus* are rare, and of 326 loci analyzed (Smith *et al*., 1996), 12.6% had *Alu* densities greater than 20%. Only three genes have been identified with higher levels of *Alus*; apolipoprotein c-I, *Blym* transforming gene, and apolipoprotein c-IV. Association of *Alu* sequences with genomic rearrangements, deletions and insertions is not uncommon and have been reported in various genes (reviewed in Smith *et al*., 1996) such as β-globin; lysyl hydroxylase (Ehlers-Danlos syndrome Type VI); low-density lipoprotein receptor (familial hypercholesteremia); apolipoprotein B (hypobetalipoproteinemia); adenosine deaminase (ADA-SCID) and complement component C1 (hereditary angioedema).

1.3 BRCA1 PROTEIN STRUCTURE AND FUNCTION

The *BRCA1* gene encodes a nuclear phosphoprotein of 220 kDa that does not display homology to other proteins, and has a cell-cycle-regulated pattern of expression. Expression of *BRCA1* mRNA and protein rises in mid to late G1, prior to the start of DNA synthesis, and peaks at the G1/S boundary or early-mid S phase. Levels then drop by late S or early G2 (Chen *et al*., 1996).

1.3.1 STRUCTURAL MOTIFS

This large protein is able to undergo numerous protein-protein interactions and is characterized by the existence of three structural motifs, one at each terminus, and a third located within exon 11 (figure 1.2).

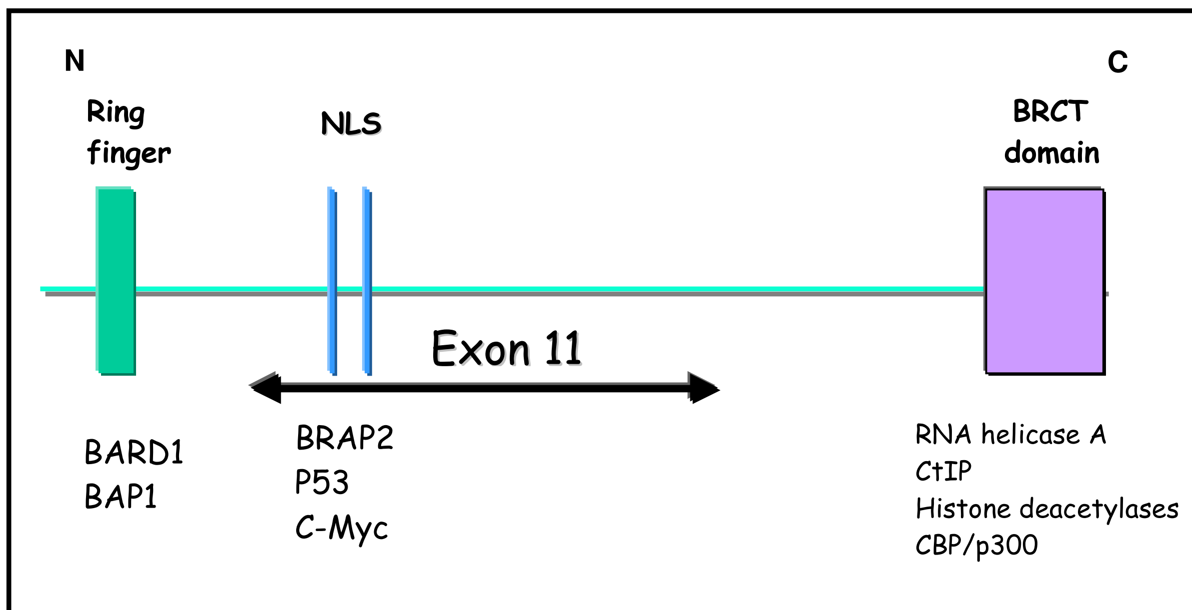

Figure 1.2: Schematic representation of the structural and functional motifs of BRCA1 and interacting proteins.

The RING finger domain located near the N-terminus of the protein was the first motif to be identified (Miki *et al*., 1994). This region interacts with a variety of proteins, such as BARD1, BAP1 (a deubiquitinating enzyme), and various cell cycle proteins such as cyclins, cyclin-dependent kinases as well as E2F (reviewed in Rosen *et al*., 2003). Disruption of the structure of this region by certain missense mutations results in the inhibition of the following: repression of

estrogen receptor-α signaling (Fan *et al*., 2001), modulation of DNA repair and ubiquitin ligase activity (Ruffner *et al*., 2001), in addition to apoptosis (Fan *et al*., 2001).

The C-terminal region of the proteins contains a transactivation domain (Chapman and Verma, 1996), which includes two tandem BRCT (BRCA1 C-terminal) domains, encompassing amino acids 1640-1863. This domain is believed to mediate the interaction between BRCA1 and various other proteins as shown in figure 1.2 (reviewed in Zheng *et al*., 2000a).

The third recognizable motif is a functionally relevant protein-protein interaction surface located at the 5' region of exon 11 (Chen *et al*., 1999), known as a nuclear localization signal (NLS). This region mediates the interaction of BRCA1 with a variety of proteins (figure 1.2). This protein-protein interaction surface includes two putative nuclear localization signals, [NLS1 (^{501}KLKRKRR) and NLS2 (^{607}KKNRLRRK)]. The BRCA1 splice variant Δ(11), which lacks these signals, was found localized in the cytoplasm (Thakur *et al*., 1997). Some controversy later developed when some other groups detected splice variants lacking these signals within nuclei. This controversy later abated when it was found that the proteins lacking exon 11 could enter the nucleus through their BRCT, as well as RING finger associations with other nuclear proteins, which can behave as chaperones and shuttle these variants into the nucleus. Also, the protein-protein interactions that are mediated by this region are non-existent in splice variants lacking this region. This suggests that these variants may have different functions in the nucleus (Reviewed in Orban and Olah 2003).

It is interesting to note that normal cells as well as other tumor cells (besides breast and ovarian cancer cells) essentially have BRCA1 located within the nucleus, while breast and ovarian cancer cell lines often display cytoplasmic localization of both endogenous isoforms and exogenous labeled full length protein (Chen *et al*., 1995; Scully *et al*., 1996).

1.3.2 BRCA1 FUNCTION

The normal cellular function of BRCA1 has proved difficult to characterize (Welcsh *et al*., 2000), and endeavors to elucidate BRCA1 function have been focused on identifying functional domains as well as interacting proteins. A large number of functions have been revealed for BRCA1, while a large number of mysteries regarding function and carcinogenesis have also been revealed. We are still a long way from understanding the role of BRCA1 in the genetics of breast cancer. It also seems as if BRCA1 is involved in almost every pathway, and perhaps the

question should rather be "what doesn't BRCA1 do?", instead of " what does this protein do?". It is however also clear that the majority of these functions are performed in association with a plethora of other proteins, or as a result of affecting transcription of genes, which act downstream of BRCA1 in a variety of pathways. In fact, over-expression of BRCA1 induces expression of many genes implicated in cell growth control, cell cycle regulation as well as DNA replication and repair (Harkin *et al*., 1999; MacLachlan *et al*., 2000).

1.3.2A DNA Repair

BRCA1 repairs DNA lesions in a manner which is dependent on other proteins, but absence or reduction of the BRCA1 protein is sufficient to compromise this repair. It appears as if BRCA1 maintains genomic integrity, as well as stability through its involvement in homologous recombination, transcription-coupled repair and the Fanconi anemia pathway of repair. Mutations that cause loss of BRCA1 function often lead to an overall decrease in the cell's DNA repair ability, which subsequently leads to increased somatic mutation, chromosome breakage, abnormal chromosome segregation, centrosome amplification, and aneuploidy (Welcsh *et al*., 2000).

Role in double strand break repair

BRCA1 was originally implicated in DNA repair by Scully *et al.* (1997a) as a result of the finding that in response to DNA damage, BRCA1 becomes hyperphosphorylated and relocates to sites of replication forks that are marked by PCNA (proliferating cell nuclear antigen). This hyperphosphorylation is facilitated by the protein kinase activity of nuclear phosphoinositide (PI) kinases, such as ATM and ATR, in response to DNA damage (reviewed in Rosen *et al*., 2003). These kinases play a role in a signaling pathway, which causes the activation of downstream components such as p53, Chk1, Chk2 as well as others. Subsequent to damage, BRCA1 becomes phosphorylated by ATM (in response to ionizing radiation) or ATR (in response to UV irradiation), and in this manner the cascade that leads to DNA repair is activated (reviewed in Rosen *et al*., 2003).

BRCA1 has been shown to directly participate in DNA double strand break (DSB) repair through both non-homologous end joining (NHEJ) and homology directed repair (HDR) (Moynahan *et al*., 1999; Zhong *et al*., 2002). BRCA1 associates directly with the DNA repair complex, MRE11-RAD50-NBS1 (MRN), in discrete nuclear foci following ionizing radiation. This interaction occurs directly through RAD50 (Zhong *et al*., 1999). The MRN complex is involved in both HR and

NHEJ in response to DNA damage (Haber, 2000), and displays both endo- and exonuclease activities (Paull and Gellert, 1998; Trujillo *et al*., 1998), and specifically functions in end-processing, an early stage in HR and NHEJ. MRE11, a subunit of the complex, has the ability to join the ends of DSB and form ssDNA (Haber, 1998). BRCA1 seems to regulate the MRN complex by inhibiting this nuclease activity of MRE11 (Paull *et al*., 2001). BRCA1 could therefore play a critical role in HDR by inactivating MRE11, thereby avoiding the more mutagenic repair process of NHEJ (Rosen *et al*., 2003; Yoshida and Miki, 2004). The choice between these two repair processes (NHEJ and HDR) is unclear at present, since in some situations BRCA1 seems to try to favor the less mutagenic HDR while Brca1-deficient mouse embryonic fibroblasts show severely retarded NHEJ (Zhong *et al*., 2002). BRCA1 must therefore function in some sort of "decision making process" in determining whether to ablate the exonuclease activity of MRE11 or not, and thereby driving one of the two repair processes.

BRCA1 colocalizes with RAD51, BRCA2 and BARD1 and migrates to sites of DNA synthesis and becomes hyperphosphorylated when replication-arrest is induced in cultured cells, or when S phase cells are treated with ionizing radiation (Chen *et al*., 1998; Scully *et al*., 1997b). Together these proteins activate the repair of double strand breaks and initiate homologous recombination. Rad51 coats single stranded DNA and facilitates pairing with a homologous region. Stand exchange is subsequently activated and a cross over between the two DNA strands occurs (reviewed in Venkitaraman 2001). This complex therefore functions in strand exchange, while the MRN complex plays a role in end-processing, an early stage in HDR. The fact that embryonic stem cells from BRCA1 knock-out mice show an extreme defect in double-strand break repair by homologous recombination (Moynahan *et al*., 1999), confirms the role for BRCA1 in homology-directed repair (HDR). The role of BRCA1 in DSB repair is outlined in figure 1.3.

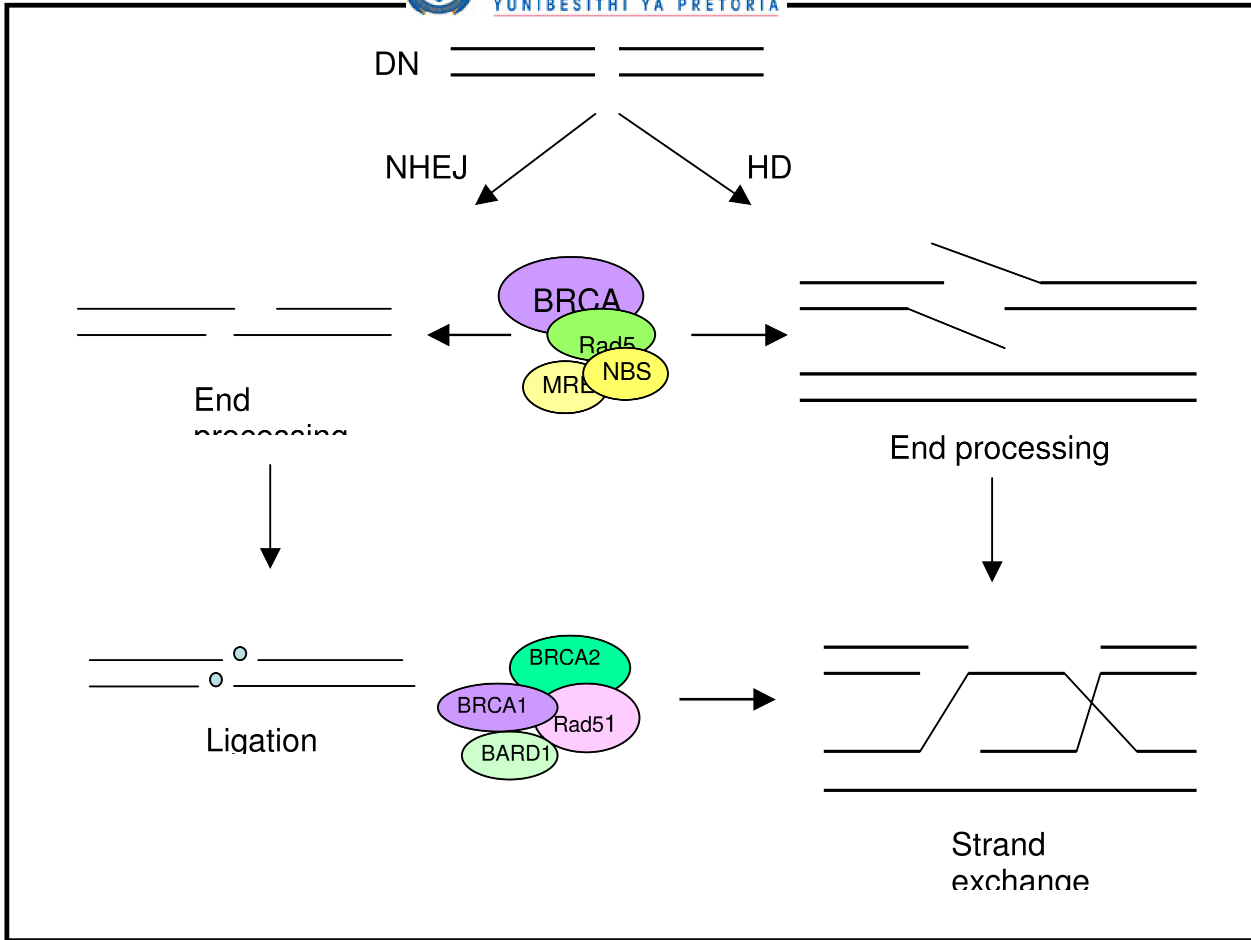

Figure 1.3: Role of BRCA1 in DNA double strand break repair.
In response to DSB BRCA1 becomes phosphorylated by ATM and subsequently activates HDR or NHEJ by recruiting the MRN complex. Adapted from Zheng *et al.*, 2000a.

Role in transcription-coupled repair

In addition to DSB repair, BRCA1 has also been implicated in transcription coupled repair (TCR). During this process, DNA damage is repaired more rapidly in transcriptionally active DNA than in non-transcriptionally active DNA (Gowen *et al.*, 1998). Both UV induced damage and oxidative damage can be repaired by this process. Mouse embryonic fibroblasts lacking Brca1 (Gowen *et al.*, 1998) and a human breast cancer cell line that has a single mutant BRCA1 allele (Abbott *et al.*, 1999) display faulty transcription coupled repair following oxidative DNA damage. A central role for BRCA1 in DNA repair was proposed by Wang *et al.* (2000) because of its association in a massive complex of proteins, all of which are associated with the recognition of abnormal or damaged DNA, and suggest that this complex may act as a radar that detects damaged DNA. This complex named BASC (BRCA1-associated-genome surveillance complex) includes proteins such as MSH2, MSH6, MLH1, ATM, BLM, the MRN complex, and DNA replication factor C (facilitates loading of PCNA onto DNA). The MSH proteins are DNA mismatch repair

proteins (MMR) and are believed to be key players in the TCR pathway. It is further known that BRCA1 occurs in a key transcriptional regulatory complex, Pol II holoenzyme (Scully *et al*., 1997c). The proposed model for TCR is that the polymerase complex halts at regions of DNA damage (Parvin 2001). After transcription is halted as a result of damage, BRCA1-BARD1 mediates ubiquitination of the Pol II holoenzyme. The ubiquitinated enzyme subsequently dissociates from the DNA, leaving BRCA1 bound to the lesion. BRCA1 is then capable of recruiting factors, such as Rad51 and the MRN complex, capable of repairing damaged DNA.

Role in Fanconi Anemia Repair pathway

BRCA1 has also been associated with the Fanconi anaemia pathway of DNA repair. Fanconi anemia (FA) is a rare autosomal recessive disease in which patients present with birth defects, reproductive defects, haematological deficiencies in addition to cancer susceptibility (Joenje and Patel, 2001). Interestingly, BRCA2 was unexpectedly identified as FANCD1 (Howlett *et al*., 2002) and biallelic inactivation of *BRCA2* results in a rare form of Fanconi anaemia. Folias *et al.* (2002) revealed that the central portion of BRCA1 and the amino terminal section of FANCA interact directly, regardless of the occurrence of DNA damage, directly connecting BRCA1 to the FA pathway of DNA repair. Briefly, in the FA pathway the Fanconi anaemia core complex (FANCA, FANCC, FANCE, FANCF, FANCG, FAAP90, FAAP100, FAAP250 AND PHF9) detects exogenous DNA damage or errors in DNA replication. PHF9 subsequently monoubiquitinates FANCD2 (Meetei *et al*., 2003). This monoubiquitination does not target FANCD2 for destruction, but rather following this, FANCD2 targets the BRCA1 and RAD51 nuclear foci (Garcia-Higuera *et al*., 2001) and the damaged DNA is repaired.

1.3.2B Cell cycle checkpoints

Mouse embryonic stem cells with homozygous deletion of exon 11 of *Brca1* display deficient G_2-M cell cycle checkpoint control subsequent to ionizing radiation (Xu *et al*., 1999). Involvement of BRCA1 in the G_2-M pathway was confirmed by Yamane *et al*. (2003), where they demonstrated that both BRCA1 and the human DNA topoisomerase II-binding protein 1 (TopBP1) co-ordinate the G_2-M checkpoint. Reduced levels of TopBP1 or the occurrence of non-functional BRCA1 causes partial arrest of this checkpoint, while reduced expression of TopBP1 in association with defective BRCA1 results in an increase in apoptosis subsequent to ionizing radiation and remarkable failure of the G_2-M checkpoint (Yamane *et al*., 2003). Yarden *et al*. (2002) have demonstrated that BRCA1 performs its checkpoint effect through Chk1. Their findings indicate

that BRCA1 does not contribute to cellular transformation directly, but rather that loss of the functional protein allows cells to bypass the G_2- M checkpoint and in this manner, accumulate DNA damage (Yarden *et al*., 2002). The regulation of expression of various genes involved in the cell-cycle checkpoints (such as cyclin D1, $p21^{WAF/Cip1}$ etc.) may explain an alternate manner in which BRCA1 controls the cell-cycle checkpoints.

1.3.2C Transcription control

The identification of an acidic domain near the carboxyl-terminus, that activates basal transcription machinery and is sensitive to cancer predisposing mutations, prompted the notion that BRCA1 is involved in transcriptional regulation (Chapman and Verma, 1996; Monteiro *et al*., 1996). The absence of demonstrable sequence specific DNA binding activity, together with this autonomous transactivation function led Haile and Parvin (1999) to suggest that BRCA1 functions as a co-activator of transcription, while also possessing a transcriptional repression ability. These abilities implicate BRCA1 in a variety of pathways. BRCA1 is known to strongly inhibit transcriptional activity of the estrogen receptor (ER-α) in both human breast and prostate cancer cell lines (Fan *et al*., 1999). ER-α receptor is believed to function in the stimulation of proliferation of mammary epithelial cells during the development of cancer (Dickson and Stancel, as cited by Rosen et al., 2003), and the fact that BRCA1 then inhibits its transcription indicates that BRCA1 has a mammary tissue-specific function.

BRCA1 physically interacts with p53 (Ouchi *et al*., 1998; Zhang *et al*., 1998) and behaves as a co-activator of p53 (Ouchi *et al*., 1998 and Zhang *et al*., 1998). This is not true for all p53 target genes, and MacLachlan *et al.* (2002) have shown that BRCA1 affects only a subset of genes involved in DNA repair and growth arrest, but surprisingly not apoptosis. Although BRCA1 is not involved in the p53 apoptotic pathway, it is known to induce the JNK/SAPK (c-Jun N-terminal kinase / stress-activated protein kinase) apoptotic pathway (Harkin *et al*., 1999), by positively regulating GADD45 (a DNA damage response gene) expression, by interacting with specific sites within the GADD45 promoter and the associated transcription factors. In addition to positive regulation, BRCA1 has also been shown to mediate transcription repression of GADD45 through binding of ZBRK1 (zinc-finger and BRCA1-interacting protein with a KRAB domain which is a DNA binding transcription factor) at a sequence specific site within an intronic sequence in the gene (Zheng *et al*., 2000b). Additionally, BRCA1 may be a significant component of the Interferon-γ-mediated apoptotic response (Andrews *et al*., 2002), by synergistically upregulating

a novel subset of genes; IRF-7, MxA and ISG-54 in the presence of interferon γ, but not interferons α or -β. The BRCA1 mutant cell line, HCC1937, as well as the p53 mutant cell line, MBR62-bcl2 were sensitized to IFN-γ-apoptosis in the presence of BRCA1 induction. IFN-γ-mediated induction of IRF-7 and MxA was attenuated in the HCC1937 cell line. This attenuation was subsequently rescued when exogenous wild-type BRCA1 was introduced into this cell line (Andrews *et al*., 2002). The authors hypothesize that tumor suppresser genes such as BRCA1, may play a role in regulating an immune surveillance pathway, which functions parallel to their tumor suppressor function. Inactivation of BRCA1 in breast epithelial cells would then possibly disrupt the natural tumor suppressor function of the immune system, and in this way lead to tumor formation (Andrews *et al*., 2002).

Rosen *et al*. (2003) validate the role of this protein in both DNA repair, as well as apoptosis by postulating a caretaker function where, when possible BRCA1 mediates DNA damage signaling and repair, but when the damage is too great to repair, BRCA1 will push the cells into apoptosis.

1.4 *BRCA1* AND SPORADIC BREAST CANCER

Since only 5-9% of breast cancer cases are "familial" (Ford and Easton., 1995), the majority of cases do not occur as a result of the inheritance of a high penetrance defective allele. Since *BRCA1* plays a rather substantial role in familial breast cancer, the original train of thought was that it would play a similar role in sporadic breast cancer cases.

Initial studies of sporadic breast cancers revealed loss of heterozygosity at the *BRCA1* locus in approximately 30-60% of sporadic breast cancers (Borg *et al*., 1994; Cropp *et al*., 1994; Futreal *et al*., 1992). These data lead to the assumption that somatic mutations in this gene would be responsible for a sizable portion of sporadic breast/ovarian cancers. The magnitude of small somatic mutations in this gene (in samples displaying LOH at this locus) was however completely overestimated and only three sporadic breast cancer cases have been found to contain *BRCA1* somatic mutations (Frolov *et al*., 2002; Khoo *et al*., 1999; van der Looij *et al*., 2000). Somatic mutations in *BRCA1* have however been detected in a small number of sporadic ovarian cancers (Berchuck *et al*., 1998; Hosking *et al*., 1995; Merajver *et al*., 1995). The absence of *BRCA1* alterations in sporadic breast cancer cases led to the suggestion that silencing of this gene by means of primary sequence alterations is not a significant mechanism in sporadic breast tumors, but rather that epigenetic events most likely inactivate the gene, and thereby cause cancer (van der Looij *et al*., 2000).

On many occasions, mention has been made concerning reduction or absence of the *BRCA1* transcript in a subgroup of sporadic breast and ovarian tumors as well as breast cancer cell lines (Catteau and Morris, 2002; Rice *et al*., 2000; Thompson *et al*., 1995). Reduced immunoreactivity and complete absence of this protein was later reported by Miyamoto *et al*. (2002), Wilson *et al.* (1999) and Yoshikawa *et al.* (1999). Subsequent to these findings, many studies have focused on hypermethylation of the promoter, and the results indicate that this type of change occurs in 11-31% of sporadic breast and 5-15% of ovarian cancer tumors (Reviewed by Catteau and Morris 2002). A detailed study by Miyamoto *et al*. (2002), which analyzed immunoreactivity, mRNA expression as well as promoter alpha hypermethylation, revealed that hypermethylation was responsible for 38% of samples that displayed a marked reduction of *BRCA1* reactivity. This indicates that other mechanisms of post-translation modification must play a role and they suggest that these may include reduced translation efficiency, decreased protein stability or alternatively an increase in protease activity. Blagosklonny *et al*. (1999) demonstrated that an equilibrium between *BRCA1* transcription and degradation of the protein by a cathepsin-like

protease is accountable for the maintenance of the *BRCA1* levels in several cell lines. Disruption of this equilibrium could possibly result in the development of sporadic breast cancer.

It has also been suggested that there may be an excess production of the *BRCA1* Δ11b splice variant in sporadic breast cancers, together with reduced full-length *BRCA1* production (Fraser *et al*., 2003). As yet, no function has been ascribed to the *BRCA1* Δ11b splice transcript, which locates cytoplasmically. Since elimination of exon 11 by differential splicing also occurs in the mouse, it suggests that this transcript encodes a protein of functional significance (Hakem *et al*., 1996). Fraser *et al*. (2003) propose that an alteration in intracellular localization of the *BRCA1* species may be a critical physiological regulatory mechanism.

1.5 CANCER PREDISPOSING MUTATIONS IN *BRCA1*

Since the discovery of *BRCA1* in October 1994, more than 800 different germline mutations have been reported in breast / ovarian cancer families (BIC). No mutational hot-spots exist within this gene and mutations are found scattered throughout the entirety of the gene. Pathogenic mutations identified within *BRCA1* can be grouped into five categories: (1) missense mutations, most of which have been reported within the RING finger domain (2) nonsense mutations (3) splice site mutations which result in the deletion of exons or insertion of intronic sequences (4) small deletions and insertions which alter the reading frame, and (5) large genomic rearrangements.

1.5.1 SMALL MUTATIONS WITHIN *BRCA1*

Approximately 80% of mutations reported to the BIC are truncating non-sense or frameshift mutations, which eliminate the C-terminal BRCT domain of the protein. This high percentage may however be indicative of the ease with which these mutations are detected, as well as the unambiguous nature of their effect on the protein. Of the disease-causing mutations reported, a few are missense mutations, occurring most notably in the RING finger domain, where they replace cysteine residues, which are required to keep the zinc finger intact (BIC). The role of missense and silent mutations reported in other regions of the gene have been difficult to verify and often these have been assumed to be polymorphic in nature. According to Cartegni *et al*. (2002) translationally silent mutations, as well as nonsense, missense and even coding region single-nucleotide polymorphisms might affect splicing accuracy and efficiency. Ladopoulou *et al*. (2002) report that a single base pair change at the last base of exon 23, originally believed to be a missense mutation actually results in abnormal RNA splicing, ultimately causing a protein lacking the corresponding amino acids from exon 23 and 24. A study on humanized mouse models of a missense mutation in codon 64 by Yang *et al*. (2003) indicated that it in fact causes aberrant splicing and a functionally null protein. They identified two additional missense mutations that they predict will affect splicing. Analysis by Williams *et al*. (2003) indicates that approximately half the missense mutations in the BRCT region lead to destabilization of the protein and loss of function.

Contribution of small *BRCA1* mutations to familial breast cancer

Certain mutations recur in different population groups, some of which have been shown by haplotype analysis to represent founder effects. In Ashkenazi Jews, as much as 47% of high-risk families have one of 2 mutations (185delAG and 5382insC) within *BRCA1* (Levy-Lahad *et al*., 1997). These two mutations appear in the general Ashkenazi Jewish population at a frequency of 1.09% and 0.13% respectively (Roa *et al*., 1996). In Dutch and Belgian breast/ovarian cancer families, 18 previously unidentified mutations are specific to this region (Peelen *et al*., 1997). Twelve of these are recurrent and account for 80% of the families in which a *BRCA1* mutation was identified. Haplotype analysis of the 2800delAA mutation, which accounted for 24% of the detected mutations, revealed a strong founder effect within this population.

Another striking example is Russia, where *BRCA1* mutations are responsible for 75% of familial ovarian cancers. In fact, 86% of the affected Russian families carry one of two common alleles (Gayther *et al*., 1997). The most common of these, 5382insC is also the most common among Europeans as a whole. The other, 4153delA has remained confined to Russia and has not been observed elsewhere. In Poland, *BRCA1* mutations account for 51.5% of breast/ovarian cancer families, and three specific alterations are collectively responsible for 82% of these mutations (Górski *et al*., 2000). A more dramatic example than Israel, of a founder mutation occurs in Iceland. Generally *BRCA1* mutations are found to occur at a frequency of 1.5 to 2.0 fold higher than *BRCA2* mutations within affected families (as a result of founder effects), but in Iceland one *BRCA2* mutation, 999del5 is responsible for almost all (64%) inherited breast and ovarian cancers (Thorlacius *et al*., 1996). A more recent report by Tulinius *et al*. (2002) indicates that the *BRCA2* 995del5 mutation is responsible for 40% of the Icelandic familial breast cancer risk. Although this seems particularly high, *BRCA1* mutations are almost absent, leaving more than half of the breast/ovarian cancer families unaccounted for. In contrast, in Italian breast/ovarian cancer families almost all observed *BRCA1* mutations are unique (Santarosa *et al*., 1999).

A study of *BRCA1* in white South African breast/ovarian cancer families revealed that small mutations within this gene were responsible for 20% (18/90) of the breast cancer burden (Reeves *et al*., 2004). In the South African Afrikaner group specifically, *BRCA1* small mutations were identified in 15% (9/60) of the families, where seven of these families had one of two unique mutations. Both these mutations occur in exon 11 and are a nonsense mutation (E881X), and a frameshift mutation (1493delC). The founder effect identified in *BRCA1* is not surprising considering the ancestry of the Afrikaner. This population was founded about 350 years ago by a small number of Dutch, German and French couples.

The breast cancer risk caused by *BRCA1* in South Africa follows the general trend within other populations, where *BRCA1* is responsible for a larger portion of breast / ovarian cancer families, and gene usually accounts for approximately 20% of familial cases (Figure 1.4).

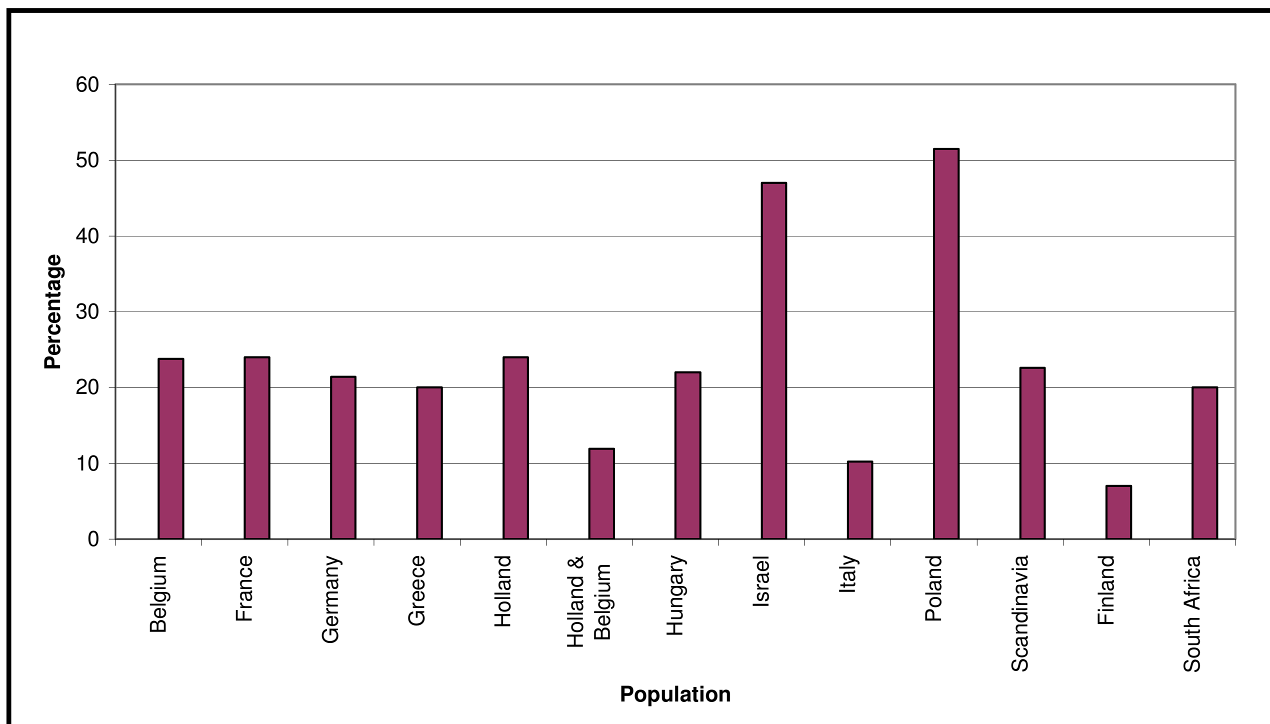

Figure 1.4: Contribution of small *BRCA1* mutations to hereditary breast/ovarian cancer in different populations.
Adapted from Reeves *et al*., 2004, values for Finland from Huusko *et al*., 1998.

1.5.2 LARGE REARRANGEMENTS WITHIN *BRCA1*

Germline mutations within either of the BRCA genes have been found to be lower than expected, with a large proportion of breast/ovarian cancer families being unaccounted for. In 1998, Ford *et al*. (1998) showed that only 63% of families linked to *BRCA1*, in fact had *BRCA1* mutations that were detected by standard PCR-based techniques. These PCR-based techniques are incapable of detecting large genomic rearrangements. Following reports of large rearrangements within various other genes and because *BRCA1* contains such a large proportion of *Alu* repeats, researches considered the possibility that such mutations may occur within *BRCA1*. A variety of methods were subsequently utilized in order to determine whether these mutations occur within *BRCA1*. Puget *et al*. reported the first germ-line large genomic rearrangement within *BRCA1* in 1997. Following this report, Swensen *et al*. (1997) announced that they had found a 14kb deletion, which removes exons 1a, 1b and 2 of *BRCA1*. Many other

groups subsequently employed quantitative techniques, and it is now clear that the fraction of genomic rearrangements is population dependent, and varies from 0% to 36% of *BRCA1* mutation positive families in Finnish and Dutch populations respectively (Lahti-Domenici *et al*. 2001; Laurila *et al*., 2005; Petrij-Bosch *et al*., 1997). In total, at least 69 unique large genomic rearrangements have been described (Table 1.2).

Hotspots seem to occur at exons 1, 17 and 20, where each of these is involved in 10 large genomic rearrangements. Interestingly, five distinct deletions have been described in exon 17, where two of these are the same size (3kb) but have different breakpoints, due to the recombination of different *Alu* sequences (Hartmann *et al*., 2004). Please note that in cases where authors have detected deletions/duplications of the same exons but have not determined the size/breakpoints, the rearrangements are groups together. These may in fact represent different rearrangements or, may be the same as others already reported.

In total, 38 of the reported rearrangements are known to be *Alu*-mediated, 9 have unspecified breakpoints, 15 have breakpoints which could not be determined, two are the result of homologous recombination and 3 due to a non-homologous recombination event (Table 1.2). It is interesting that the majority of rearrangements are deletions (54/69). Of the remaining rearrangements, 10 are duplications, one is a triplication, and three include both deletions and insertions. The remaining mutation was detected in a sporadic breast tumor and involves a deletion together with another complex rearrangement, of which the exact nature has not been determined.

The rearrangements that include both deletions and insertions were found to include exon 3 (2 mutations) and exons 17-20. The complex rearrangement described by Payne *et al*. (2000) involving exon 3 is an inverted duplication of nucleotides g.12965-g.12974 (GenBank accession # L78833), in addition to a deletion of 1039 base pairs. This change ultimately causes the creation of a premature stop at codon 27, due to the skipping of exon 3, since the deletion removes the 5' splice site for intron 3 (Payne *et al*., 2000). The second complex rearrangement reported in exon 3 also results in the formation of a stop in codon 27, but involves the deletion of 1049 base pairs, together with an insertion of 7 base pairs (Walsh *et al*., 2006). The complex in-frame deletion of exon 17 reported by Rohlfs *et al*. (2000a) is characterized by the deletion of 8.2kb (breakpoints at g.58668 & g.66883, exons 17-19), an insertion of 420bp (breakpoints at g.61320 & g.61740) in addition to a 3.7kb deletion (breakpoints at g.68669 & g.72379; exon 20). The inserted sequence was found to be homologous to an area in intron 17.

Table 1.2: Reported rearrangements in *BRCA1*

Description	*Size*	*Method used*	*Reference*
Deletion of *BRCA1* promoter & upstream regulatory region (*Alu*-mediated)	Not determined	Southern blot	Unger *et al*., 2000
Exon 1a, 1b and 2 deletion (homologous recombination between *BRCA1* & pseudo-*BRCA1*)	37kb deletion	Color bar coding MLPA MLPA MLPA	Puget *et al*., 2002 (originally detected by Southern blot but not characterised by Puget *et al*., 1999b) Montagna *et al*., 2003 Preisler-Adams *et al*., 2006 Walsh *et al*., 2006
Exon 1a, 1b and 2 deletion (*Alu*-mediated)	14kb deletion	Southern blot	Swensen *et al*., 1997
Exon 1a, 1b and 2 deletion	Not determined	MLPA	Montagna *et al*., 2003 Hartmann *et al*., 2004 Agata *et al*., 2006
Exon 1a-3 deletion (*Alu*-mediated)	23.4kb deletion	MLPA	Walsh *et al*., 2006
Exon 1a-12 deletion (*Alu*–mediated)	88.5kb deletion	MLPA	Walsh *et al*., 2006
Exons 1a-15 deletion	>169.6kb breakpoints not determined	MLPA	Walsh *et al*., 2006
Exons 1 – 17 deletion	Not specified	MLPA	Bunyan *et al*., 2004
Exons 1-22 deletion	Not determined	QMPSF	Casilli *et al*., 2002
Exons 1a-23 deletion (*Alu*-mediated)	86.9kb deletion	MLPA	Walsh *et al*., 2006
Exons 1 – 24 deletion	Not determined	MLPA	de la Hoya *et al*., 2006
Inverted duplication and deletion, which partially deletes exon 3 and intron 3. (nonhomologous event)	Del1039ins10	LR- PCR	Payne *et al*., 2000
Exon 3 deletion (nonhomologous event)	Del1049ins7	MLPA	Walsh *et al*., 2006
Exon 3 deletion	Not specified	MLPA	Woodward *et al*., 2005
Exon 3 – 5 duplication	Not specified	MLPA	Bunyan *et al*., 2004
Exon 3-8 duplication (*Alu*-mediated)	17.2kb duplication	Color bar coding QMPSF	Gad *et al*., 2001a and 2002a Casilli *et al*., 2002
Exons 3 – 16 deletion (*Alu*-mediated)	46.5kb deletion	MLPA	Thomassen *et al*., 2006
Exon 5 deletion (homologous recombination of a stretch of 9bp that flank exon 5)	244bp deletion	MLPA	Preisler-Adams *et al*., 2006
Exon 5 deletion	Not determined	MLPA	Agata *et al*., 2006 Woodward *et al*., 2005
Exon 5 – 7 deletion (*Alu*-mediated)	5kb deletion	MLPA	Preisler-Adams *et al*., 2006
Exon 5 – 7 deletion	Not determined	MLPA	Agata *et al*., 2006
Exons 5 – 8 deletion	Not determined	MLPA	Agata *et al*., 2006
Exons 8-9 deletion (non-homologous recombination)	3.9kb deletion	MLPA	Walsh *et al*., 2006

Description	***Size***	***Method used***	***Reference***
Exon 8 deletion (*Alu*-mediated)	1.5kb deletion	MLPA	Hogervorst *et al*., 2003
Exons 8-9 deletion (*Alu*-mediated)	7.1kb deletion	Southern blot/PTT Southern blot	Rohlfs *et al*., 2000b Unger *et al*., 2000
Exon 8 – 13 deletion	Not specified	MLPA	Bunyan *et al*., 2004
Exon 8-13 deletion (*Alu*-mediated)	23.8kb deletion	Southern blot Color bar coding QMPSF MLPA	Puget *et al*., 1999b Gad *et al* 2001b; 2002a and 2002b. Casilli *et al*., 2002 de la Hoya *et al*., 2006
Exons 8 – 24 deletion (*Alu*-mediated)	65.5kb deletion	MLPA	Walsh *et al*., 2006
Exon 9 – 12 deletion	Not specified	MLPA	Bunyan *et al*., 2004
Exon 9-19 deletion (*Alu*-mediated)	3.6kb deletion	MLPA	Montagna *et al*., 2003 Agata *et al*., 2006
Exon 11-15 deletion	Not specified	MLPA	Peixoto *et al*., 2006
Exon 11-15 deletion	23kb deletion, breakpoints not confirmed	MLPA	de la Hoya et al., 2006
Exon 13 deletion (*Alu*-mediated)	3.8kb deletion	Southern blot	Petrij-Bosch *et al*., 1997
Exon 13 duplication (*Alu*-mediated)	6kb duplication	RT-PCR LR- PCR MLPA SMP LR-PCR MLPA	Puget *et al*., 1999a The *BRCA1* Exon 13 Duplication Screening Group (2000). Hofmann *et al*., 2002 Hogervorst *et al*., 2003 Hofmann *et al*., 2003 Robinson *et al*., 2000 Unger *et al*., 2000 Preisler-Adams *et al*., 2006 Walsh *et al*., 2006
Exon 13-15 deletion (*Alu*-mediated)	11.6 kb deletion	Color bar coding	Gad *et al*., 2001b Thomassen *et al*., 2006
Exons 13-16 deletion; in frame Breakpoints not determined	14kb deletion	RT-PCR	Petrij-Bosch *et al*., 1997
Exon 14 deletion (*Alu*-mediated)	4.9kb deletion	MLPA	de la Hoya *et al*., 2006
Exons 14 – 20 deletion (*Alu*-mediated)	26.4kb deletion	MLPA	Walsh *et al*., 2006
Exon 15 deletion (*Alu*-mediated)	3kb deletion	Southern blot	Puget *et al*., 1999b
Exon 15-16 deletion (*Alu*-mediated)	~6kb deletion	QMPSF	Casilli *et al*., 2002
Exon 16 – 20 deletion (*Alu*-mediated)	18.3kb deletion	MLPA	Agata *et al*., 2006
Exon 17 deletion (*Alu*- mediated)	1kb deletion	LR- PCR MLPA	Puget *et al*., 1997 Walsh *et al*., 2006
Exon 17 deletion (*Alu*- mediated)	2.7kb deletion	MLPA	Walsh *et al*., 2006
Exon 17 deletion (*Alu*- mediated)	3kb deletion	Southern blot MLPA	Montagna *et al*., 1999 Preisler-Adams *et al*., 2006 Agata *et al*., 2006 Walsh *et al*., 2006

Description	***Size***	***Method used***	***Reference***
Exon 17 deletion (*Alu* -mediated)	5.1kb	MLPA	Hartmann *et al*., 2004
*Exon 17 deletion (*Alu*-mediated, but distinct from deletion reported by Montagna *et al*., 1999)	3 kb deletion	DNA array-based analysis	Frolov *et al*., 2002
Exon 17-19 triplication (*Alu*-mediated)	8.3kb insertion	MLPA	Hogervorst *et al*., 2003
Exons 17-20 deletion; in-frame (*Alu*-mediated)	8.2kb deletion, 420bp insertion, 3.7kb deletion	Southern blot/PTT	Rohlfs *et al*., 2000a
Exon 17-19 deletion	Not determined	Southern blot	Unger *et al*., 2000
*Exons 17-23 deletion??	~20kb deletion, + another complex rearrangement. Exact nature not determined	Southern blot	Van der Looij *et al*., 2000
Exons 18 – 19 duplication (*Alu*-mediated)	5.9kb duplication	MLPA	Walsh *et al*., 2006
Exon 18-19 deletion; (*Alu*-mediated)	4.8kb deletion	MLPA	Montagna *et al*., 2003
Exons 18-20 duplication (*Alu*-mediated)	8658 bp duplication	Color bar coding QMPSF	Gad *et al*., 2002a Casilli *et al*., 2002
Exons 19-20 duplication	Not determined	MLPA	de la Hoya *et al*., 2006
Exon 20 deletion. (non-homologous event)	3.2kb deletion	MLPA	Belogianni *et al*., 2004
Exon 20 deletion; in frame (*Alu*-mediated)	4.3kb deletion	MLPA	Montagna *et al*., 2003
Exon 20 deletion (*Alu*-mediated)	4kb deletion	MLPA	Walsh *et al*.., 2006
Exon 20 deletion	Not specified	MLPA	Bunyan *et al*.., 2004
Exon 20 duplication (*Alu*-mediated)	8.7kb duplication	MLPA	Agata *et al*., 2006
Exon 20 duplication	Not determined	MLPA	de la Hoya *et al*., 2006
Exon 20 - 22 deletion (*Alu*-mediated)	11.4kb deletion	MLPA	Hogervorst *et al*., 2003 Walsh *et al*., 2006
Exon 21 – 22 deletion (*Alu*-mediated)	3.4kb deletion	Southern blot/PTT	Rohlfs *et al*., 2000a
Exon 21 – 22 duplication	Not determined	MLPA	de la Hoya *et al*., 2006
Exon 21-23 duplication (*Alu*-mediated)	7.6kb tandem duplication	MLPA	Hogervorst *et al*., 2003
Exon 21-23 deletion	Not specified	MLPA	Woodward *et al*., 2005
Exon 21 - 24 deletion	Not specified	MLPA	Bunyan *et al*., 2004
Exons 21 – 24 deletion (*Alu*-mediated)	19.2kb deletion	MLPA	Walsh *et al*., 2006
Exon 22 deletion (non-homologous recombination)	510bp deletion	Southern blot LR- PCR MLPA	Petrij-Bosch *et al*., 1997 Hofmann *et al*., 2002 Walsh *et al*., 2006
Exon 23 - 24 deletion	Not determined	MLPA	Agata *et al*., 2006 de la Hoya *et al*., 2006

* Somatic large rearrangement

Contribution of large rearrangements to familial breast cancer

The frequency of *BRCA1* genomic rearrangements varies widely in different populations. In the German population genomic rearrangements were detected in six percent of *BRCA1/2* mutation negative breast/ovarian cancer families and three percent of all high-risk families (Hartmann *et al*., 2004). In the Italian population 22% of *BRCA1/2* mutation negative breast/ovarian cancer families and 13.5% of all high risk families presented with large genomic rearrangements (Montagna *et al*., 2003). In contrast, large genomic rearrangements in *BRCA1* play no role in the etiology of this disease in Finland (Lahti-Domenici *et al*., 2001; Laurilla *et al*., 2005) as well as in the French-Canadian population (Moisan *et al*., 2006). Unger *et al*. (2000) determined that in the American population, 11.9% of breast/ovarian cancer families testing negative for *BRCA1*/*BRCA2* coding mutations had large rearrangements within *BRCA1*. In France, large rearrangements within *BRCA1* account for 3.3% of breast-ovarian cancer cases and 9.5% of *BRCA1* mutations in breast/ovarian cancer families (Gad *et al*., 2002a). Interestingly, such alterations represent founder effects within the Dutch population, where 2 large rearrangements (510bp deletion of exon 22 and 3 835bp deletion of exon 13) represent 36% of all *BRCA1* mutations, where Ex22del510 was found in 6.39% of Dutch breast cancer families (Petrij-Bosch *et al*., 1997). The 6kb insertion in exon 13 (nucleotides g.44369-g.50449), originally described by Puget *et al*. (1999a) is now also considered a founder. Puget *et al*. (1999a) found the mutation in 3 American families (all of whom had mixed European ancestry) in addition to a Portuguese family. All of these families share the same haplotype at nine markers (Puget *et al*., 1999a). Following the screening of 3580 unrelated individuals with a positive family history by The *BRCA1* Exon 13 Duplication Screening Group (2000), it was determined that 11 additional families presented with this mutation. Haplotype analysis indicated the occurrence of a common ancestor, possibly of northern British origin (The *BRCA1* Exon 13 Duplication Screening Group., 2000). The mutation was detected in three other families from non-English speaking countries (Belgium, Portugal and Germany) that have trading or other historical links with Britain, which may explain their occurrence. The breakpoints for this mutation are located in two Sx *Alu* sequences with 23bp of perfect identity.

Large rearrangements in *BRCA1* may be responsible for a significant portion of familial breast / ovarian cancer, and screening is suggested to become routine. It still seems however as if point mutations splice site and frame shift mutations heavily outnumber the occurrence of these complex rearrangements (BIC). This impression may however be false, due to the preferred use of qualitative PCR-based techniques in the past.

1.6 OBJECTIVES

Accumulating evidence on the occurrence of large genomic rearrangements within different populations has highlighted the importance of using alternate techniques that will detect such alterations. The contribution of large genomic rearrangements in *BRCA1* to familial breast / ovarian cancer in South Africa is unknown. The aim of this particular study is to assess the frequency of *BRCA1* genomic rearrangements in South African and in particular Afrikaner breast/ovarian cancer families.

CHAPTER 2
MATERIALS AND METHODS

Seventy-four samples from 58 South African breast and/or ovarian cancer families were investigated for large genomic rearrangements within *BRCA1* using Southern Blot analysis as well as Multiplex Ligation-dependent Probe Amplification (MLPA).

2.1 PATIENTS

All patients included in this study had previously been found negative for coding-region and splice-site mutations within *BRCA1* and *BRCA2* by means of SSCP / Hetroduplex Analysis and PTT. These patients were referred by private practitioners, or attended the Familial Cancer Clinic of the Department of Human Genetics at the University of Pretoria. In each case, informed consent was obtained prior to enrolment into the study. Approval for the study was granted by the Ethics Committee of the University of Pretoria, Medical School (Protocol number 18/98). A copy of the ethics approval is available in Appendix A.

The selection criterion for the families included in the study was based on the occurrence of 3 or more breast and / or ovarian cancer cases within a single family, where bilateral disease was scored as two cases (see Appendix B). Two families did not fulfill these criteria but were nevertheless included. These two families (BRC66 and BRC119) only had two affected individuals each. In the case of BRC66 the two affected individuals were sisters and developed cancer of the breast at the ages of 41 and 42 years. They were included due to their young age at diagnosis and them being siblings. In family BRC119, two sisters were diagnosed with ovarian cancer (diagnosis at ages 64 and 68 years) and since ovarian cancer is not that common for two sisters to develop, it could be possible that this is due to inherited susceptibility. Furthermore, two of the families evaluated had previously been found to carry a disease causing mutation within *BRCA2* (BRC46 and BRC56). Each family did however have one individual who developed breast cancer and did not carry the family mutation. The mutation identified in family BRC46 was *BRCA2* 4781delA, while family BRC56 presented with *BRCA2* 1024delT. Individual BRC46.2 was diagnosed with cancer of the breast at age 55 and BRC56.2 at age 38. These persons could be phenocopies or may actually have another unique mutation, which may have been inherited from the paternal side of the family, for which no information is available. These persons were fully screened for *BRCA1* and *BRCA2* by PCR-SSCP/HA as well as PTT and were

found to be mutation negative. They were therefore included in the present study to exclude the possibility of the occurrence of a large rearrangement.

The families included in the study are of mixed ancestry (Table 2.1) but are South African, while a single Dutch patient who immigrated to South Africa from Holland was also included.

Table 2.1: Ancestral links of families included in the study

Ancestry	Number of families
Afrikaner	40
English	4
Ashkenazi Jewish	3
Dutch	3
German	1
Greek	1
Polish	1
Belgian	1
Portuguese	1
English/Welsh	1
English/Scottish	1
English/Australian	1

Clinical details of the families screened are available in Appendix B.

2.2 METHODS

Unless stated otherwise, all reagents and chemicals used in this study were obtained from E Merck Darmstadt, BDH Laboratory Supplies Poole, Roche Molecular Biochemicals, United States Biochemicals, Invitrogen, Amersham Biosciences and all primers were synthesized by Genosys Biotechnologies Inc, USA.

2.2.1 EXTRACTION OF GENOMIC DNA

Genomic DNA was isolated according to a modified method of that proposed by Johns and Paulus-Thomas (1989).

Ten ml blood, from an EDTA vacutainer was lysed with 30ml lysis buffer (0.32M sucrose; 10mM Tris-HCl pH 8; 5mM $MgCl_2$; 1% Triton X-100) and left on ice for 10 minutes. The solution was subsequently centrifuged at 8 120 *g* (Beckman model J2-21M centrifuge, JA-17 rotor) for half an hour at 4ºC. The pellet containing the nuclear material was resuspended in 9ml suspension buffer (10 mM Tris-HCl pH 8; 0.15 M NaCl; 5 mM EDTA). Following resuspension, addition of 1ml 10% SDS and 2.5ml 5M $NaClO_4$ (freshly prepared) ensured protein denaturation. An equal volume of (24:1) chloroform: Isoamyl alcohol (IAA) solution was subsequently added, and the samples shaken on a rotating platform for half an hour. Following phase separation by means of centrifugation at 20ºC for 15 minutes at 330 *g* (Beckman model J2-21M centrifuge, JA-17 rotor), the aqueous phase was removed and the previous step repeated to allow for protein removal. Subsequent addition of ethanol to the aqueous phase allowed for DNA precipitation, which was then collected on a sterile glass rod. The DNA was dried and subjected to overnight resuspension in 0.5ml Tris-EDTA buffer (10mM Tris-HCl pH 8.2; 1mM EDTA). DNA concentration was established by determining the absorbance at 260nm with a spectrophotometer (Unicam 3625 UV/VIS). Reading the optical density at 280nm revealed whether any protein contamination was present. DNA stocks were kept at -70ºC.

2.2.2 SOUTHERN BLOT ANALYSIS

Southern Blot analysis was performed according to the procedure described by Unger *et al.* (2000) in which a series of long probes amplified from genomic DNA were used. Our methodology was altered in terms of probe labeling and fragment detection, where Unger *et al.* (2000) utilized the random primed DNA labeling with ^{32}P, followed by detection using a phosphoimager. The current study involved the random labeling of probes with Digoxigenin-11-2'-deoxy-uridine-5'triphosphate (Roche) via PCR and detection using CDP-S*tar* (Roche).

2.2.2a) PROBE AMPLIFICATION AND LABELING

Probes were amplified from genomic DNA of a Afrikaner unaffected individual and randomly labeled with DIG-dUTP. The primers used, together with amplification conditions are listed in table 2.1. In order to ensure high fidelity amplification, either TaKaRa *Ex Taq* ™ or *LA Taq*™ (*TaKaRa* Bio Europe S.A.) was utilized. In the labeling process, DIG-11-dUTP replaces dTTP in a ratio of 65% dTTP and 35% DIG-11-dUTP.

All PCR reactions were prepared on ice and carried out in a total volume of 50µl. Final concentrations for the PCR reactions are as follows: 150ng DNA; 1x *LA Taq*™ buffer (Mg^{2+} free) or 1x *Ex Taq*™ buffer (2.0mM $MgCl_2$); 0.2µM of each primer; 250µM of each dNTP including 0.2 µM Digoxigenin – 11 – 2' – deoxy – uridine – 5' – triphosphate (Boehringer Mannheim) and 1.5U *TaKaRa LA Taq*™ or TaKaRa *Ex Taq*™ (*TaKaRa* Bio Europe S.A.) The varied conditions with regard to addition of $MgCl_2$, enzyme and type of buffer added are listed in table 2.1.

Success of the amplification was determined by analyzing 4µl of the product alongside 4µl of the Low DNA Mass™ Ladder (Life Technologies) on a 1% D1LE (Hispanagar) ethidium bromide stained Agarose gel at 70V for one hour. The Low DNA Mass™ Ladder allowed for the approximate determination of the concentrations of the probe.

Table 2.2: Primers and PCR conditions used to synthesize probes for Southern blot.

Probe name	Exons covered	Primer name	Primer sequence (5' – 3')	Product size (bp)	Taq (GC buffer; [$MgCl_2$])	Annealing Temperature (ºC)
Probe 1	1 & 2	BRC1 – 1F	TAG CCC TTG GTT TCC GTG[1]	1 522	LA Taq (GC buffer I; 3.0mM $MgCl_2$)	55
		BRC1 – 2R	TGT CTT TTC TTC CCT AGT ATG T[1]			
Probe 2	3	SouEx3F	GTG GAT ATG GGT GAA ACA GC[2]	1 863	LA Taq (GC buffer I)	60
		SouEx3R	CCA GAA AAA TGT ACA TGG CC[2]			
Probe 3	5 - 7	BRC1- 5F	CTC TTA AGG GCA GTT GTG AG[1]	2 626	LA Taq (GC buffer I)	58
		BRC1- 7R	AGG ACT GCT TCT AGC CTG[3]			
Probe 4	8	SouEx8F	GTT ATC AGA TGT GAT TGG AAT G[2]	768	Ex Taq	55
		SouEx8R	TCT TTT GCT CCC TTT TTA AA[2]			
Probe 5	9 & 10	BRC1- 9F	CCA CAG TAG ATG CTC AGT AAA TA[1]	1 636	LA Taq (GC buffer I)	53
		BRC1- 10R	CCC AAA TGG TCT TCA GAA TA[3]			
Probe 6	11 & 12	BRC1- 11LF	TCA ATG TCA CCT GAA AGA GAA ATG G[1]	1 657	Ex Taq	55
		BRC1- 12R	TGT CAG CAA ACC TAA GAA TGT[1]			
Probe 7	13	SouEx13F	ATT TCA TTT TCT TGG TGC CA[2]	1 645	Ex Taq	58
		SouEx13R	GGG AGA AAA AGG CTC AAA AC[2]			
Probe 8	14	SouEx15F	CCT TCT TGT GCC ATT TCA TC[2]	1 084	Ex Taq	60
		SouEx14R	ACC ATC AGT TTC CAA GCT TG[2]			
Probe 9	15	SouEx15F	AAA AGG CAG GCA ATA GGG AT[2]	1 211	Ex Taq	60
		SouEx15R	CCA AGA CTC CCT CAT CCT CA[2]			
Probe 10	16	SouEx16F	AAT TAA TGG GTG AAG AGT ACT CC[2]	1 338	Ex Taq	56
		SouEx16R	ACA GGG GTG GTA AAC TTC TC[2]			

Probe name	Exons covered	Primer name	Primer sequence (5' – 3')	Product size (bp)	Taq (GC buffer; [$MgCl_2$])	Annealing Temperature ºC
Probe 11	17	SouEx17F	TTT ATG TCT GCT GAT GTG TAC A[2]	1 124	LA Taq (GC buffer II)	52
		SouEx17R	AGA CTA TCA TCC ATG CTA TGC[2]			
Probe 12	18 & 19	BRC1- 18F	GGC TCT TTA GCT TCT TAG GAC[1]	799	Ex Taq	56
		BRC1- 19R	CAT TGT TAA GGA AAG TGG TGC[1]			
Probe 13	20	SouEx20F	CCT GAA TGC CTT TAA ATA TGA[2]	715	Ex Taq 3.0mM $MgCl_2$	53
		SouEx20R	TAA ATT TTA GCT ATT ATT GGC TG[2]			
Probe 14	21 & 22	BRC1- 21F	AAG CTC TTC CTT TTT GAA AGT C[1]	2 201	Ex Taq	56
		BRC1- 22R	GAG AAG ACT TCT GAG GCT AC[1]			
Probe 15	23 & 24	SouEx23F	TGA TGA AGT GAC AGT TCC AG[2]	2 188	LA Taq (GC buffer I)	60
		BRC1-24R	GTA GCC AGG ACA GTA GAA GGA[1]			

[1]Friedman et al., 1994
[2]Unger et al., 2000
[3]Own primer

2.2.2b) RESTRICTION DIGESTION AND BLOTTING

Three separate Restriction digestions were completed for each sample. Ten micrograms of genomic DNA was digested separately with 30U *Hind* III, *Xba* I, or *Eco* RI for five hours at 37ºC. The total product was separated on a 0.8% D1LE (Hispanagar) agarose gel (1x TBE) stained with ethidium bromide, using the Horizon 20x25cm gel electrophoresis apparatus (Life Technologies). The digested products were electrophoresed alongside a Digoxigenin – labeled DNA Molecular Weight Marker II (Roche Diagnostics) at 45V for 24 hours. Following separation, the DNA was visualized under a UV light to determine whether digestion was complete. The agarose gel was subsequently soaked in 0.25M HCl for 5 min in order to depurinate, (until the xylene cyanol turns green / bromophenol blue turns yellow), rinsed with water and submerged in the denaturation solution (0.5M NaOH / 1.5M NaCl), with gentle shaking for 30 minutes. DNA was subsequently transferred onto a nylon Hybond^{TM}-N^{+} membrane (Amersham) overnight, using the alkaline denaturation solution (0.5M NaOH / 1.5M NaCl). Once transferred, the DNA was cross-linked under a UV light for 5 minutes.

2.2.2c) PREHYBRIDIZATION / HYBRIDIZATION AND STRINGENCY WASHES

The conditions used for prehybridization, hybridization and stringency washes were optimized during the course of this investigation. Prehybridization and hybridization were carried out in a total volume of 20ml. Probes were pooled according to Unger *et al*. (2000) (table 2.2). Pre- and hybridization was carried out in either the Bachoffer hybridization oven or the Techne Hybridiser HB-1D apparatus.

Initially, both pre- and hybridization were carried out in a single solution (5X SSC; 5X Denhardt's solution; 1% SDS (w/v) and 20µg / ml denatured salmon sperm) at 65ºC for 2 hours and 16 hours respectively, with 10ng probe added prior to hybridization. Membranes were then rinsed twice with 2X SSC, washed once for 15 minutes with 2X SSC/0.1% SDS and once with 1XSSC/0.1%SDS at room temperature.

The Southern blot probes were pooled into three mixes according to Unger *et al*. (2000), determined solely on what size fragment each probe will generate. In this manner a number of probes can be used in a single hybridization reaction and the resulting fragments easily distinguished. The exons covered by each probe mix are indicated in table 2.2.

Table 2.3: Exons covered by the different probe mixes

Probe Mixes			
Mix	**1**	**2**	**3**
Exons covered	3 – 10 15 - 19	11 – 14 20 - 24	Exons 1 - 2

Optimization

In order to allow the probes to bind to the DNA, stringency of prehybridization/hybridization was decreased, by reducing the temperature to 42ºC. The pre-hybridization / hybridization buffer was also changed to two separate solutions containing 5X SSC; 10X Denhardt's solution; 0.5% (w/v) SDS; 50% Formamide and 20µg / ml denatured salmon sperm (prehybridization) and 50% Formamide; 5X SSC; 1X Denhardt's; 0.5% SDS; 5% Dextran Sulphate together with 10ng of labeled probe per ml (hybridization). This was later changed to a single buffer for both pre- and hybridization which contained 50% Formamide, 5X SSC, 1X FPG, 25mM KH_2PO_4, 0.2% SDS, 5% Dextran Sulphate and 25µg/ml denatured salmon sperm, and the amount of labeled probe added reduced to 5ng/ml of hybridization solution. Prehybridization and hybridization were allowed for 3 and 13 hours respectively. In an effort to further reduce background, the FPG concentration of the prehybridization/hybridization solution was increased to a 5X final concentration and the Dextran Sulphate concentration decreased to 2.5%. This was done since FPG results in lower background than Dextran Sulphate (Keller and Manak., 1989). Prehybridization occurred overnight and hybridization for 4 hours at 42ºC.

During the study, stringency of the washes was increased due to the occurrence of background. Final washes were performed at 65ºC in a shaking water bath, and membranes were washed twice for 15 minutes with 0.5X SSC/0.1% SDS, and twice for 15 minutes with 0.1X SSC/0.1% SDS.

2.2.2d) DIG DETECTION

Detection was carried out using the DIG Wash and Block Buffer Set and CDP-*Star* (Roche Diagnostics) with few modifications. CDP-*Star* is a very sensitive chemiluminescent substrate for alkaline phosphatase that generates a light signal very rapidly.

Following stringency washes, membranes were equilibrated for 5 minutes in 100ml 1X washing buffer, followed by blocking for 30 minutes in 160ml 1X blocking solution on a rotating platform. Filters were incubated in 100ml of antibody solution (anti-digoxigenin-AP conjugate, diluted 1:10000) for 30 minutes and then washed twice for 15 minutes in 200ml 1X washing buffer. Filters were then equilibrated in 100ml 1X detection buffer for 5 minutes, followed by incubation in 25ml CDP-Star diluted 1:100 in 1X detection buffer for 5 minutes. All reactions were facilitated at room temperature. Membranes were covered with cling film and exposed to Lumi-Film Chemiluminescent Detection Film (Roche Diagnostics) with varying exposure times and developed. The band patterns that should be visualized with the different probe mixes are listed in table 2.3.

Table 2.4: Size fragments generated with the different probe mixes

Fragment sizes (bp)			
Mix	**1**	**2**	**3**
Eco RI	18 062 3 390 5 730 9 764	8 200 5 812 2 968 1 522 16 148	7 120
Hind III	1 511 2 893 10 380 4 017 1 663 7 197 19 040	7 197 6 405 3 220 3 706 9 716	8 802
Xba I	13 091 18 485 5 330 6 545 1 659	18 485 3 465 2 237 6 172 1 082 6 922	13 091 6 500

2.2.2e) PROBE STRIPPING

In order to allow hybridization of 3 different probe mixes on a single membrane, and to facilitate optimization, membranes were stripped of probes with Alkaline Probe stripping solution (0.2M NaOH / 0.1% SDS). Membranes were first washed in dH_2O for one minute, followed by incubation at 37ºC for 10 minutes with gentle shaking in the Alkaline Probe stripping solution. The solution was removed and stripping repeated. The solution was poured off and the membrane rinsed thoroughly twice in 2X SSC. The membranes were immediately pre-hybridized or stored wet in 2X SSC.

2.2.3 MLPA ANALYSIS

MLPA or Multiplex Ligation-dependent Probe Amplification is a method utilized for the relative quantification of nucleic acid sequences (Schouten *et al*., 2002). Briefly, the basis of MLPA is as follows: Each MLPA probe is essentially made up of two oligonucleotides, one short synthetic and one long phage M13-derived oligonucleotide. The long oligo has a special “stuffer” fragment at its 3’ end to ensure that all probes have a unique size, making size separation possible. The two oligonucleotides anneal directly adjacent to each other at the target site.

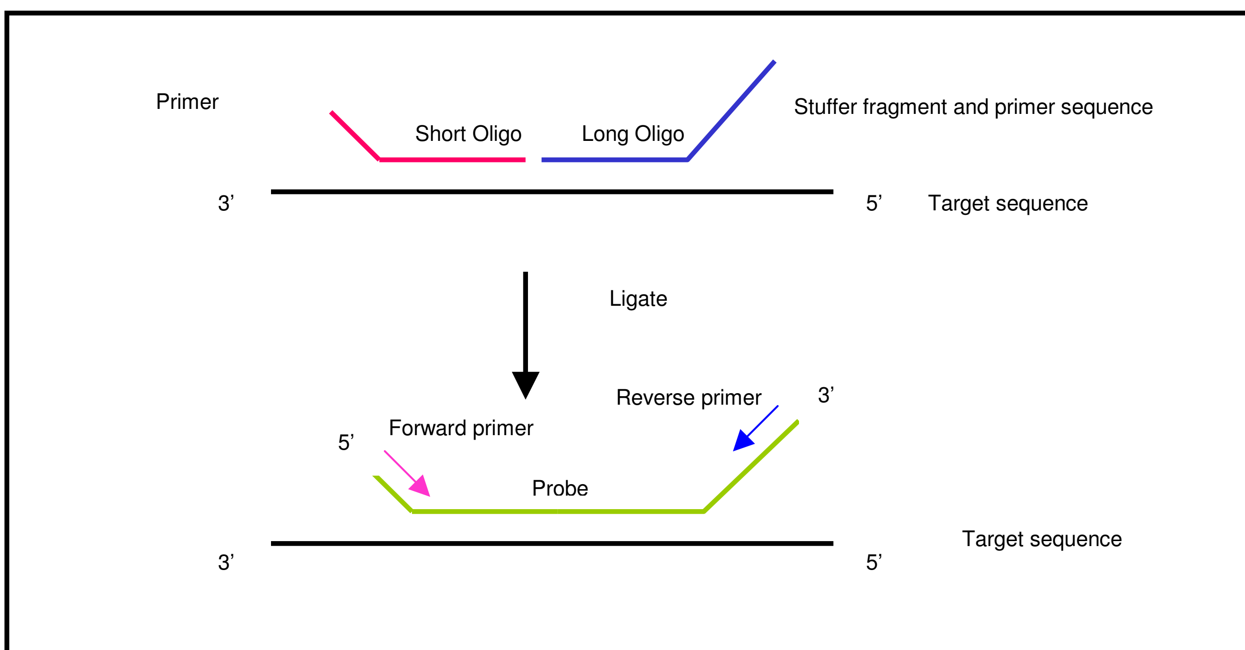

Figure 2.1: Basis of the MLPA technique.
The short and long oligonucleotides anneal directly adjacent to one another on the target sequence. Following hybridization, the oligos are ligated to form the MLPA probe. The probe can then be amplified and the amount of product quantified.

Once hybridization is achieved, these two oligos are ligated, and in this manner form the complete probe, which will ultimately be amplified. The short synthetic oligos have identical primer sequences at their 5’ ends, while all long oligos have identical primer sequences at their 3’ ends. This allows amplification of all probes to be achieved with one primer pair (Figure 2.1).

The short synthetic oligonucleotides are 21-35 nucleotides in length, with the common primer sequence at the 5' end, and the target sequence immediately adjacent to it, on the 3' end (Figure 2.2).

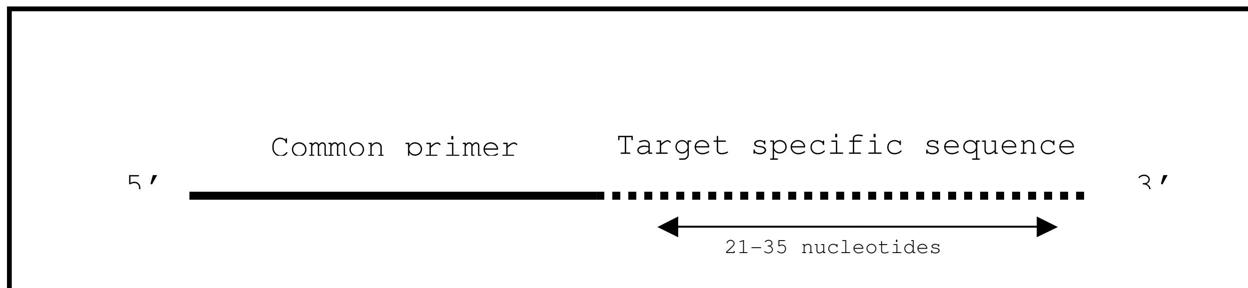

Figure 2.2 Structure of short synthetic oligonucleotide

The long oligonucleotide possesses a target specific sequence of 25-43 nucleotides at the 5' end, this portion will hybridize directly adjacent to the short oligo. The common primer sequence is located at the 3' end, and between this sequence and the target sequence a stuffer fragment of 19-370 nucleotides is positioned (Figure 2.3). This stuffer fragment ensures that each amplified fragment will be of a unique defined size.

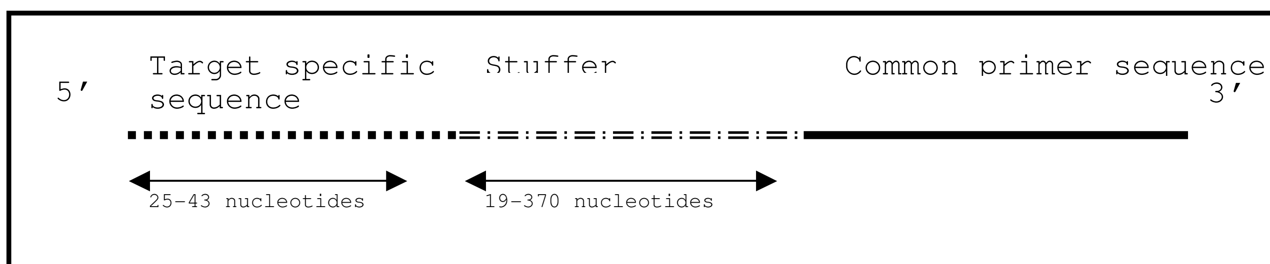

Figure 2.3 Structure of long M13 derived oligonucleotide

2.2.4 MLPA ANALYSIS OF *BRCA1*

The SALSA P002 *BRCA1* exon deletion test kit (MRC Holland) was utilized to screen the samples for large genomic rearrangements. The *BRCA1* probe mix contains 34 probes of which 9 are control probes, located on different chromosomes. Amplification products of the 34 probes differ in length, allowing effective size separation of products by electrophoresis. The difference in length between two control probes or between two *BRCA1* probes is 9bp, while the difference between a control and a *BRCA1* probe is 12bp. Nine samples that displayed aberrant MLPA profiles in addition to a single sample that was not screened previously using the P002 kit were analyzed with the new *BRCA1* P087 kit. The hybridization sequences of the P002 and P087 kits are given in Appendix C.

2.2.4a) HYBRIDIZATION AND LIGATION OF OLIGONUCLEOTIDES

One hundred nanograms genomic DNA (5µl) was denatured at 98°C in a Programmable Thermal Controller PTC-100 (MJ Research, Inc) with heated lid, for 5 minutes, and cooled to 25°C before opening the thermal cycler. One and a half microlitres of MLPA buffer (1.5M KCl, 300mM Tris-HCl pH 8.5, and 1mM EDTA) together with 1.5µl SALSA probe mix containing 1-4 fmol of each synthetic and M-13 derived oligonucleotide (MRC-Holland) was added to the denatured DNA, and mixed carefully. The sample was subsequently heated to 95°C for 1 minute, and hybridization allowed at 60°C for 16 hours. The temperature of the thermal cycler was decreased to 54°C, and 32µl ligation reaction mix, with final concentrations of 2.6mM $MgCl_2$; 5mM Tris-HCl pH 8.5, 0.013% non-ionic detergents, 0.2mM NAD and 1U Ligase-65 enzyme (MRC-Holland) was added to the samples and mixed. Ligation was allowed at 54°C for 15 minutes, followed by inactivation of the enzyme at 98°C for 5 minutes.

2.2.4b) POLYMERASE CHAIN REACTION

A total of 40µl PCR reaction mix was added to 10µl of the ligation reaction. Final concentrations were 10pmol PCR primers (one FAM-labeled and one unlabelled primer), 2.5nmol dNTPs, 2.6mM $MgCl_2$, 5mM Tris-HCL pH 8.5, 0.013% non-ionic detergents, 0.2mM NAD and 2.5U SALSA polymerase (MRC-Holland). Amplification occurred during the following cycles: 1 minute at 95°C initial denaturation, followed by 33 cycles of 30 seconds at 95°C, 30 seconds at 60°C and 1 minute at 72°C and concluded with a final extension at 72°C for 20 minutes.

Primers utilized are complementary to the universal primer sequences on all of the oligonucleotides, and sequences are as follows:
Forward, 5' - *GGGTTCCCTAAGGGTTGGA - 3' (FAM-labeled)
Reverse: 5' - GTGCCAGCAAGATCCAATCTAGA - 3'.

2.2.4c) SIZE SEPARATION OF MLPA FRAGMENTS

Amplified products were separated using either the ABI-3100 or ABI-3130 (Applied Biosystems) genetic analyzer. Eight and a half microlitres deionized formamide together with 0.5µl GeneScan-500 LIZ size standard (Applied Biosystems) was added to 1µl MLPA PCR product. Samples were denatured at 94℃ for 2 minutes cooled on ice and electrophoresed at 15kV using POP-4 (ABI-3100) or POP7 (ABI-3130) Polymer with the G5 filterset.

2.2.4d) DATA ANALYSIS

MLPA data is analyzed by determining exon copy number or dosage quotient (DQ) values for each ligation product. This was achieved by statistical analysis using one of two methods, either the Schouten or the Wallace method. The Schouten analysis is recommended by MRC-Holland for use of their kits, while the Wallace method calculates DQ in the standard manner in addition to assigning a significant probability to the dosage data. Prior to beginning with DQ calculation, either Genotyper®3.7 or GeneMapper 3.0™ (ABI PRISM®) was utilized to generate an MLPA profile in order to visually inspect the quality of the control mix fragments. This is necessary to determine whether analysis was successful (described in detail in Chapter 3).

Schouten method

Peak areas were imported into Excel spreadsheets and dosage quotients calculated as specified (Schouten *et al.*, 2002; MRC-Holland., 2003). The peak area of a specific probe was divided by the sum of all peak areas of that sample. The resultant relative peak area of each probe was divided by the relative peak area of that specific probe of an Afrikaner unaffected control sample analyzed in the same run. The resultant value should be 1, provided that no deletion / duplication has occurred. If a deletion occurred, a value of 0.5 is expected, whereas with a duplication, a value of 1.5 is expected. Samples were repeated if any probes displayed variation between 20 and 30%, and scored as a deletion or duplication if they displayed variation greater than 30%.

Wallace method

A far more robust analysis, which assigns a significant probability to dosage data, was developed in 2004 and presented at the CGMS (lecture is available at http://www.ngrl.org.uk/Manchester/Pages/mutationsload.htm). For this analysis it is imperative that five unaffected control samples be included in each particular experiment. The Excel MACRO is available at the MRC-Holland website (www.mrc-holland.com). Peak areas are imported into the "RAW DATA" spreadsheet, and the MACRO then calculates dosage quotients in the standard manner, while including the calculation of likelihood probability of concordance with one of three hypotheses (the occurrence of 1, 2 or 3 copies of a specific ligation site within the test sample) by comparing the sample to five Afrikaner unaffected control samples. The probability of variation is also calculated, using the t-statistic. In addition, the standard deviation of the dosage quotients generated for each of the control ligation products is calculated. This value then behaves as a control that determines the quality of each individual test. A standard deviation greater than 0.1 means that the analysis of that particular sample was of poor quality and should be repeated.

The Wallace method of DQ calculation was not available at the start of this study. The data generated during the initial analyses could subsequently not be analyzed with this MACRO since only a single unaffected control sample was included in each analysis. The Wallace method requires five unaffected controls obtained from the same experiment (due to variation between analyses). The analysis was however used for the last analysis, where ten samples were analyzed. Of these, one was analyzed for the first time, while 9 were repeated after displaying equivocal DQs in previous analyses.

2.2.5 FINE CHARACTERIZATION OF ABERRANT MLPA FRAGMENTS

Samples that displayed aberrant MLPA fragments were amplified for that specific region using primers which flank the exon(s) shown as aberrant and cycle sequenced using the BigDye® Terminator v3.1 Cycle sequencing kit (Applied Biosystems).

2.2.5a) AMPLIFICATION OF PUTATIVE DELETED FRAGMENTS

Long-range PCR was performed with primers that flank two to three exons over the region in which the suspected aberration was detected. Primers used for amplification are indicated in table 2.4.

Fragments 1 and 2 were produced by long-range PCR using LA *Taq* (*TaKaRa* Bio Europe S.A.) and the supplied GC buffer I in a total reaction volume of 20µl. Fragment 5 was also amplified using LA Taq, however GC buffer II was utilized. Final concentrations were as follows: 200ng genomic DNA, 0.2µM of each primer, 1X LA GC buffer I or II, 250µM of each dNTP and 2.5U TaKaRa LA *Taq*™. Amplification success was determined by electrophoresis of 5µl PCR product on a 0.5% D5 (Hispanagar) agarose gel (fragments 1 and 2) or on a 1% D1LE (Hispanagar) agarose gel at 50V for 4 hours.

The PCR reaction for fragment 3 was performed in a total volume of 20µl with 50ng of genomic DNA, 250µM of each dNTP, 2.0mM $MgCl_2$, 20mM Tris-HCl (pH 8.4), 50mM KCl and 0.5U Taq polymerase (Invitrogen). Five microlitres were electrophoresed on a 1% D1LE (Hispanagar) agarose gel at 70V for 40 minutes.

Fragment 4 was amplified by long-range PCR using DyNAzyme™ EXT DNA polymerase (FINNZYMES). A final reaction volume of 50µl was used, where the final concentrations were as follows: 100ng genomic DNA, 0.5µM of each primer, 625µM of each dNTP, 1X Optimized DyNAzyme EXT buffer and 2U EXT polymerase. Following amplification, 5µl PCR product was electrophoresed on a 1% D1LE (Hispanagar) agarose gel at 70V for 2 hours.

Table 2.5: Information for primers utilized to amplify and sequence.

Fragment / Primer set	Primer name	Primer sequence	Product size (bp)
1	[2]BRC1-17F	AGC TGT GTG CTA GAG GTA ACT C	4 516
	[1]BRC1-19R	CAT TGT TAA GGA AAG TGG TGC	
2	[2]BRC1-17F	AGC TGT GTG CTA GAG GTA ACT C	3 976
	[2]BRC1-18R	CTC AGA CTC AGC ATC AGC	
3	[1]BRC1-18F	GGC TCT TTA GCT TCT TAG GA	258
	[2]BRC18-R[*]	CTC AGA CTC AGC ATC AGC	
4	[1]BRC1-22F[*]	TCC CAT TGA GAG GTC TTG CT	1 751
	[1]BRC1-23R	ACT GTG CTA CTC AAG CAC CA	
5	[1]BRC1-22F	TCC CAT TGA GAG GTC TTG CT	3 741
	[1]BRC1-24R	GTA GCC AGG ACA GTA GAA GGA	

[1]Friedman *et al*. (1994)

[2]Breast Cancer Information Core (Bic)

[*]Primers used to sequence

2.2.5b) <u>CYCLE SEQUENCING</u>

Sample preparation for sequencing was done by either pre-treating the PCR product or by agarose gel removal. Pre-treatment was performed by adding 10U Exonuclease I to the 7.5µl PCR product and incubating at 37ºC for 15 minutes, followed by 80 ºC for a further 15 minutes. Two units of Shrimp Alkaline Phosphatase was added, incubated at 37ºC for 15 minutes, followed by 80 ºC for 15 minutes.

Samples displaying deleted fragments on initial electrophoresis were re-electrophoresed (45µl PCR product on a 0.76% FMC SeaPlaque®GTG® agarose gel at 50V for 4 hours), and the deleted band excised from the gel with a razor blade and agarose removed using the Wizard® SV Gel and PCR Clean-Up System (Promega), using the centrifugation protocol with no modifications.

Sequencing of all samples was achieved by diluting 4µl of pretreated or gel-cleaned product with 7µl ddH_2O, to which 1µl of primer (20µM) was added (Table 2.3). Seven microlitres of a BigDye

v3.1 dilution was added to 3µl of DNA together with primer. The DNA was cycle sequenced with 10 seconds at 96ºC to allow for denaturation, followed by the annealing step at 55ºC for 5 seconds and an extension phase at 60ºC for 4 minutes. This cycle was repeated 25 times.

Following cycle sequencing, the DNA was precipitated in a final volume of 100µl with final concentrations of 0.09M Sodium Acetate and 65% EtOH. After addition of the precipitation mix, the sample was incubated in the dark for 15 minutes, centrifuged at 14 000 rpm for 30 minutes, and the supernatant removed. The resultant pellet was washed with 70% EtOH, dried and dissolved in 10µl formamide, heated at 96ºC for 2 minutes and cooled on ice. Sequence analysis was subsequently performed on the ABI 3130 (Applied Biosystems) using the POP 7 polymer and filterset D.

CHAPTER 3

RESULTS AND DISCUSSION

Seventy-four samples from 58 breast and / or ovarian cancer families were analyzed by Southern blot and multiplex ligation-dependent probe amplification (MLPA).

Analysis of samples by Southern blotting, using a modified method of that described by Unger *et al.* (2000) proved laborious and ineffective. This method could not be optimized successfully.

3.1 <u>SOUTHERN BLOT ANALYSIS</u>

Attempts were made to optimize this technique, but it remained difficult to achieve reliable results. The different methodologies utilized in an effort to optimize this technique, together with a few of the autoradiographs are illustrated in figures 3.2 to 3.5.

3.1.1 <u>PROBE AMPLIFICATION AND LABELING</u>

Probes were labeled randomly by incorporating DIG-dUTP via PCR. Four microlitres of each probe was electrophoresed on a 1% D1LE agarose gel (figure 3.1) together with 4µl Low DNA Mass™ Ladder to determine that the correct product size was generated and that no non-specific product was present. Furthermore, this ladder allows the approximate determination of probe concentration. Electrophoresis of 4µl of this ladder results in bands containing 200, 120, 80, 40, 20 and 10ng of DNA respectively for the fragments 2 000, 1 200, 800, 800, 200 and 100bp.

Although all the probes were amplified, only those which make up probe mix 2 were actually used during this study while this technique was optimized. The electrophoresed products of these probes are indicated in figure 3.1.

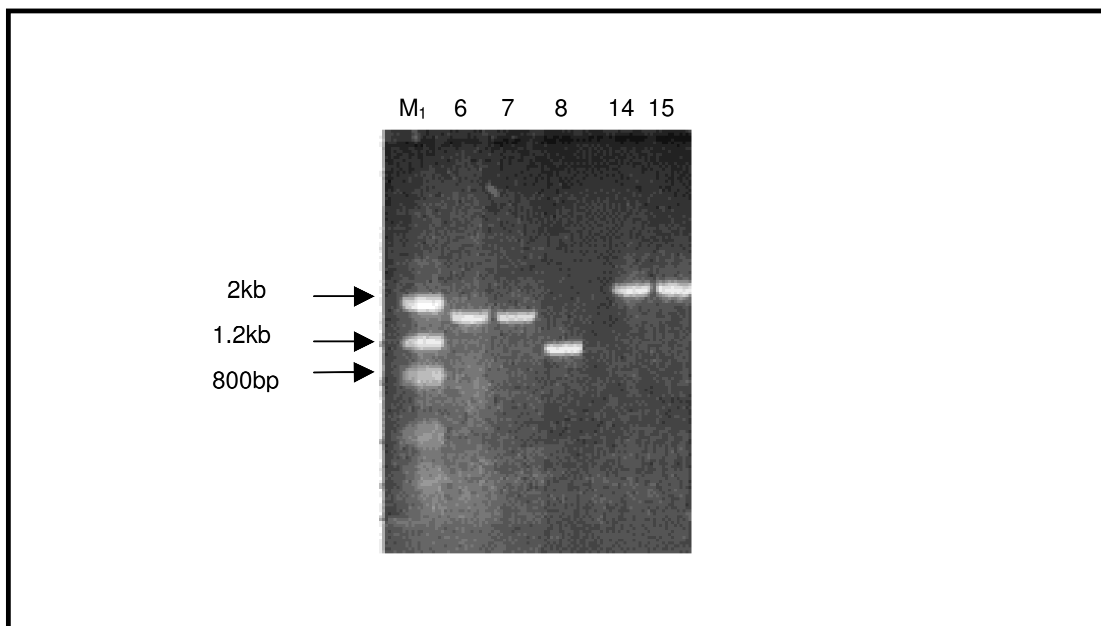

Figure 3.1: Agarose gel electrophoresis of labeled probes in probe mix 2
Probe number is given above gel.
M_1: Low DNA Mass^{TM} Ladder (Life Technologies)

The concentration of the 5 probes depicted was determined to be approximately 25ng/µl (100ng/4µl). Since the intensity of the bands are approximately equal, the same quantities of each probe could be used during hybridization. Probe 13 was unsuccessfully amplified during this round of PCR. Probe 13 was later re-amplified, and the correct product resulted. This probe was however eliminated from the mix during optimization, due to its low concentration.

3.1.2 SOUTHERN BLOT RESULTS

The first Southern blot analysis was carried out using prehybridization of 2 hours and hybridization of 16 hours at 65ºC in a single aqueous prehybridization/hybridization (APH) buffer (5X SSC; 5X Denhardt's solution; 1% SDS (w/v); 20µg/ml denatured salmon sperm DNA). Ten nanograms of denatured labeled probe was added per milliliter of solution just before hybridization. Following hybridization, membranes were rinsed twice with 2X SSC, washed once for 15 minutes with 2X SSC / 0.1% SDS and once for 15 minutes with 1X SSC / 0.1% SDS at room temperature. The result is shown in figure 3.2.

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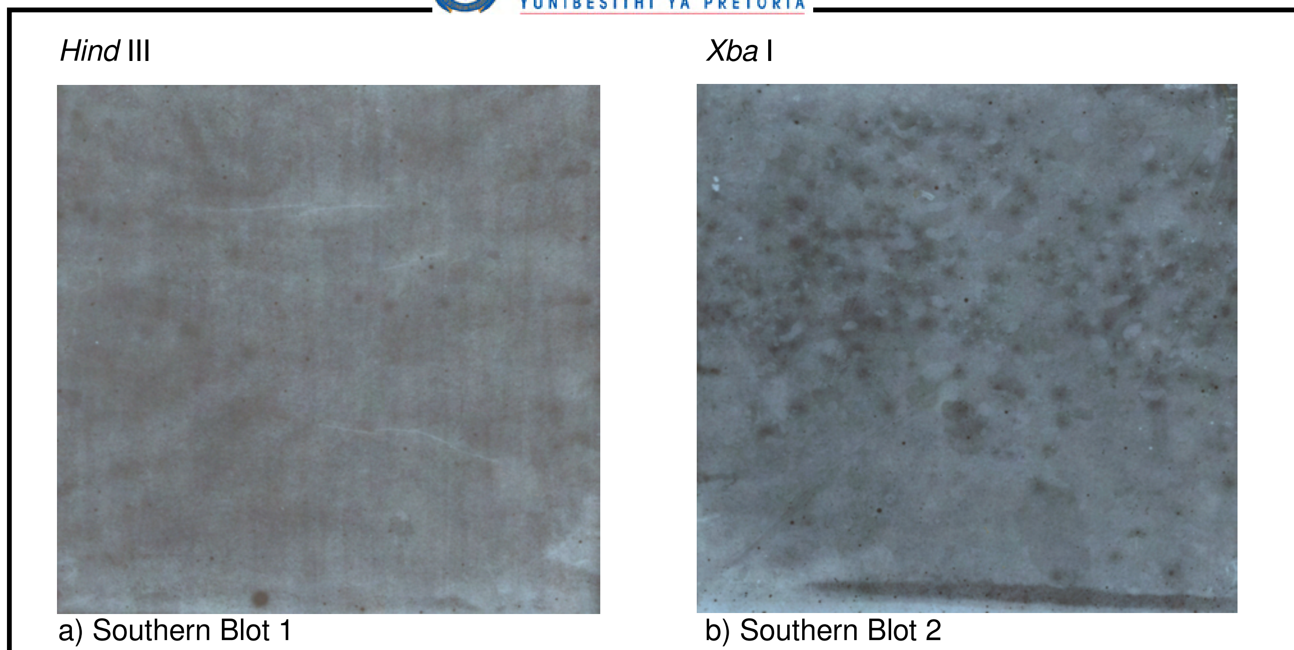


Figure 3.2: Southern Blot 1 (*Hind* III) and Southern Blot 2 (*Xba* I) hybridized with probe mix 2.
3 night exposure

The conditions utilized did not allow for the probes to bind to the digested genomic DNA. Various factors could be responsible for this absence of signal, i.e. hybridization rate was too low; the stringency of the hybridization as well as the washes may have been too high. To address this, the stringency was reduced by reducing the pre/hybridization temperature to 42ºC and reducing the stringency of the washes. In order to increase the rate of hybridization a buffer was chosen which includes a hybridization accelerator. Two separate buffers were used for prehybridization (50% formamide; 5X SSC; 10X Denhardt's; 0.5% SDS; 20μg/ml denatured salmon sperm DNA) and hybridization (50% formamide; 5X SSC; 1X Denhardt's; 0.5% SDS; 5% Dextran Sulphate). The membranes were washed twice for five minutes in 2X SSC/0.1% SDS at 65ºC (figure 3.3).

These alterations resulted in hybridization of the probes at the cost of a low signal to noise ratio. Interestingly, the noise is particularly high at the top portion on the membrane. Four bands are clearly visible at the bottom of the gel. Size separation of the digested DNA on an agarose gel together with a molecular weight marker showed that these fragments range from 850bp to 2.5kb. In fact the dark bands (indicated * in figure 3.3) were visible on the UV illuminated ethidium-bromide stained agarose gel following electrophoresis. The bands that should be seen here are 18 485, 3 465 and 2 231bp in size. The top most fragment may actually represent the 2.2kb fragment. When this analysis was completed by Unger *et al.* (2000), only the expected bands were visualized. It should however be noted that they only illustrated membranes hybridized with probe mix 1 and 3. The occurrence of four fragments (only 3 should be

visualized) and the high background indicates that the probes are binding non-specifically to the target DNA. This could be caused by factors such as too high probe concentration, inadequate prehybridization, insufficient blocking during detection or inadequate DNA transfer.

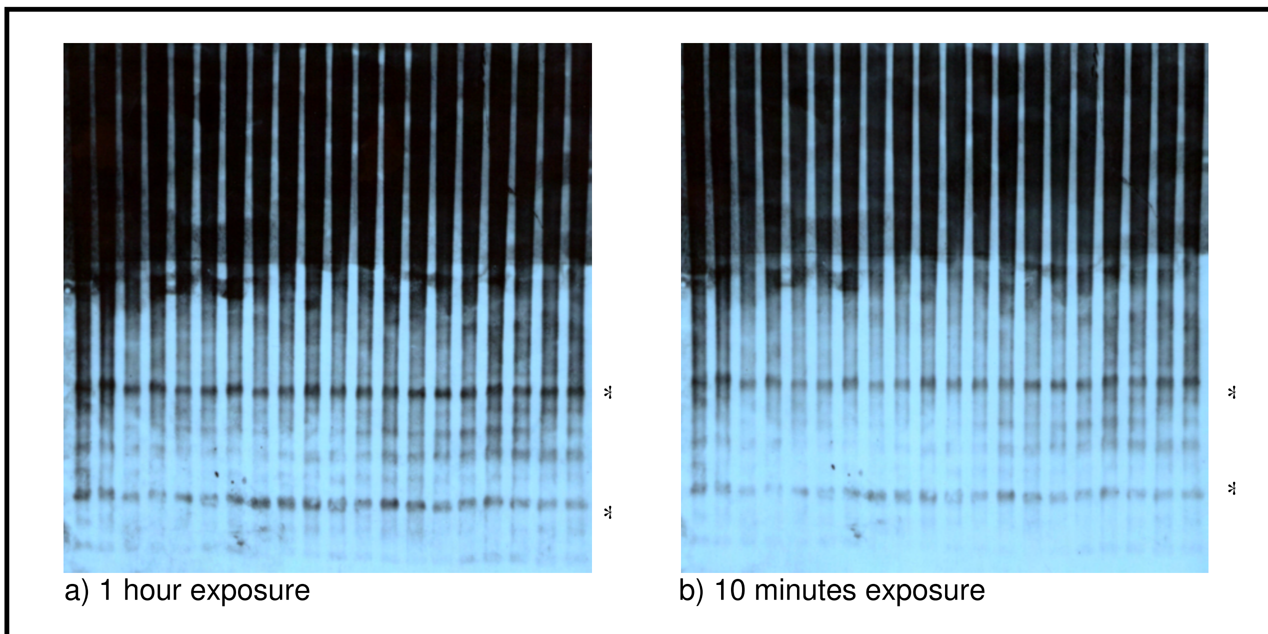


Figure 3.3: Southern Blot 3 (*Xba* I) hybridized with probes 6, 7 and 8.

In order to reduce the amount of background, probe concentration was reduced to 5ng/ml hybridization solution so that the probes would have to compete with the blocking agents for DNA binding sites. Stringency washes were increased to remove probes non-specifically bound to the membrane by washing twice for 10 minutes with 2X SSC/0.1% SDS and once for 10 minutes with 1X SSC/0.1% SDS. These washes were completed by heating the wash buffer to 65ºC, placing the membrane and heated buffer in a closed container, and shaking at room temperature. This did not lead to an increase in signal to noise ratio. The hybridization was repeated and the same washes carried out using the hybridization oven heated to 65ºC. Again there was no visible reduction in background. During this time it was noticed that the background was always more prevalent either at the top or on one vertical side of the membrane. It was considered that this may be due to the use of hybridization bottles with a small diameter, so that during hybridization the membranes overlapped on themselves, either along their widths or lengths. In order to determine whether this could be the cause, a membrane previously displaying high background on the left hand side was stripped and re-hybridized in the Bachoffer hybridization oven (bottle diameter: 7.3cm) as opposed to the Techne HB-1D apparatus (bottle diameter: 3.3cm). After probe stripping, the detection protocol was repeated to ensure that all

probes were removed. The stringency of the washes were increased to washing twice for 15 minutes with 0.5X SSC/0.1%SDS and once with 0.1XSSC/0.1%SDS for 15 minutes. These washes were carried out in a shaking water bath heated to 65ºC (figure 3.4). The shaking water bath was used for the remaining Southern blot analyses.

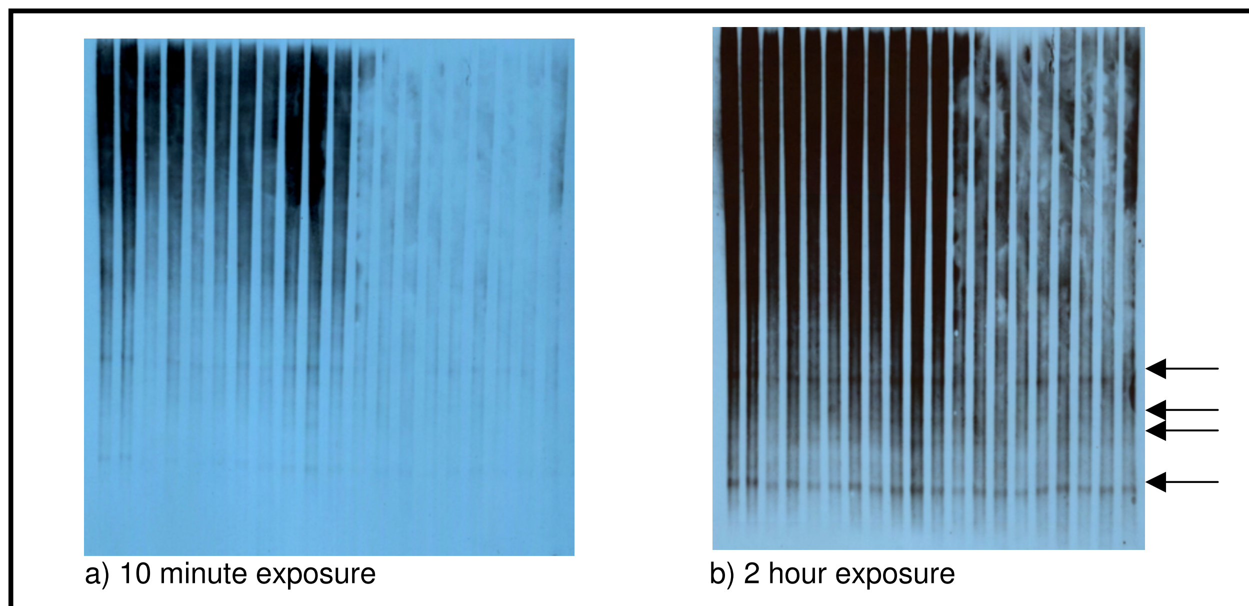


Figure 3.4: Southern Blot 11(*Xba* I) hybridized with probes 6, 7 and 8.
Arrows indicate bands visible on autoradiograph.

The background is clearly worse on the left hand side of the membrane. Since this membrane previously showed higher background on this side, and the membrane did not overlap during this hybridization, and membrane stripping was determined successful, the uneven background could be attributed to uneven DNA transfer. Nevertheless, the signal to noise ratio remained high even though the stringency of the washes was increased.

The bands expected from this analysis are 18 485, 3 465 and 2 237bp in size. There are again clearly 4 bands present on gel b) (indicated with arrows), as was seen in figure 3.3. Again, the two darker fragments could be seen on the agarose gel (as with Southern Blot 3, figure 3.3). The top-most band could possibly represent the 2.2kb fragment, but the presence of the other 3 fragments indicates that the specificity of the analysis is compromised. Since probes can attach to nucleic acid binding sites present on the membrane surface (present to bind DNA during the procedure of blotting), reagents are included in the hybridization/prehybridization buffers to block these sites. Increase in the concentration of such reagents in hybridization buffers should therefore result in a reduction of the amount of background. Keller and Manak (1989) noted that although both Denhardt's and FPG are both blocking agents, FPG results in less background.

Their pre/hybridization solution was therefore used (50% de-ionized formamide, 5X SSC; 1X FPG; 25mM KH_2PO_4; 0.2% SDS, 25µg/µl salmon sperm DNA, 5% Dextran Sulphate). Additionally, the prehybridization time was increased to 3 hours and the hybridization time reduced to 13 hours. These alterations did not result in a reduction of background signal and the resultant autoradiograph in fact displayed more background than that of Southern Blot 11 (figure 3.4).

Since uneven DNA transfer was considered a causative factor in the occurrence of varied background, DNA samples were re-digested and electrophoresed with the DIG-labeled DNA Molecular weight Marker II (Roche). Special attention was then given to even weight distribution during the transfer process. Subsequent to DNA transfer, the gel was visualized under a UV light to ensure that all DNA had been transferred. For hybridization, the concentration of the blocking agents in the same pre/hybridization buffer was increased to 1.25X FPG and 40µg/ml denatured salmon sperm DNA. The blocking time during detection was also increased to 45 minutes as opposed to 30 minutes. Additionally, the stringency washes were again increased. These modifications now resulted in "consistent" background across the membrane.

The membrane was stripped and the concentration of the FPG and salmon sperm DNA in the buffer further increased to 5X and 100µg/ml respectively. The concentration of the hybridization accelerator (Dextran Sulphate) was reduced from 5 to 2.5%. Since it was clear that blocking was insufficient, prehybridization time was increased to 16 hours and hybridization time further reduced to 4 hours, to limit the time during which probe could non-specifically bind to the membrane. Following hybridization, the membrane was exposed to the blocking solution for 1 hour and to further reduce signal intensity, the concentration of the antibody in the detection step was decreased from 7.5µl to 5µl (of a 75mU/ml solution) per 100 µl of blocking solution (figure 3.5). It is clear that the sample in lane 9 was not completely digested. This was also visible on the ethidium bromide stained agarose gel.

These modifications seemed to further decrease the signal: noise ratio. The amount of background is so high that the appropriate bands cannot be visualized. Interestingly, there is no background in lane 1, which contains the Digoxigenin-labeled DNA Molecular weight Marker. This could perhaps mean that the quantity of genomic DNA on the membrane is too high. Nevertheless, it is well known that the major disadvantage of using nylon membranes is the occurrence of high background signals. This is particularly bad when non-radioactive probes are utilized (Brown, 1993). The analysis by Unger et al. (2000) was completed using radioisotopic

labeled probes, as opposed to DIG-labeled probes and chemiluminescent detection. This could explain why, in this study the analysis was unsuccessful.

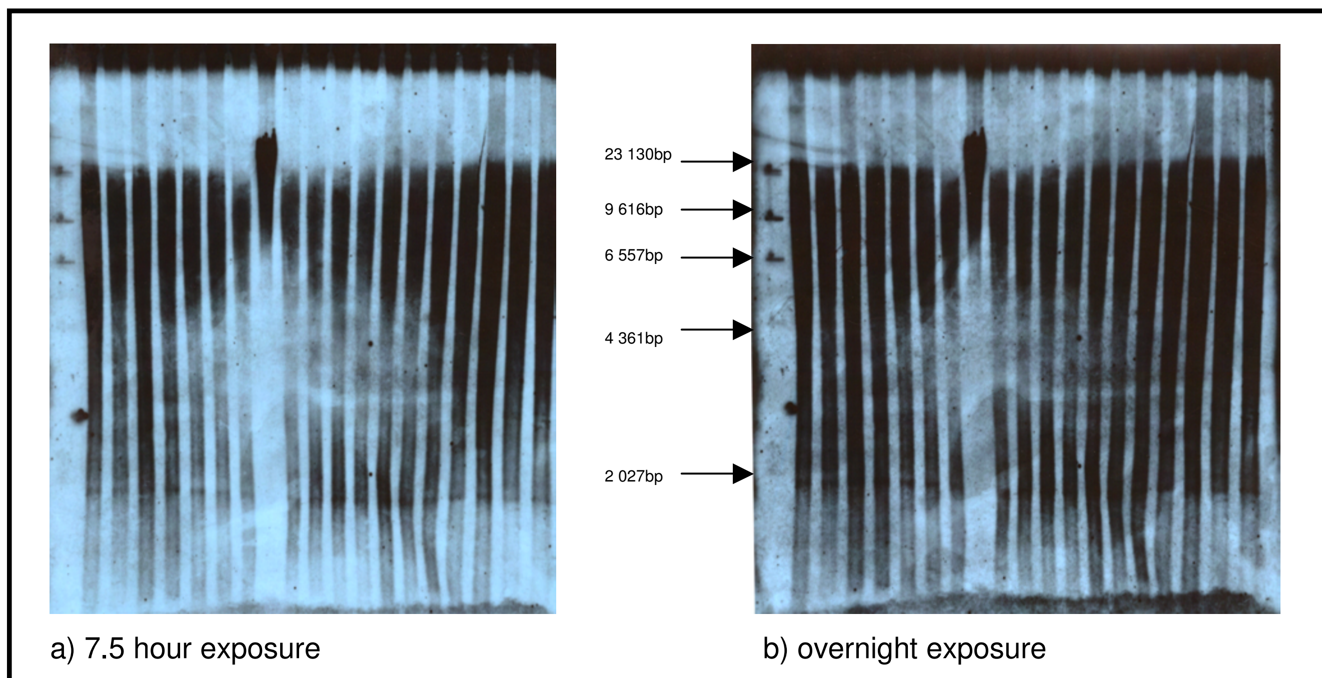


Figure 3.5: **Southern Blot 15 (*Hind* III) hybridized with probe mix 2.**

At this time the MLPA method was developed (Schouten *et al*., 2002). This method was deemed more effective for the detection of large deletions/duplications in *BRCA1*. Southern blotting was therefore abandoned and MLPA utilized.

3.2 MLPA DATA ANALYSIS

The 74 breast / ovarian cancer samples were analyzed using the *BRCA1* kit produced by MRC-Holland, SALSA MLPA KIT P002 *BRCA1*, nine of these samples were reanalyzed with the P087 kit in addition to a sample that was newly acquired at this time. Both probe mixes include a probe for each exon of the gene (2 probes for exon 11 because of its size), nine additional control probes for other human genes situated on different chromosomes in addition to the MLPA control mix. This control mix produces fragments which indicate whether ligation was successful, and whether sufficient quantities of DNA were included in each sample.

3.2.1 DETERMINATION OF ANALYSIS SUCCESS

In each run, an Afrikaner unaffected control, water and a deletion positive control were analyzed. Following analysis on the ABI-3100 or ABI-3130, peak areas were imported into either Genotyper®3.7 or GeneMapper 3.0™ (ABI PRISM®) for fragment analysis. This was done to visualize the control mix fragments (64-94bp) as well as the MLPA profile of each sample (127-454bp). Both these profiles should be inspected to determine whether the analysis was successful.

a) Reviewing the MLPA control mix fragments

The control mix generates five control fragments. The chromosome 2q14 specific MLPA control mix probe produces an amplification product of 94bp that is ligation-dependent (figure 3.6). If ligation is successful, this peak will have a similar area to that of the other MLPA amplification products. The remaining four amplification products generated by the MLPA control mix are 64, 70, 76 and 82bp in size, are ligation-independent, but DNA concentration – dependent.

The peak areas of these fragments should be small when sufficient DNA (50ng or more) has been utilized, and will be hardly visible when 100ng or more sample DNA is analyzed. Should these peaks have a peak area similar to that of the 94bp ligation-dependent peak (figure 3.6), the amount of DNA included is inadequate for reliable analysis. This may result in consistently low signals for all probes, since the 33 PCR cycles will probably not be sufficient to produce an adequate amount of amplicon.

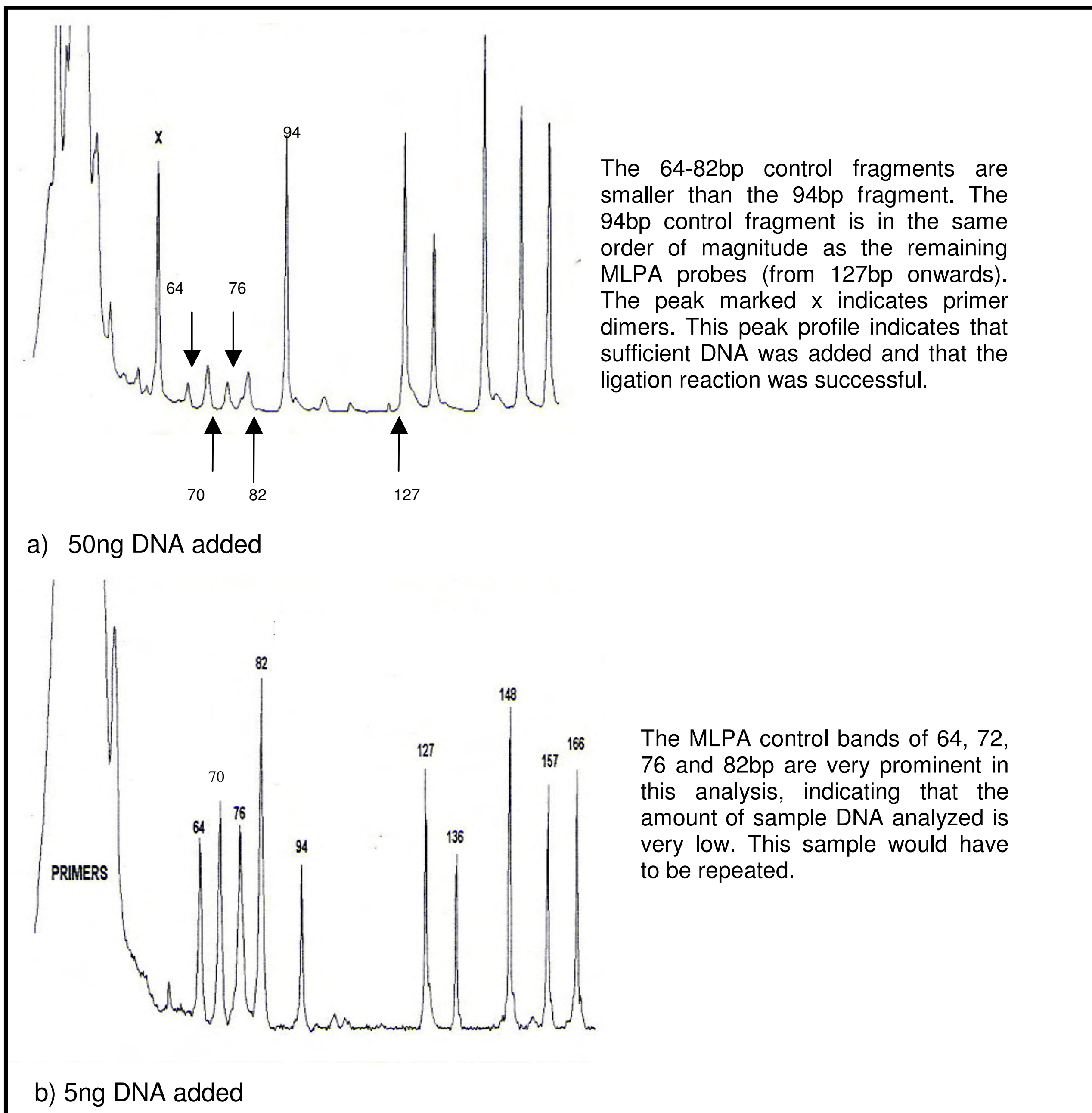


Figure 3.6: Partial MLPA profiles to illustrate the effect of DNA concentration on the MLPA control mix fragments (64 – 94bp).
Numbers above and below peaks indicate peak size (bp)

Should any of the five control mix fragments have uncharacteristic sizes, analysis of that sample should be repeated. Apart from the control mix fragments, the MLPA fragments (127bp and greater) can also be utilized to determine whether analysis of the sample was successful.

b) Reviewing MLPA profiles

In an MLPA profile, the peak area of each fragment is not equal. This is because the efficiency of amplification is different for each probe. The PCR efficiency is affected by polymerase and KCl concentration within the reaction, as well as the identity of the first nucleotide following the forward PCR primer. The fact that the PCR efficiency is affected by polymerase activity is rather interesting. One would expect that changes in polymerase concentration would affect amplification of all probes within a reaction uniformly. Nevertheless, Schouten *et al*. (2002) demonstrated that when 2.5 times less polymerase was utilized, 5-10% of the probes showed a reduction in relative peak area greater than 25%. The authors also determined that when an adenine residue followed the forward PCR primer sequence, average signal strength decreased more than two-fold. Signal strength decreased in the order C > G > T > A (Schouten *et al*., 2002).

Although relative signal strengths differ between probes, a certain probe will have a stronger / weaker signal strength when compared to others in all reactions in a single analysis (if polymerase and KCl concentration is equal between reactions).

The MLPA profile generally exhibits a slope (figure 3.7), where the smaller fragments (bp) exhibit larger peak areas while the larger fragments exhibit smaller areas. This slope is explained in terms of the PCR reaction. Since a single primer pair is utilized to generate all fragments, annealing time should be more or less the same. However, the time taken to generate larger fragments will be longer since more nucleotides must be incorporated. Shorter fragments will be produced more rapidly, resulting in more copies of the smaller fragments, and fewer of the larger fragments.

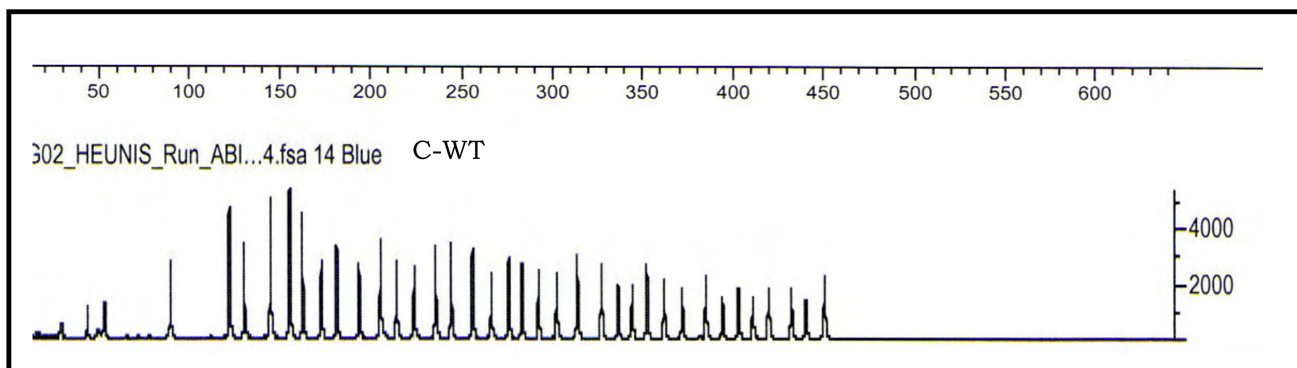


Figure 3.7: MLPA profile of wild-type control sample.
The smaller fragments in general have a larger peak area than the larger fragments, giving the MLPA profile a “sloped” appearance.

The MLPA profile may also be utilized to determine whether a particular analysis was successful. In figure 3.8, examples of aberrant MLPA profiles are illustrated.

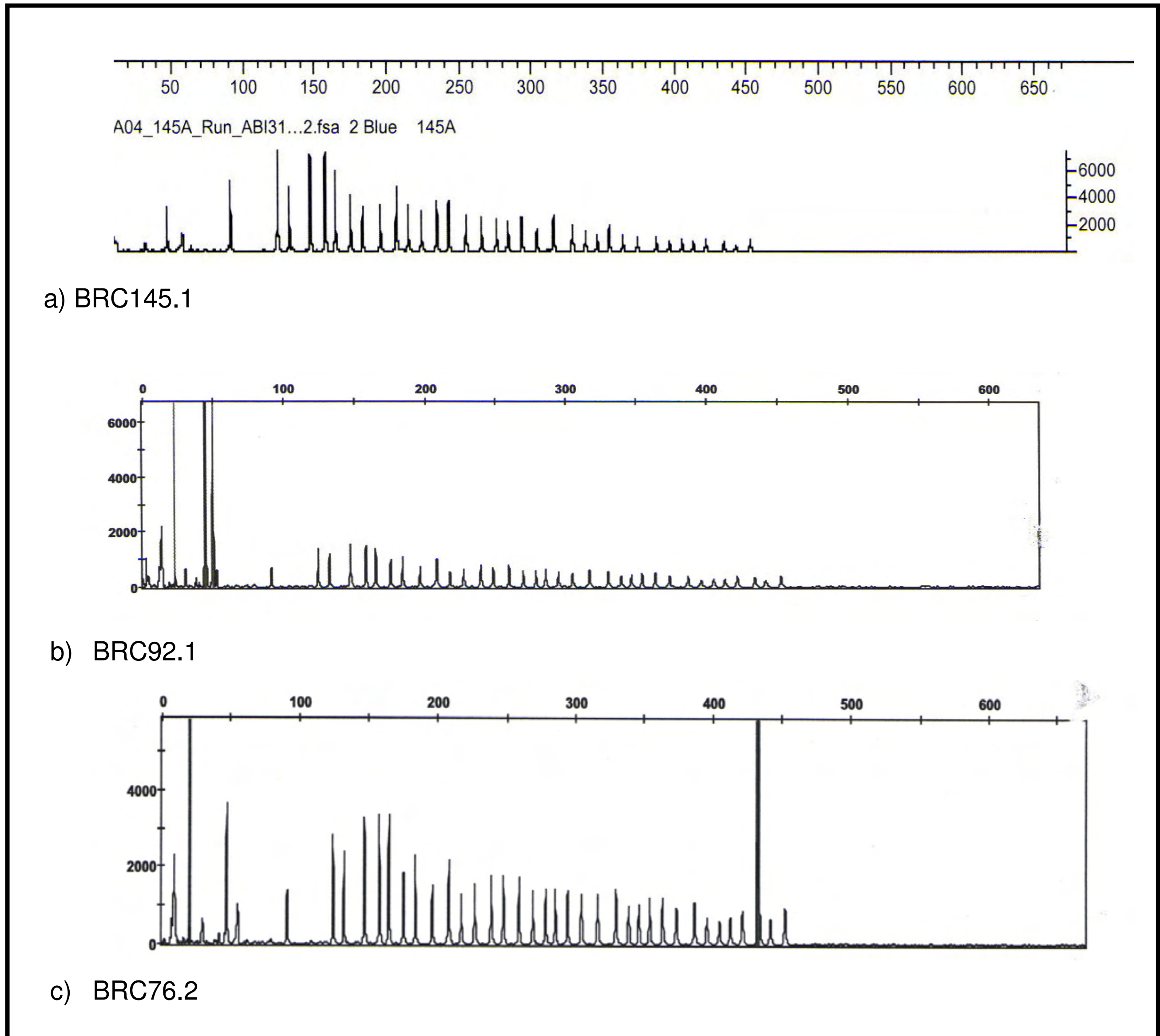


Figure 3.8: MLPA peak profiles of samples that displayed aberrant profiles.

Although the control mix peak areas (64-94bp) of BRC145.1 are the appropriate sizes (figure 3.8a), there is a distinct reduction in peak area as the MLPA fragments become larger (bp), i.e. the peaks slope dramatically. Sloped profiles are normal, as is seen in figure 3.7, but the decline is generally not as dramatic as is seen in figure 3.8a). The severe slope in figure 3.8a) is most likely caused by inadequate PCR reaction, and the analysis was repeated.

An exceptionally small 94bp ligation-dependent peak occurred for sample BRC92.1 (figure 3.8b). This peak should be similar in size to the MLPA amplification products greater than 127bp. Although this 94bp peak is small, so are all the MLPA peaks. The problem in the analysis is therefore not that ligation was inadequate, but rather that the PCR amplification was insufficient. Nevertheless, since all peak areas are undersized, all normalized values following the Schouten statistical analysis will be less than one. If the Wallace method is however used, a standard deviation greater than 0.1 is generated, indicating that the analysis was of poor quality.

The third aberrant profile (figure 3.8c) has an exceptionally high 436bp peak. This peak represents the control probe on 11p13 (LMO2). There are therefore no abnormalities in the *BRCA1* peaks, nor in the initial 5 control peaks. Impurities were most likely present in this DNA sample. When the analysis was later repeated, this did not recur and all peaks were normal.

3.2.2 DETERMINATION OF THE PRESENCE OF A MUTATION

The presence of a large genomic rearrangement can be determined by either visual inspection or statistical analysis.

a) Visual Inspection

Since each fragment's peak area varies, identifying a duplication/deletion from a single MLPA profile is very difficult, but can be achieved when compared to an unaffected control. In figure 3.9 the MLPA profiles of an exon 13 deletion positive control and an unaffected control are shown.

For the mutation positive sample (figure 3.9a), it is clear that the exon 13 fragment (indicated with an arrow) is smaller than the adjacent fragments, but when compared to the rest of the fragments (in the same sample), it is larger than the 388bp (exon 20) and 445bp (control 3p21) peaks (indicated with *). Identification of deletions / insertions in this manner is therefore not reliable. This is due to the fact that all peaks do not have a standard area. Peak areas can however be utilized to draw dosage conclusions if they are compared to the peaks of unaffected control samples.

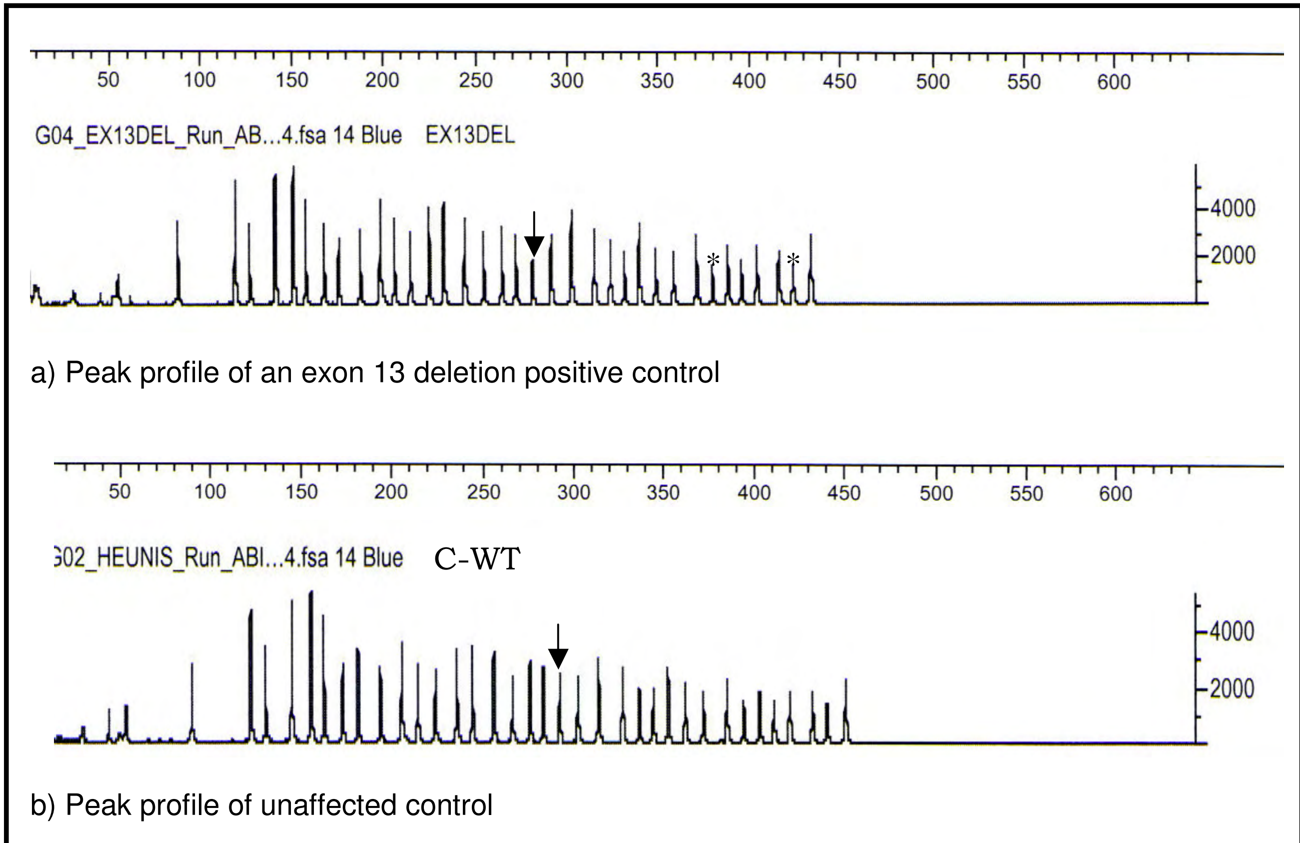


Figure 3.9: MLPA Peak Profiles of control samples
Exon 13 fragment is indicated with an arrow head, and fragments with a smaller area than the deleted fragment are indicated by *.

When comparing the peak areas of the positive control (figure 3.9a) to the unaffected control, it is apparent that there is a reduction in the peak area of exon 13 of the positive control. This sort of analysis is clearly not very reliable. It can however be completed by superimposing the patient's electrophoresis peak profile over that of a control. This is achieved by using two different colors for the control and patient samples with Genotyper®3.7 software (Applied Biosystems), and changing the size marker length by 2 nucleotides for the control sample. The superimposed profile of the exon 13 positive control and unaffected control is indicated in figure 3.10.

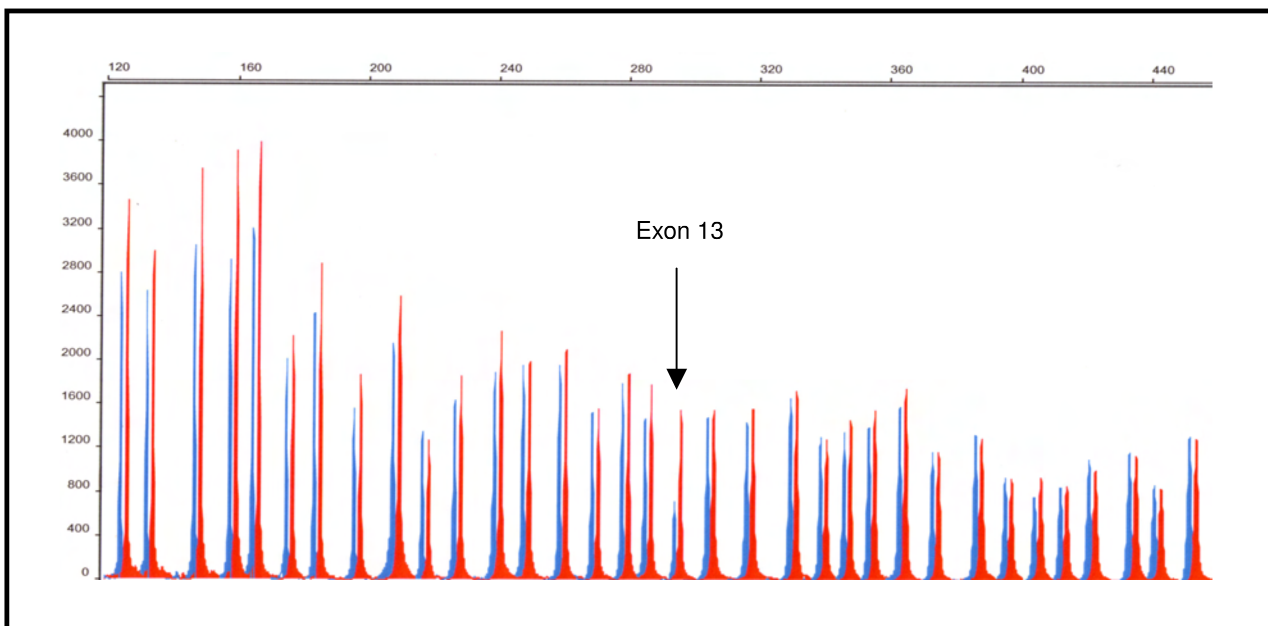


Figure 3.10: **Superimposed electrophoresis profile of an unaffected (red) and mutation positive control (blue).**

With this analysis, there is a marked reduction in peak area of exon 13 (indicated with an arrow head) for the mutation positive control compared to the unaffected control. Although use of this method clearly indicates a decrease in copy number it is far less reliable than statistical analysis, since with this visual analysis each sample is compared to only a single unaffected control. This analysis should therefore be confirmed by statistical calculation of copy number. This visual inspection is also far more time consuming when analyzing a large number of samples.

b) <u>Statistical analysis of MLPA results</u>

Dosage quotients (DQ) or exon copy number can be calculated in various ways. A number of Excel MACROS have been developed, many of which can be down-loaded from the MRC-Holland website (www.mrc-holland.com). In this study, one of two methods was used, either the Schouten or Wallace method. One of the problems with dosage analysis is its continuous variability due to its quantitative nature, while in diagnostics, a “binary” answer, (in other words, the patient sample is either normal or not) is required. Dosage data must therefore be analyzed in such a way so as to supply unambiguous yes / no answers. The problem with dosage analysis is made more complex by an increasing number of analyses in newer tests. When this occurs together with the use of arbitrary cut-offs, variability between loci is not taken into account, and if a standard statistical measure of significance for each tested exon is used, the likelihood for the occurrence of a type I error is increased. Furthermore, there exists a great amount of variability between experiments, which essentially means that analysis of different experiments must be

done separately. These factors were discussed by Andrew Wallace from the National Genetics Reference Laboratory in Manchester, at the CGMS in 2004 (lecture available at http://www.ngrl.org.uk/Manchester/Pages/mutationsload.htm).

With copy number analysis, a dosage quotient (DQ) of 1.0 is expected for normal sequences and the occurrence of a deletion or duplication will result in a DQ of 0.5 and 1.5 respectively (Schouten et al., 2002). Analysis of a series of control samples by Bunyan *et al.* (2004) revealed that normal sequences resulted in a mean DQ of 1.04 (range 0.79 - 1.27, standard deviation=0.06), duplicated sequences resulted in a mean DQ of 1.60 (range 1.32 - 1.73, standard deviation=0.06) while a deleted sequence gave a mean DQ of 0.5 (range 0.34 - 0.67, standard deviation=0.07). Samples could therefore be considered acceptable if the DQs fall within 0.8-1.2 (Bunyan *et al*., 2004). If peaks have values greater than 1.3 or less than 0.7 a duplication or deletion should be scored. Samples that display intermediate values should be repeated (Bunyan *et al*., 2004). These results were used in this study for the analysis of the generated DQs.

Schouten analysis

The Schouten method as described in Chapter 2 is recommended and used at MRC-Holland, where the kit is produced. Briefly, relative peak areas are compared to a single unaffected control to produce fragment DQs. This is dangerous since a comparison is made to a single control that may in fact, although unlikely, contain an aberration or have undergone a faulty analysis. This can however be checked by comparing relative peak area of each probe to the mean relative peak area of all samples in the analysis. This could however have negative implications should there be a founder rearrangement in the sample group. The average relative peak area will then automatically be increased or decreased by a founder duplication or deletion.

Although the Schouten method is recommended by MRC-Holland, it has a number of flaws, as with most dosage analyses, as described above. These discrepancies led to the development of the Wallace method of dosage quotient analysis which is far more robust and eliminates many of the previous major concerns. The Wallace method was developed at the National Genetics Reference Laboratory in Manchester and attaches objective and significant probabilities to dosage data.

Wallace analysis

This analysis still produces dosage quotients in the standard manner, while integrating two innovative features to assist with data interpretation. A likelihood probability of concordance with one of three hypotheses is constructed. These hypotheses include the occurrence of 1, 2 or 3 copies of a specific ligation site within the test sample. This is achieved by comparing the sample to five unaffected control samples. The t-statistic is subsequently utilized to calculate the probability of variation. The second innovative feature behaves as a control that determines the quality of each individual test. This is achieved by determining the standard deviation of the dosage quotients generated for each of the control ligation products. A standard deviation greater than 0.1 means that analysis of that particular sample was of poor quality. This feature means that a manual visualization of the control mix fragments is no longer required, drastically reducing the analysis time and increasing the reliability of the test.

At the start of this study, the Wallace analysis was not yet available and samples were therefore analyzed using the Schouten method. Once the Wallace method became available nine samples that needed to be repeated in addition to one sample that had been newly acquired were analyzed with this method. Samples were either repeated because of the occurrence of aberrant electrophoresis profiles or because certain probes displayed equivocal DQs (equivocal DQ defined as $0.8 > DQ > 0.7$; or $1.2 > DQ > 1.3$, as described above). The Wallace method could not be used to analyze the previous data, since five control samples were not included in a single MLPA analysis. The inclusion of 5 unaffected controls increases the reliability of the test, and the DQs of samples are calculated against the mean relative areas of these five controls, reducing the risk of error. This analysis was completed as described in Chapter 2, where peak areas of the MLPA fragments were exported from GeneMapper into the *BRCA1* MLPA regression “Raw data” spreadsheet. The MACRO then automatically calculates the DQs, odds ratios, deviation probabilities and internal quality control standard deviations, which are displayed in the “RESULTS” spreadsheet. The MACRO is specifically designed to assist with MLPA analysis with use of the kits from MRC-Holland, and has been created in Microsoft Excel 2000.

The results of the analysis are given in three key ways. 1) As dosage quotients for each ligation product against each control ligation product, 2) a mean DQ for each ligation product, and 3) as a likelihood probability and odds ratio for each ligation product. This probability is calculated for one of three hypotheses: normal dosage (2 copies), deleted dosage (1 copy) and duplicated dosage (3 copies). The program highlights any samples that are of poor quality (standard deviation > 0.1) as an aberrant result.

The results are presented in tabular format (figure 3.11), with the internal quality control standard deviation (Int QC Stand Dev) indicated on the left hand side, below the sample information (column B). This value is highlighted in green if analysis of that sample was of good quality (stdev < 0.1), and highlighted in red if the analysis was poor (stdev > 0.1). Samples that have standard deviations with a value less than 0.1 display no overlap between normal, deleted and duplicated ranges (GR Taylor personal communication to Andrew Wallace; presented at the CMGS in 2004). The DQ values are presented in the columns to the right of the sample information (columns D-AK, please note that not all columns are represented in figure 3.11). The DQ of each MLPA ligation product is calculated against each control ligation product, i.e. a DQ series is given for each ligation product, since each probe is compared to every control probe. The last row of each column represents the mean DQ for each ligation product which is used to generate the DQ histogram (figure 3.12).

DQs that lie within the normal range (0.85 - 1.15) have a white background. DQs that lie within the range 0.35-0.65 are shaded aqua, while equivocal or deleted DQs (0.65-0.85 and >1.15) have a cream background. Just below the DQ series, the two odds ratios for the alternative hypotheses (normal: deletion and normal: duplicated) are indicated. If the normal hypothesis is favored, then these two cells will have a green background. If the odds ratio is clearly in favor of the abnormal hypothesis (>20:1), the cells are highlighted in magenta. Equivocal/ vague results have a cream background. Below the odds ratios three rows of absolute probabilities are given (green). These are calculated by the t-statistic of the variation between the mean DQ of that specific ligation product and the expected DQ from 5 unaffected controls. These absolute probabilities are indicated for the normal, deleted and duplicated hypotheses. A probability of 60% for the normal hypothesis indicates that any other random wild-type sample would be expected to deviate by the same amount in 60% of the tests. Normal values have a white background, abnormal values a magenta background and equivocal values a cream background.

a)

B	C	D	E	F	G	H	I	J	K	L	M	Y	Z	AA	AB	AC	AD	AE
	NORMALISED DELETION CONTROL RESULTS																	
Lab No		C5q31	C6p21	C15q21	C2q14	C12p12	C4q26	C11p13	C12p13	C3p21	BRCA1Ex1A	BRCA1Ex12	BRCA1Ex13	BRCA1Ex14	BRCA1Ex15	BRCA1Ex16	BRCA1Ex17	BRCA1Ex18
Ex13Del	C5q31	1.00	1.04	0.95	0.95	0.97	1.00	0.97	0.99	1.03	1.00	0.97	0.48	0.98	1.02	0.99	1.00	1.02
Operator	C6p21	0.96	1.00	0.91	0.91	0.93	0.96	0.93	0.95	0.99	0.96	0.93	0.46	0.94	0.98	0.95	0.96	0.98
michelle	C15q21	1.05	1.09	1.00	1.00	1.02	1.05	1.02	1.04	1.08	1.05	1.02	0.50	1.03	1.07	1.04	1.05	1.07
Worksheet	C2q14	1.05	1.10	1.00	1.00	1.02	1.05	1.02	1.05	1.09	1.05	1.02	0.50	1.03	1.07	1.04	1.05	1.08
4	C12p12	1.03	1.08	0.98	0.98	1.00	1.03	1.00	1.03	1.07	1.03	1.00	0.49	1.01	1.05	1.02	1.03	1.05
Int QC Stand Dev	C4q26	1.00	1.05	0.96	0.95	0.97	1.00	0.98	1.00	1.04	1.00	0.97	0.48	0.98	1.02	0.99	1.00	1.03
0.033046663	C11p13	1.03	1.07	0.98	0.98	1.00	1.03	1.00	1.02	1.06	1.03	1.00	0.49	1.01	1.05	1.02	1.03	1.05
	C12p13	1.01	1.05	0.96	0.96	0.97	1.00	0.98	1.00	1.04	1.00	0.98	0.48	0.99	1.03	1.00	1.01	1.03
	C3p21	0.97	1.01	0.92	0.92	0.94	0.96	0.94	0.96	1.00	0.97	0.94	0.46	0.95	0.99	0.96	0.97	0.99
	MEAN	1.01	1.05	0.96	0.96	0.98	1.01	0.98	1.01	1.04	1.01	0.98	0.48	0.99	1.03	1.00	1.01	1.03
ODDS NORMAL	:DEL	583885:1	31058:1	4594:1	20547:1	36701:1	71684:1	22346:1	81552:1	65354:1	1134:1	8568:1	1:1973	11220:1	8937:1	13648:1	15331:1	5682:1
ODDS NORMAL	:DUP	6184:1	179:1	123:1	515:1	680:1	866:1	412:1	1012:1	426:1	22:1	172:1	3:1	192:1	86:1	201:1	189:1	56:1
PROB OF DEVIATION	NORMAL	62.4767%	22.1705%	58.1213%	34.3096%	62.4822%	87.8222%	70.4491%	91.3308%	21.8246%	95.0859%	75.2046%	0.0236%	97.5991%	66.2880%	99.8837%	86.0360%	68.0338%
PROB OF DEVIATION	DELETED	0.0001%	0.0007%	0.0127%	0.0017%	0.0017%	0.0012%	0.0032%	0.0011%	0.0003%	0.0839%	0.0088%	46.6449%	0.0078%	0.0074%	0.0073%	0.0056%	0.0120%
PROB OF DEVIATION	DUP	0.0101%	0.1236%	0.4741%	0.0666%	0.0918%	0.1014%	0.1709%	0.0903%	0.0512%	4.3355%	0.4376%	0.0081%	0.4569%	0.7695%	0.4961%	0.4545%	1.2076%

b)

B	C	D	E	F	G	H	I	J	K	L	M	Y	Z	AA	AB	AC	AD	AE
	TEST SAMPLE RESULTS																	
Lab No		C5q31	C6p21	C15q21	C2q14	C12p12	C4q26	C11p13	C12p13	C3p21	BRCA1Ex1A	BRCA1Ex12	BRCA1Ex13	BRCA1Ex14	BRCA1Ex15	BRCA1Ex16	BRCA1Ex17	BRCA1Ex18
BRC59.1	C5q31	1.00	0.99	0.94	0.92	0.94	0.94	0.99	0.96	1.00	0.98	0.94	0.89	0.98	1.07	0.93	1.04	1.02
Operator	C6p21	1.01	1.00	0.95	0.93	0.95	0.94	1.00	0.97	1.01	0.98	0.95	0.90	0.99	1.08	0.94	1.05	1.03
michelle	C15q21	1.06	1.06	1.00	0.98	1.00	0.99	1.05	1.02	1.06	1.04	1.00	0.95	1.04	1.14	0.99	1.11	1.08
Worksheet	C2q14	1.09	1.08	1.02	1.00	1.03	1.02	1.08	1.05	1.09	1.06	1.03	0.97	1.07	1.17	1.01	1.13	1.11
4	C12p12	1.06	1.05	1.00	0.97	1.00	0.99	1.05	1.02	1.06	1.03	1.00	0.95	1.04	1.14	0.98	1.10	1.08
Int QC Stand Dev	C4q26	1.07	1.06	1.01	0.98	1.01	1.00	1.06	1.03	1.07	1.05	1.01	0.96	1.05	1.15	0.99	1.11	1.09
0.032807499	C11p13	1.01	1.00	0.95	0.93	0.95	0.94	1.00	0.97	1.01	0.99	0.95	0.90	0.99	1.08	0.94	1.05	1.03
	C12p13	1.04	1.03	0.98	0.95	0.98	0.97	1.03	1.00	1.04	1.02	0.98	0.93	1.02	1.12	0.97	1.08	1.06
	C3p21	1.00	0.99	0.94	0.92	0.95	0.94	0.99	0.96	1.00	0.98	0.94	0.90	0.98	1.07	0.93	1.04	1.02
	MEAN	1.04	1.03	0.98	0.95	0.98	0.97	1.03	1.00	1.04	1.01	0.98	0.93	1.02	1.11	0.96	1.08	1.06
ODDS NORMAL	:DEL	181415:1	55932:1	6239:1	14942:1	36608:1	27826:1	26563:1	79482:1	83111:1	1136:1	7814:1	4731:1	12699:1	3553:1	5729:1	6864:1	4730:1
ODDS NORMAL	:DUP	1290:1	473:1	137:1	425:1	680:1	604:1	238:1	1103:1	610:1	21:1	166:1	200:1	150:1	10:1	148:1	30:1	33:1
PROB OF DEVIATION	NORMAL	16.0334%	47.7445%	70.9938%	26.7557%	62.3628%	46.2651%	57.9676%	94.2548%	29.3243%	91.7066%	70.7895%	15.3468%	81.8252%	14.7531%	56.7506%	23.2968%	47.2650%
PROB OF DEVIATION	DELETED	0.0001%	0.0009%	0.0114%	0.0018%	0.0017%	0.0017%	0.0022%	0.0012%	0.0004%	0.0807%	0.0091%	0.0032%	0.0064%	0.0042%	0.0099%	0.0034%	0.0100%
PROB OF DEVIATION	DUP	0.0124%	0.1010%	0.5167%	0.0629%	0.0918%	0.0766%	0.2435%	0.0854%	0.0481%	4.4687%	0.4261%	0.0766%	0.5456%	1.5094%	0.3828%	0.7841%	1.4539%

Figure 3.11: Layout of Wallace "RESULTS" spreadsheet

Only columns B-M and Y-AE are represented to supply an idea of what the "RESULTS" sheet looks like.

a) Normalized deletion control results
b) Test sample results

In figure 3.11, the results of an exon 13 deletion positive control and one test sample are shown. For the mutation positive sample (figure 3.11a), the DQ values for the exon 13 ligation products all have an aqua background (indicated within the blue circle), and range from 0.46-0.50. This is indicative of the deleted hypothesis (single copy of exon 13). The odds ratio for the normal: deleted hypothesis has a magenta background, where this sample has a one in 1 973 chance of being normal. The odds ratio for normal: duplicated is shaded in yellow, indicating that this is an equivocal result. Furthermore, the absolute probabilities indicate that a deletion has occurred in this exon. The histogram for this deletion control indicates the occurrence of the deletion (figure 3.12a), where the mean DQ of this exon is shown as less than 0.5.

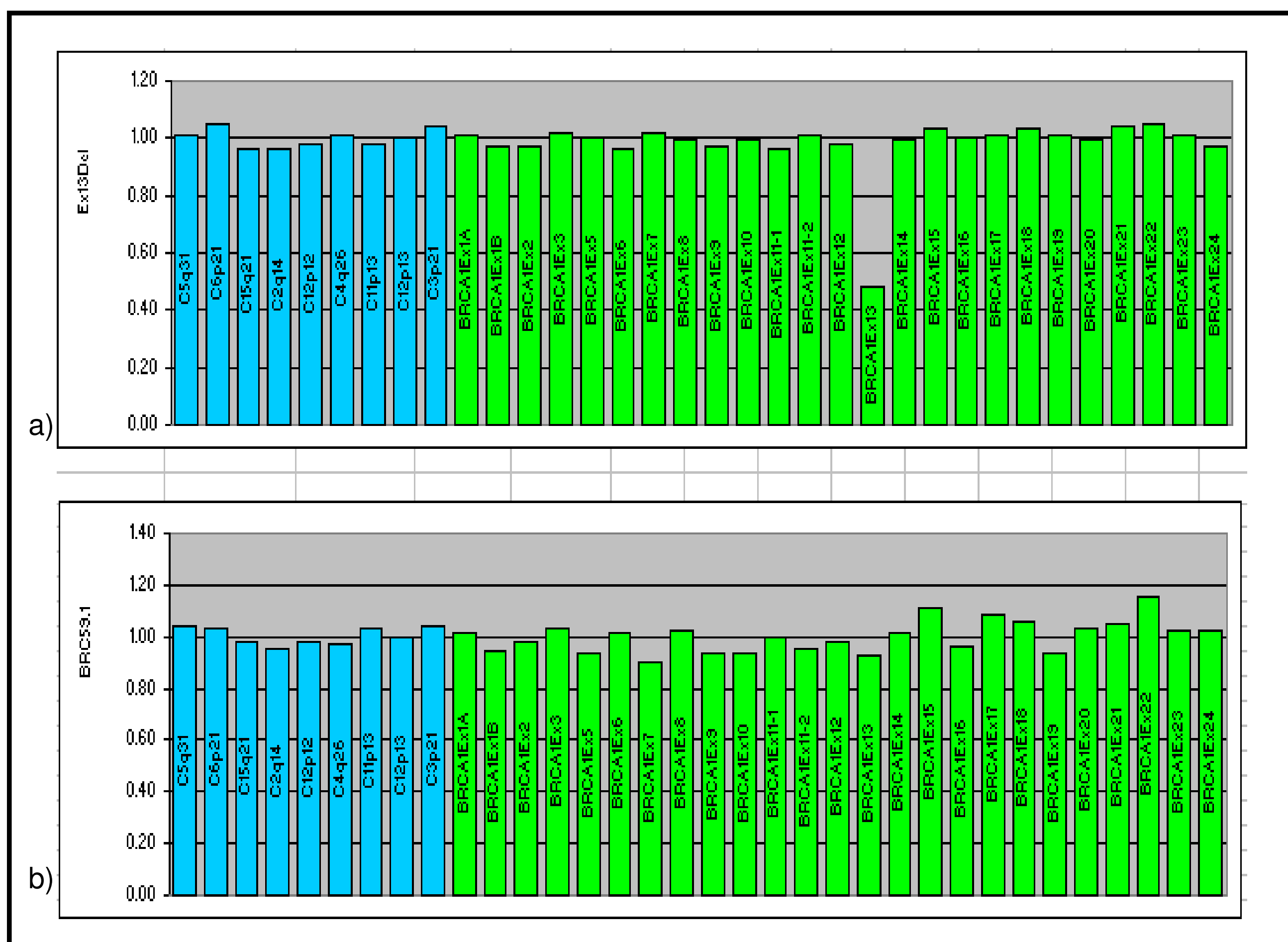


Figure 3.12: Histogram representation of Dosage Quotients

a) Exon 13 deletion control
b) Test sample BRC59.1

The test sample results (figure 3.11b), display DQ values which all fall within the normal range and have white backgrounds, save for a single DQ in the series for the exon 15 ligation product (circled in red). The value 1.17 is given as an ambiguous/duplicated result. Since no other equivocal DQs occur when this exon is compared to the remaining control probes, exon 15 can be regarded as having 2 copies. If however all DQs within this series were highlighted together with aberrant deviation probabilities it would be advisable to repeat the analysis. The histogram of the mean DQs for this sample is given in figure 3.12b.

Although this program indicates "normal" and "abnormal" results, it is essentially designed to supplement and not replace professional judgment. One should not simply look out for highlighted values, and discard equivocal values as being normal. In instances where this occurs it is imperative to review the results and determine whether the DQs fall within the normal ranges and identify reasons for any aberrant results obtained.

3.3 MLPA RESULTS

The DQs of the MLPA analysis for all samples included in this study are indicated in appendix D. Four samples from 3 families displayed aberrant copy number values or DQs. A reduction in copy number was detected for exon 18 for samples BRC119.1 and 119.2, exon 22 for sample BRC100.1, and exons 23-24 for sample BRC158.1.

3.3.1 CHARACTERIZATION OF THE SUSPECTED EXON 18 DELETION

Samples BRC119.1 and BRC119.2 presented with 1.59 and 1.72 fold reductions in relative copy number respectively (figure 3.13), indicating that this exon is deleted in these samples.

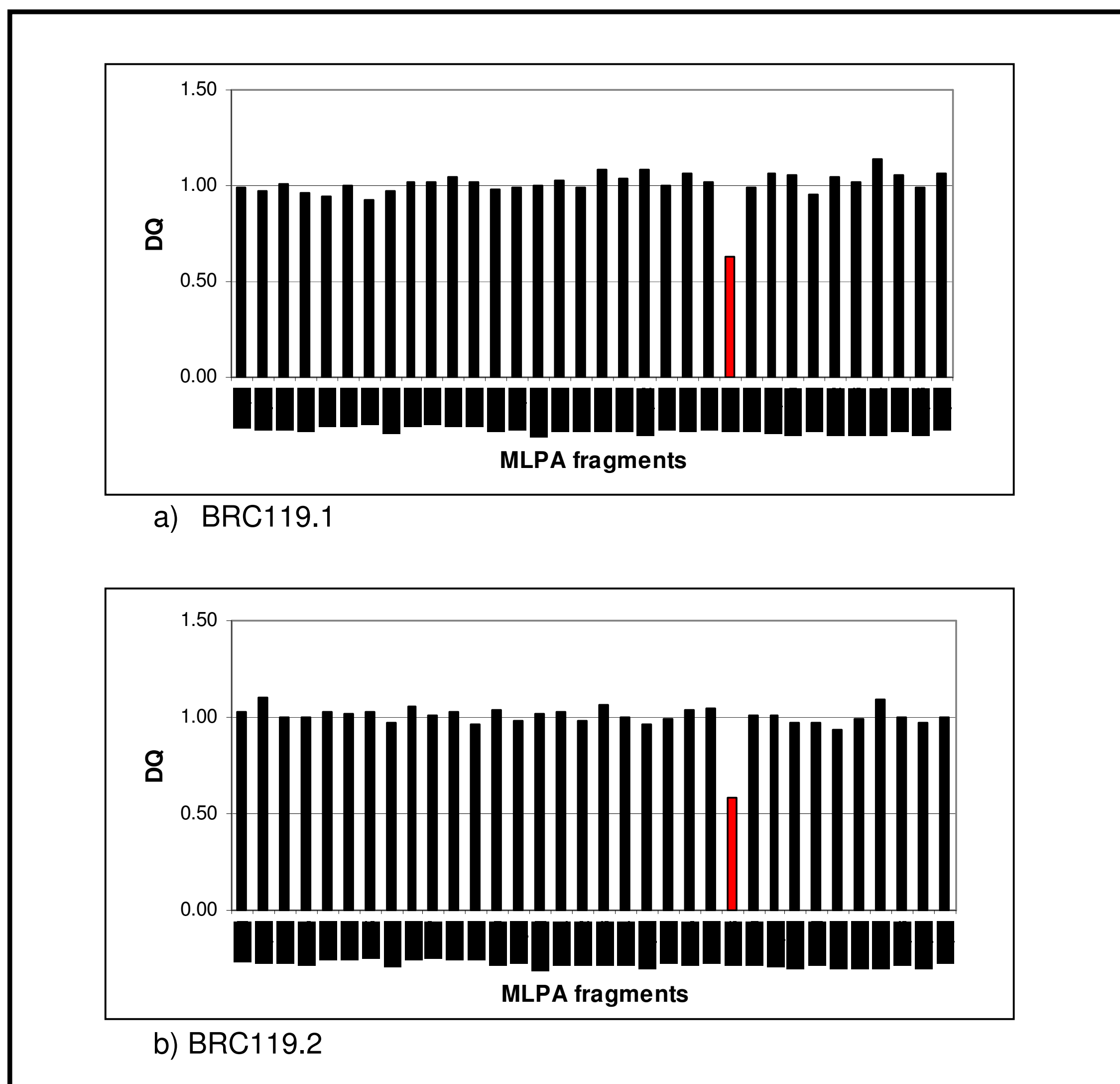


Figure 3.13: Histogram representation of MLPA fragment DQs for samples BRC119.1 and BRC119.2.

The family identified with this exon 18 abnormality is a South African Afrikaner family with no reported cases of breast cancer, only two cases of ovarian carcinoma (figure 3.14). The individuals presenting with ovarian cancer were diagnosed at relatively late ages, and are sisters. There is one individual who was diagnosed with colon cancer, and two of her sons died within their twenties from cancer, one who presented with leukemia, while the primary was unknown in the other son.

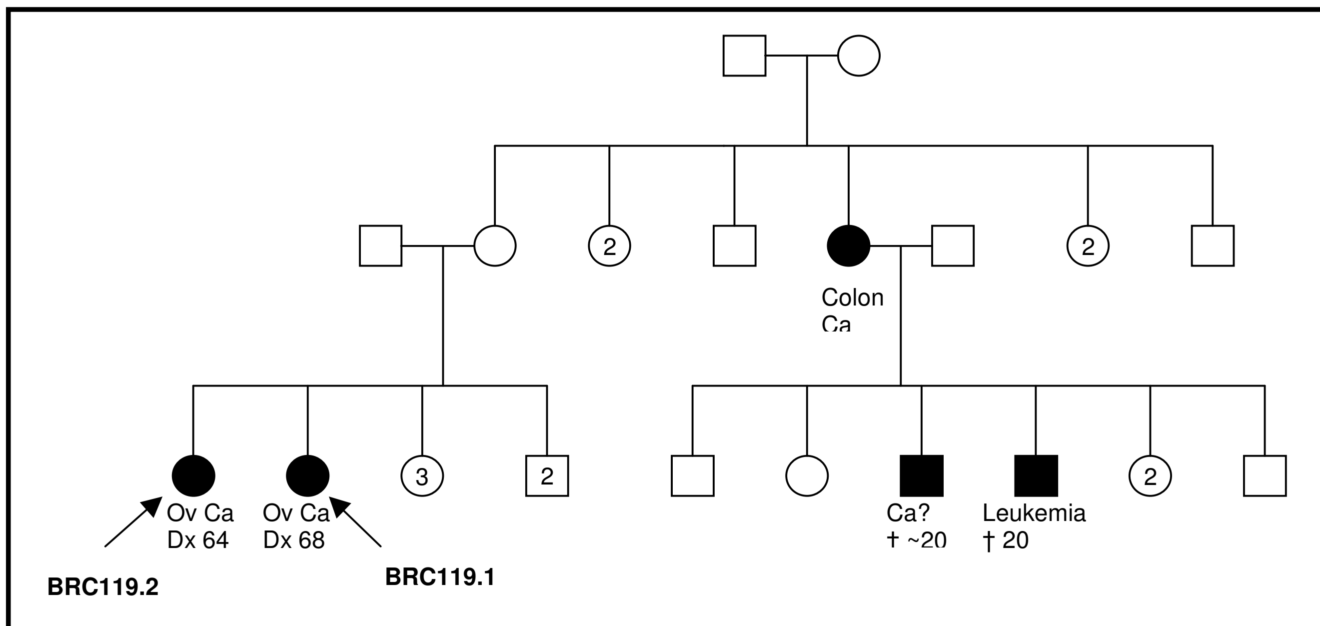


Figure 3.14: Pedigree of family BRC119.
Individuals affected with cancer are indicated by black blocks / circles. The index cases are indicated with arrow heads.

In order to characterize this mutation, two long-range PCR reactions were performed using primers located in introns 16 and 19, as well as 16 and 18 to produce amplification products of 4 516 and 3 979bp respectively (as described in Chapter 2). Five microlitres of the PCR product was electrophoresed on a 0.5% D5 Agarose gel (Hispanagar) at 50V for 4 hours (figure 3.15).

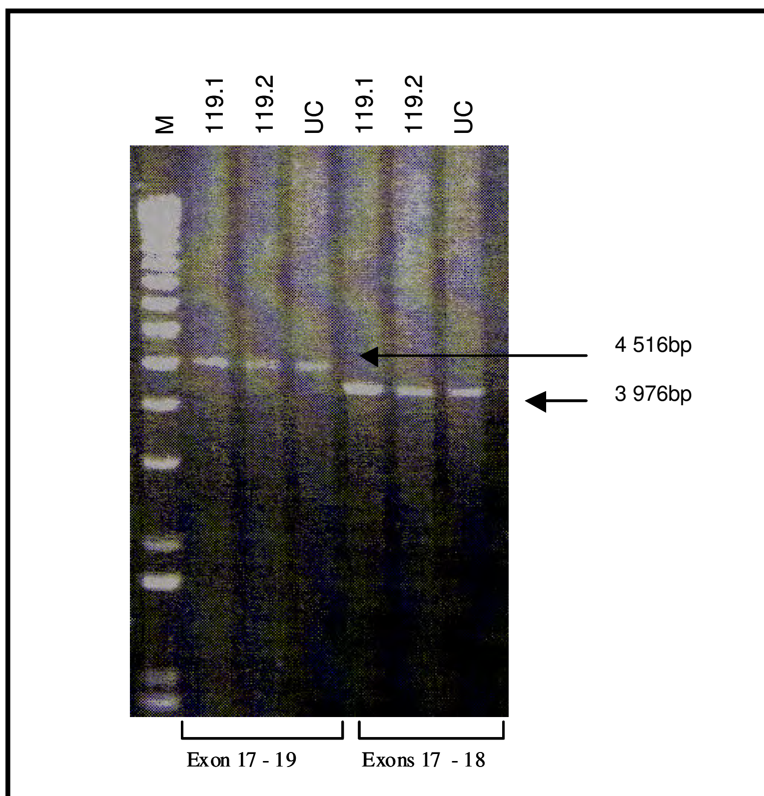
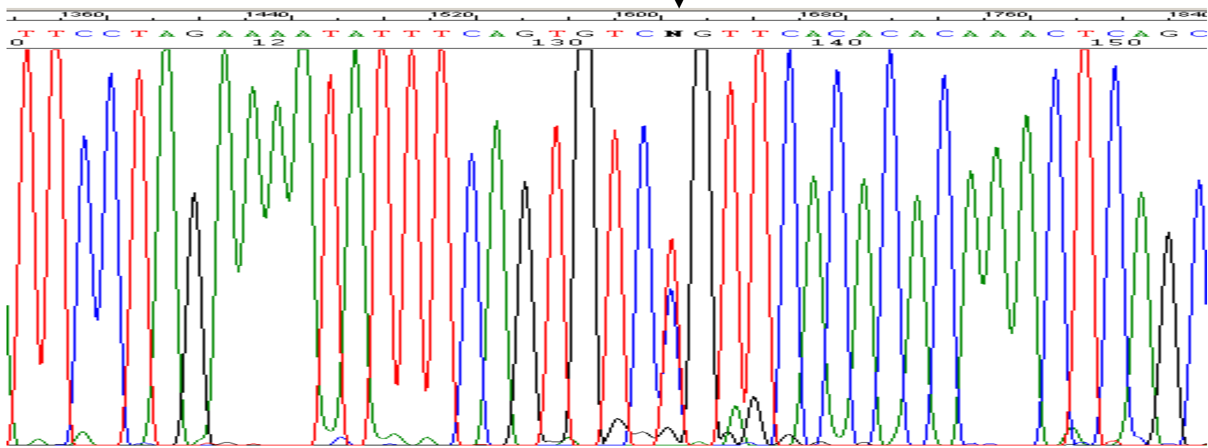
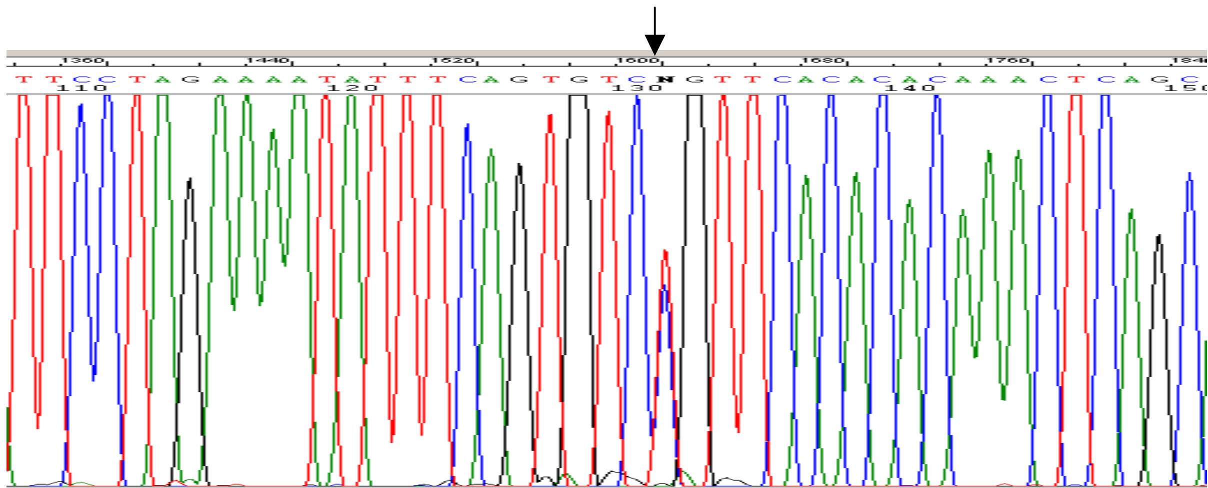


Figure 3.15: Agarose gel electrophoresis of long-range PCR products of BRC119.1, 119.2 and unaffected control.
M: 1kb Plus molecular weight marker (Invitrogen).
UC: Unaffected control
Exons amplified are indicated below the figure

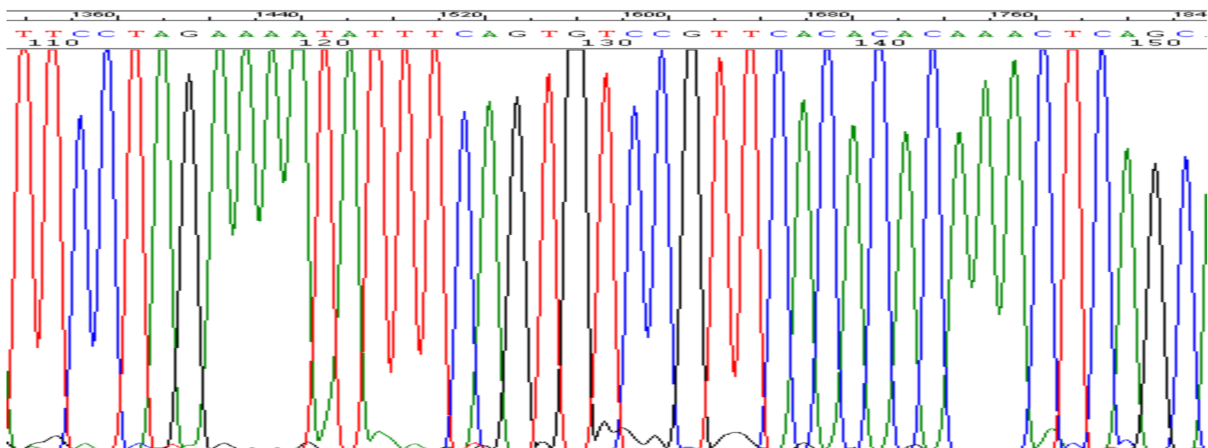
Since a reduction in copy number occurred only for exon 18 and primers were utilized which flank exons on either side of exon 18, the deletion fragment should have been amplified. Since no deletion fragment was produced (figure 3.15), it was suspected that the reduced DQ may occur as a result of a mutation at either the hybridization site of one of the two oligos, or at the ligation site, thereby abrogating either hybridization or ligation of the two oligonucleotides. In both these cases the complete probe would not be formed therefore not be amplified, causing a reduced DQ. Exon 18 of BRC119.1, BRC119.2 and an unaffected Afrikaner control were subsequently amplified and the anti-sense strand sequenced (figure 3.16), to determine whether either of these factors could be the cause for the reduced dosage quotient value.



a) BRC119.1.



b) BRC119.2.



c) Unaffected control

Figure 3.16: Electropherograms of the anti-sense strand of exon 18 of samples BRC119.1, BRC119.2 and control.
The heterozygous C→T transition at c.5215 is indicated by arrow heads.

From the antisense strand sequence of BRC119.1 and 119.2, it is clear that a C→T nucleotide change (indicated with arrow heads), which is absent in the wild-type sequence, has occurred. This G→A transition (sense strand) at nucleotide c.5215 results in the occurrence of a mismatch at the 5' end of the long oligo, 2bp away from the ligation site. This single base alteration prevents complete hybridization of the long oligonucleotide and therefore ligation of the short and long oligos. The probe can therefore not be amplified and the dosage quotient is reduced.

The sequence of exon 18 and surrounding intronic regions is shown in figure 3.17.

```
64681 atcttgggag tgtaaaaaac tgaggctctt tagcttctta ggacagcact tcctgatttt
64741 gttttcaact tctaatcctt tgagtgtttt tcattctgca gATGCTGAGT TTGTGTGTGA
64801 ACGGACACTG AAATATTTTC TAGGAATTGC GGGAGGAAAA TGGGTAGTTA GCTATTTCTg
64861 taagtataat actatttctc ccctcctccc tttaacacct cagaattgca tttttacacc
64921 taacgtttaa cacctaaggt ttttgctgat gctgagtctg agttaccaaa aggtctttaa
```

Figure 3.17: Genomic sequence of *BRCA1* exon 18 and the flanking intron sequences. The exon sequence is indicated in uppercase and the intronic regions in lower case. The short MLPA oligo is highlighted in yellow and the long MLPA oligo is highlighted in green. The changed nucleotide (G→A) is bold and underlined.

The c.5215G→A transition results in the non-conservative substitution of an Arginine with a Glycine residue at codon 1699 (R1699Q). This basic to polar uncharged amino acid change has been reported twice previously to the BIC. This variant falls within the BRCT domain (composed of two BRCT repeats) at the C-terminal end of the protein, specifically within the first BRCT repeat, amino acids 1649 – 1736 (Vallon-Christersson *et al*., 2001). The BRCT domain of the protein is associated with a large number of BRCA1's functions, since BRCA1 associates with a variety of proteins through this region. In particular, the BRCT repeats are known to play an important role in transcription activation (reviewed in Mirkovic *et al*., 2004). The functional significance of the BRCT repeats is emphasized by the high degree of sequence conservation within this region among Xenopus, mammalian as well as avian BRCA1 homologues (reviewed in Williams *et al*., 2003). The amino acid at position 1699 is a conserved Arginine and is known to participate in a salt bridge between the BRCT repeats (WIliams *et al*., 2001).

The R1699Q variant was first detected in a single affected Swedish individual in 1997, out of a test group of more than 450 index cases from affected families (Hakansson *et al*., 1997). It was

then determined that this variant was not present in a set of 50 healthy Swedish control samples (100 alleles). Disease association of this variant is however complicated by the fact that the individual positive for this mutation had no family history of breast or ovarian cancer, although she was diagnosed with breast cancer at the age of 39. A personal communication by T.S Frank informed Vallon-Christersson and co-workers that they had found this same variant in an unaffected individual whose mother had been diagnosed with premenopausal breast cancer and whose grandmother had been diagnosed with ovarian cancer at the approximate age of 60 years. The grandmother's mutation status was unknown and the mother was considered to be an obligate carrier of this particular variant. The inconclusive functional and family data of this mutant, together with another variant in the same codon (R1699W), led researchers to examine their effect on protein functionality.

Vallon-Christersson *et al.* (2001) utilized a transcription activation assay to determine the effect of unique variants found in Scandinavian breast / ovarian cancer families. Following expression of these variants in three *Saccharomyces cerevisiae* strains, the authors found that R1699Q as well as R1699W had transcription activity rates equal to that of the wild-type. These results were in disagreement with the family data for the R1699W variant, where this allele was found to segregate with the disease. The same assay was then performed in mammalian 293T cells. In the mammalian cell line, both variants displayed a loss of function phenotype (Vallon-Christersson *et al.*, 2001).

Both these variants were later analyzed by Williams *et al.* (2003) and Mirkovic *et al.* (2004), based on two opposing conclusions drawn from the study by Vallon-Christersson *et al.* (2001). Williams *et al.* (2003) concluded from Vallon-Christersson *et al.* (2001) that R1699Q had no effect on transcription (a conclusion drawn solely from the table showing an effect on transcription activation in yeast cells, and not from the results given in the text). Williams *et al.* (2003) made use of a proteolysis-based assay together with computational predictive methods to determine aberrant protein conformations caused by missense mutations. Their analysis of R1699Q revealed that this change has little or no effect on the structure of the BRCT repeats. They further state by referencing Vallon-Christersson *et al.* (2001) that this alteration has little effect on transcription activation. Williams *et al.* (2003) did however determine that the R1699W variant destabilizes the protein.

Mirkovic *et al.* (2004) utilized the 3-D structure of the human BRCA1 BRCT domains to determine the effect that missense mutations have on the transcriptional activation functions of the protein. The authors predict that the R1699Q change is cancer-associated since the location

represents a predicted binding site and the amino acid substitution results in a large volume change as well as charge change. Their prediction is further grounded by the fact that there is "no detectable activity in transcription assays at 37°C" (referencing Vallon-Christersson *et al.*, 2004). The location of the two R1699 variants within the BRCT domain is illustrated in figure 3.18.

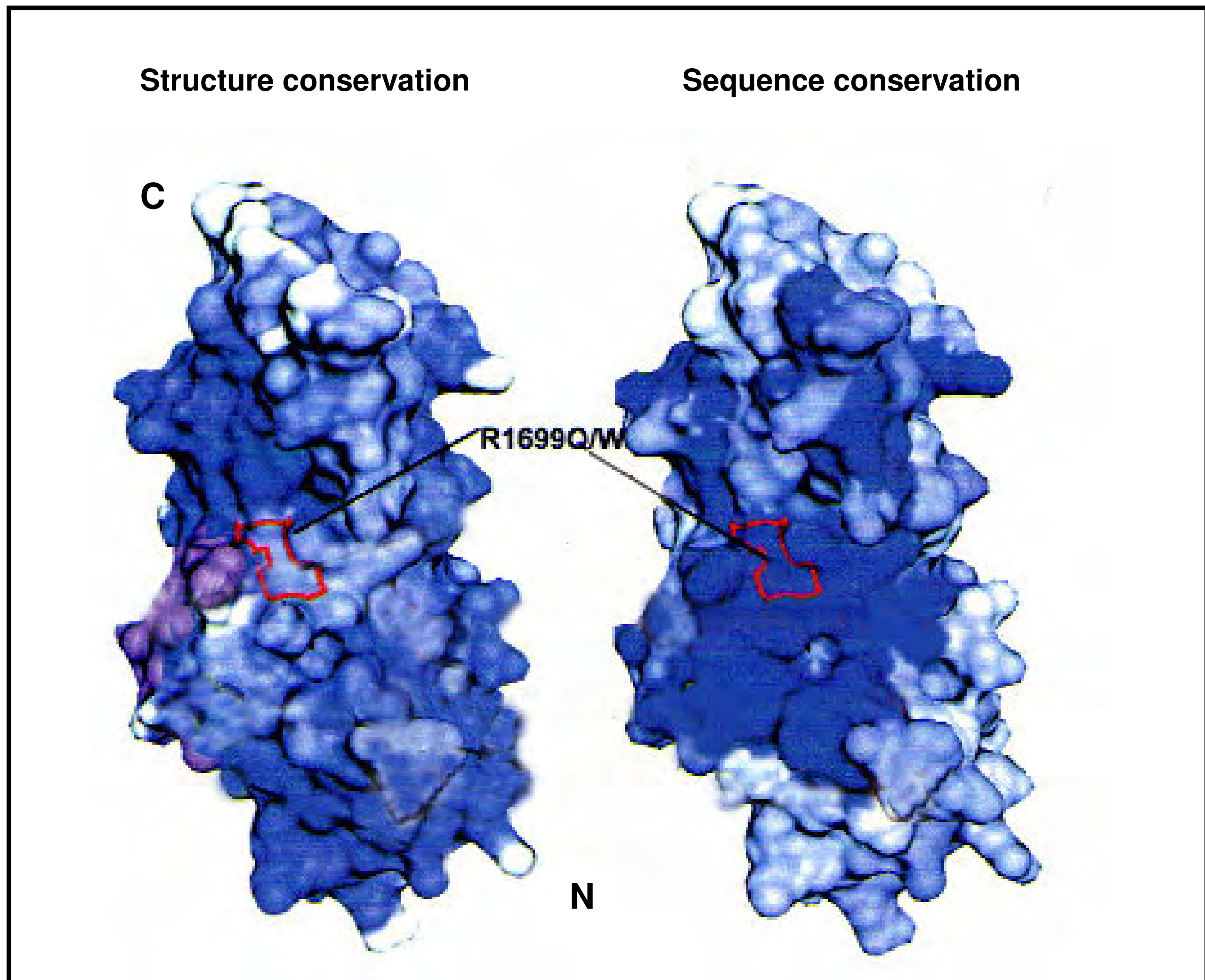


Figure 3.18: Human BRCA1 BRCT domains.
Solvent exposed missense mutations (R1699Q and R1699W) on the surface of the protein are illustrated (outlined in red). Conservation of structure of the BRCT domain is indicated by the intensity of blue on the protein shown on the left hand side. Conservation of sequence is illustrated by the intensity of blue on the protein depicted on the right hand side (The more intense the blue, the more conserved the sequence). The region shown in purple in the left panel represents the linker between the two BRCT repeats. Adapted from Mirkovic *et al.* (2004).

3.3.2 CHARACTERIZATION OF THE EXON 22 DELETION

Following DQ analysis, sample BRC100.1 presented with a relative copy number of 0.69 for exon 22, a 1.45 fold reduction in relative copy number (figure 3.19), indicating a possible deletion of exon 22.

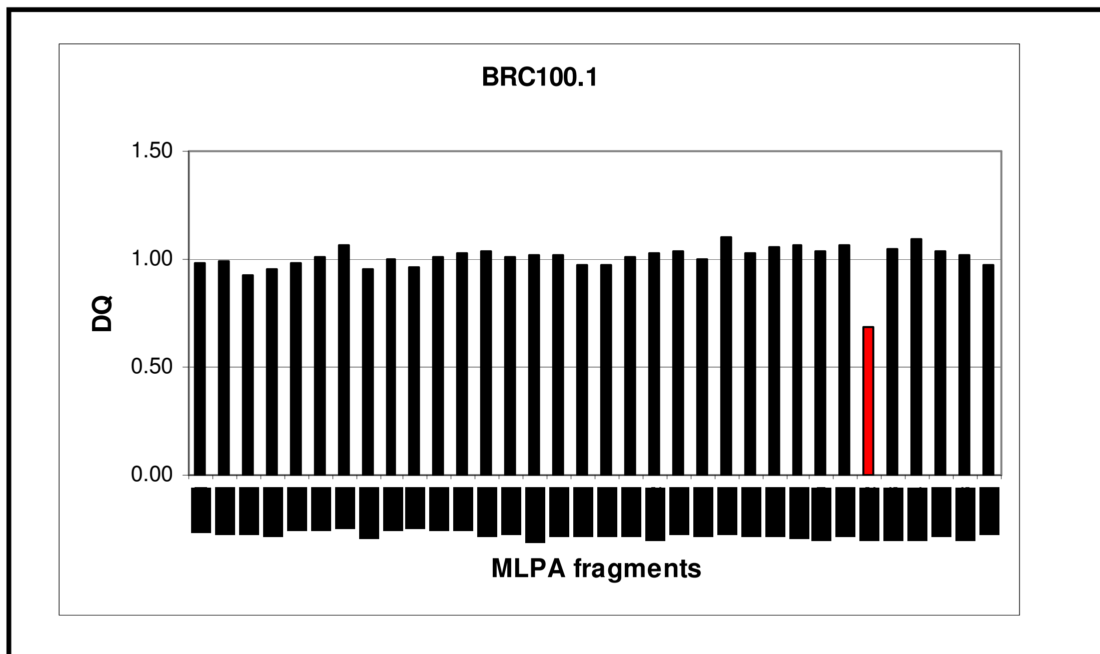


Figure 3.19: Histogram representation of MLPA fragment DQs for sample BRC100.1

Individual BRC100.1 is Dutch and recently immigrated to South Africa. She was diagnosed with breast cancer and ovarian cancer at age 34 and 45 respectively. Three of her aunts have been diagnosed with ovarian cancer (all at or before the age of 70), two of whom were also diagnosed with breast cancer, one of whom has bilateral cancer of the breast. Her mother was diagnosed with breast cancer at the age of 39, and her cousin presented with bilateral breast cancer at the ages of 27 and 32 years (figure 3.20). In this family there are a total of 9 breast cancer cases, and 4 cases of ovarian cancers.

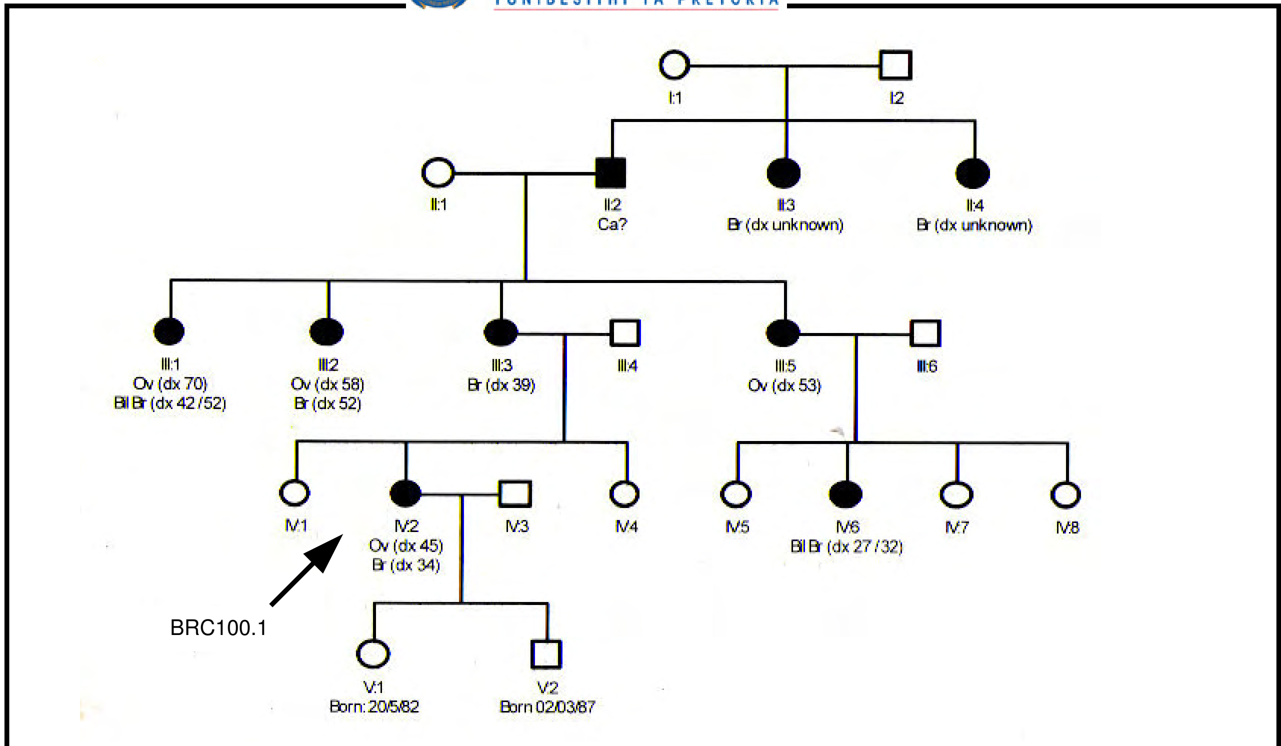


Figure 3.20: Pedigree of family BRC100

Since one of the Dutch founder mutations is a 510bp deletion, which completely removes exon 22 (Petrij-Bosch *et al*., 1997), it was suspected that this individual might harbor this mutation. Primers flanking exons 22 and 23 were utilized in a long-range PCR reaction (Chapter 2) to determine whether this specific mutation was present.

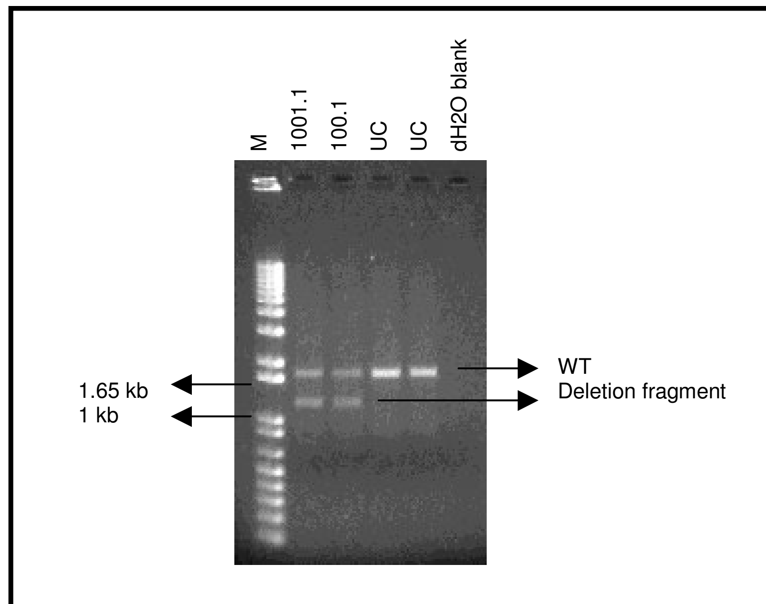


Figure 3.21: Agarose gel electrophoresis of long-range PCR product of BRC100.1.
M: 1kb Plus Molecular Weight Marker (Invitrogen); UC: Unaffected control; WT: Wild-type

This amplification resulted in the production of an aberrant genomic fragment (figure 3.21), together with the wild-type 1 751bp fragment. The presence and size of this deleted fragment indicated that the mutation was most likely the 510bp deletion of exon 22. In order to confirm this, the deleted fragment was excised from the gel, purified and sequenced (Chapter 2). The electropherogram of the sense strand is depicted in figure 3.22.

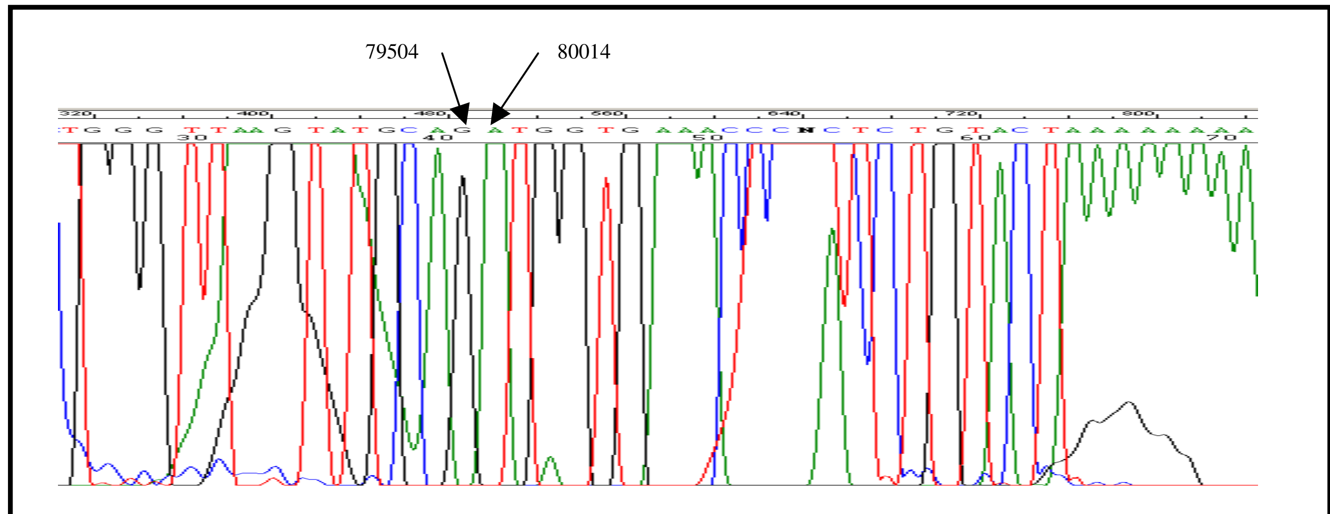


Figure 3.22: Electropherogram of the sense strand of the exon 22 deletion fragment.
The nucleotides flanking the deletion are indicated by arrow heads.

The deletion detected is indeed the Dutch founder mutation, IVS21-38del510. The sequence change of this variant is indicated in figure 3.23.

```
79441 agaggtcttg ctataagcct tcatccggag agtgtagggt agagggcctg ggttaagtat
79501 gcagattact gcagtgattt tacatctaaa tgtccatttt agATCAACTG GAATGGATGG
79561 TACAGCTGTG TGGTGCTTCT GTGGTGAAGG AGCTTTCATC ATTCACCCTT GGCACAgtaa
79621 gtattgggtg ccctgtcaga gagggaggac acaatattct ctcctgtgag caagactggc
79681 acctgtcagt ccctatggat gcccctactg tagcctcaga agtcttctct gcccacatac
79741 ctgtgccaaa agactccatc tgtaagggat gggtaaggat ttgagaactg cacatattaa
79801 atatactgag ggaagacttt ttccctctaa ctctttttcc catatgtccc tccccctcct
79861 ctctgtgact gccccagcat actgtgtttc aacaaatcat caagaaatga tggctggag
79921 gctgggcatg gtggctcatg tctgtaatcc cagcactttg ggaggccgag gcaggtggat
79981 cacttgtcag gagtttgaga ccagcctggc caacatggtg aaaccccatc tgtactaaaa
80041 aaaaaaaaac aaaaagtagc caggcctggt ggagcatgcc tgtaatgcca gctatttggg
80101 aagttgaggt gtgagcatcg cttgaacgtg ggaggcagag gttgcagtga gccaagattg
```

Figure 3.23: Genomic sequence of exon 22 and the flanking intron sequences.
The exon sequence is indicated in uppercase and the intronic regions in lower case. The first 241bp of an *Alu* element is shown in italics. The sequence highlighted in yellow represents the 510bp deletion (IVS21-38del510).

This deletion begins within intron 21 and terminates within the most upstream copy of four *Alu* elements within intron 22 (figure 3.24). The breakpoint in intron 21 does not lie within an *Alu* repeat, and begins 1 165bp away from the *Alu*Y repeat in intron 21.

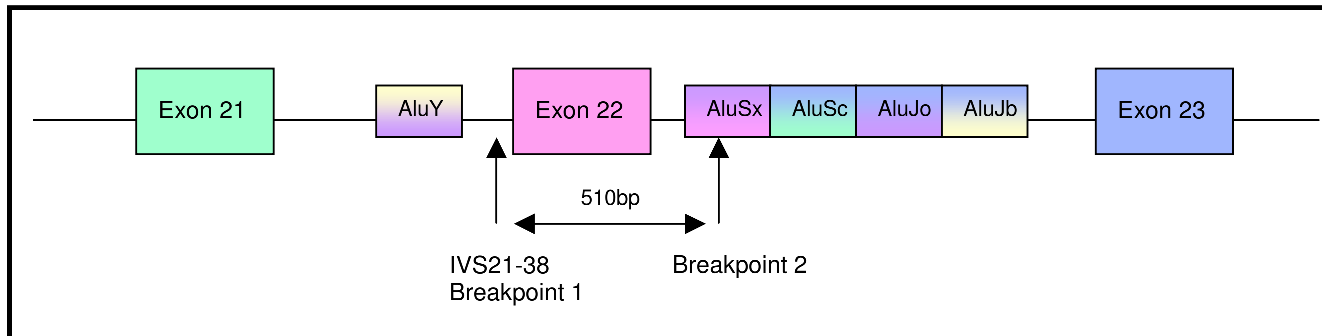


Figure 3.24: Genomic structure of intron 21-22.

The breakpoint in intron 22 is within the *Alu*Sx family repeat. The four *Alu*s in intron 22 are all between 3 and 10 bp away from each other. The deletion results in removal of the out-of-frame exon 22 and premature termination of translation at codon position 1805, removing the last 60 amino acid residues of the protein. This particular mutation was detected in 6.39% of Dutch breast cancer families (Petrij-Bosch *et al*., 1997).

3.3.3 DESCRIPTION OF THE EXON 23-24 DELETION

Sample BRC158.1 was acquired after the completion of screening all other samples with the MLPA P002 kit. All samples that displayed aberrant MLPA profiles in that batch were subsequently screened using the newly developed *BRCA1* confirmation P087 MLPA kit, and sample BRC158.1 was included.

The P087 kit was designed for the confirmation of deletions and insertions detected using the P002 kit. The P087 probe mix includes 36 probes, 10 of which represent control probes. In addition, the MLPA control mix, which consists of 7 probes is also included. This P087 probe mix includes two probes for exon 13, since a silent polymorphism (Ser1436Ser) at the ligation site of the P002 probe occurs commonly in the United Kingdom. One of these 2 probes detects the same sequence as the P002 probe, but the hybridization sequence is longer, and therefore the polymorphism is not expected to result in a reduced DQ. The new probe is positioned upstream of this probe, to confirm any apparent DQ changes, should they occur. Apart from the one exon 13 probe, all probes hybridize to different sequences when compared to the P002 kit. This then allows for the confirmation of suspected deletions / duplications. It is recommended that the P002 kit be utilized for initial screening, and that the P087 probe mix be used only for confirmation. It may therefore be advantageous that sample BRC158.1 be re-screened using the P002 kit in future.

Following the use of the P087 probe mix to screen sample BRC158.1, relative copy numbers of 0.54 and 0.55 were identified for exons 23 and 24 respectively (figure 3.25).

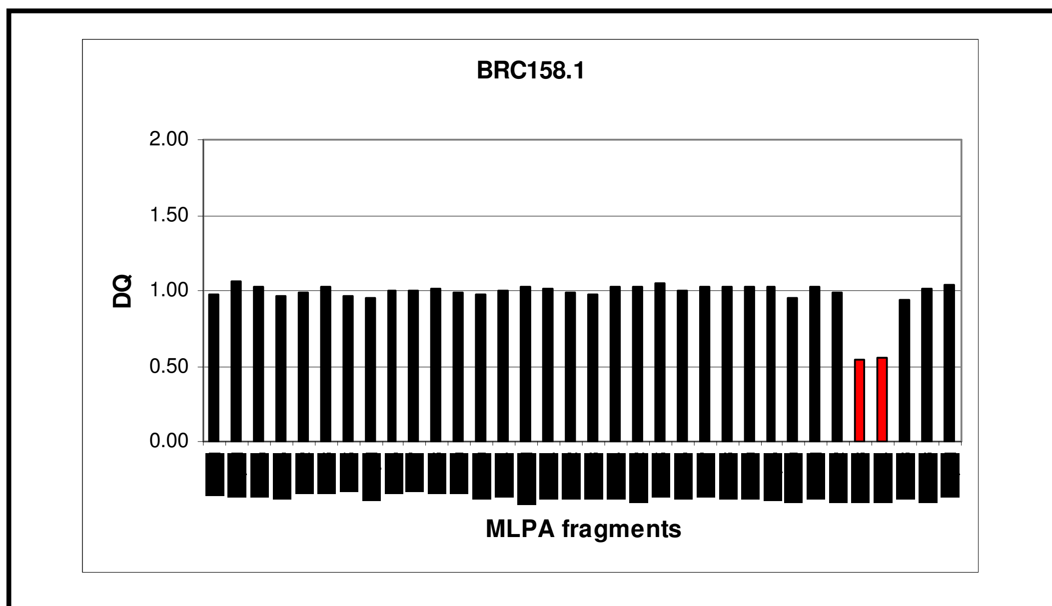


Figure 3.25: Histogram representation of MLPA fragment DQs for sample BRC158.1

The family identified with this deletion has three individuals diagnosed with breast cancer, with an average age of 38 at diagnosis. The mutation is clearly inherited from the maternal side of the family (figure 3.26). This side of the family is of Greek ancestry, where the Grandparents of the proband immigrated to South Africa.

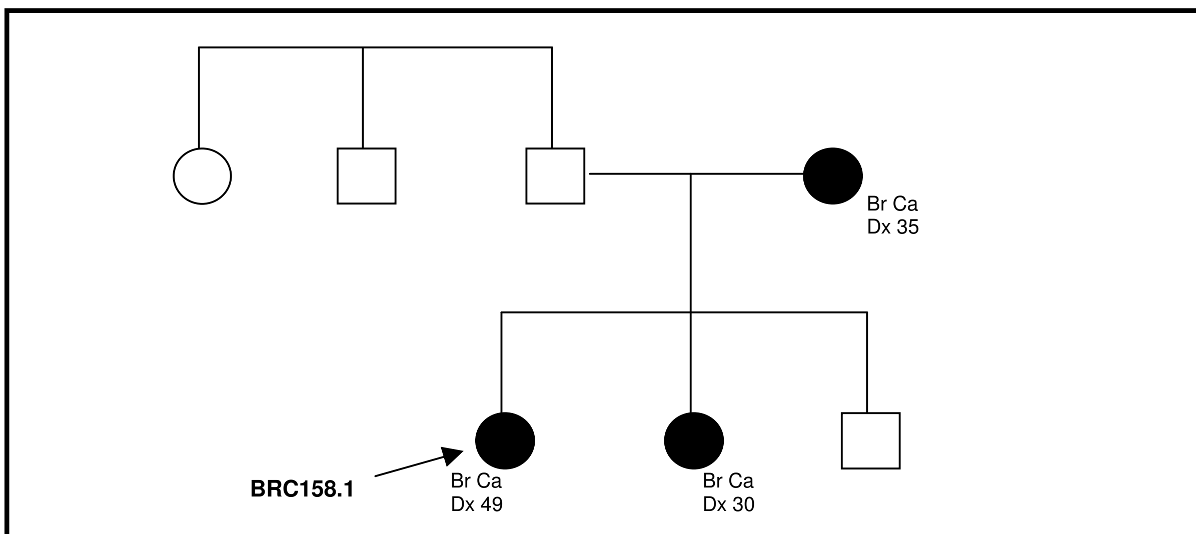


Figure 3.26: Pedigree of family BRC158.

The oligos for detecting relative copy number for exon 24 of kit P087 is located within the 3'UTR, where the ligation site is positioned 450bp away from the stop codon. The only primer available at this time was located 93bp downstream of exon 24. This primer is therefore located within the deleted section (deletion extends from exon 23 – at least 450bp downstream of exon 24). PCR with this primer would therefore not amplify the deletion fragment as shown in figure 3.27 on the following page. The PCR product generated from the unaffected Afrikaner control is clearly more intense than that of sample BRC158.1 (figure 3.27). This possibly indicates that a single allele was amplified for the patient, i.e. the deletion fragment was not amplified.

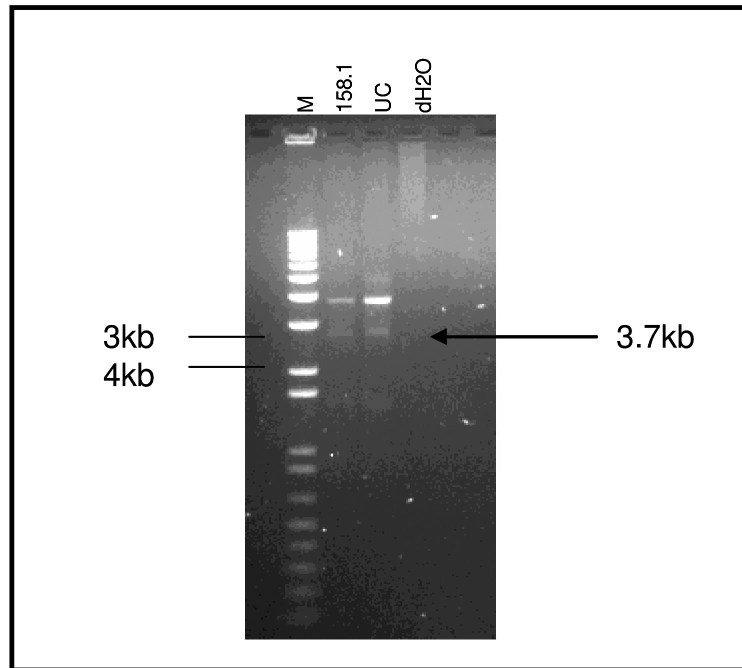


Figure 3.27: Agarose gel electrophoresis of long-range PCR products of BRC158.1 and unaffected control.
M: 1kb plus molecular weight marker (Invitrogen)
UC: Unaffected control

Unfortunately, due to time constraints, amplification of the deleted fragment detected in this study was not achieved, and therefore the breakpoints of this particular deletion have not been characterized. This is because primers further downstream of exon 24 were unavailable at the time. Such primers will however be designed and ordered, to allow for characterization of this deletion. Nevertheless, this large rearrangement removes a sizeable portion of the *BRCA1* BRCT domain, abrogating critical protein-protein interactions.

Four *Alu* sequences are located within intron 22, one within the 3'UTR and 13 within intron 24 (figure 3.28). Two of the same *Alu* families occur in introns 22, 24 and the 3'UTR (*Alu* Sx and *Alu* Jb).

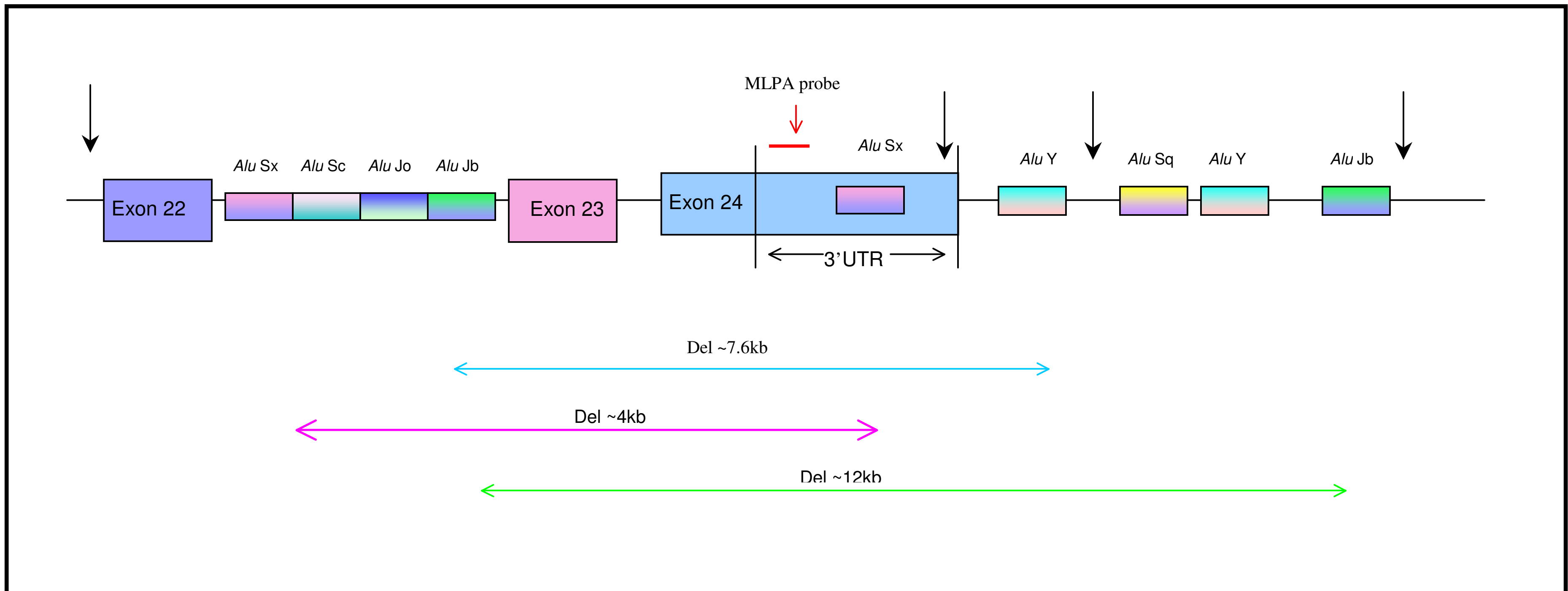


Figure 3.28: Genomic structure of the 3' region in which the DelEx23-24 mutation is located.
Solid colored boxes represent exons 22-24. Shaded boxes represent *Alu* repeats. Although 13 *Alu* sequences occur in intron 24, only the 4 most 5' are indicated. The pink and green horizontal arrows represent the size of the deletion should the same family of *Alu*s be involved. The blue horizontal arrow represents the deletion and possible *Alus* involved in the rearrangement reported by de la Hoya *et al*. (2006). Black vertical arrows indicate the location were primers will be designed to characterize the mutation. The red band and arrow indicates the location of the MLPA probe that suggests the deletion of exon 24.

Deletions of both exons 23 and 24 have been reported in two Italian families (Agata *et al.*, 2006) and one Spanish family (de la Hoya *et al.*, 2006), but the breakpoints of these deletions have not been characterized. In an attempt to characterize this mutation, de la Hoya *et al.* (2006) performed long-range PCR with a forward primer located in intron 21 and a reverse primer 9.4 kb downstream of exon 24. This did not result in amplification of the expected 13.1 kb fragment in the normal control, but did produce an amplicon of `6kb in individuals displaying an abnormal MLPA profile. Further analysis indicated that an XmaII-HindIII fragment, 7.6kb in size, which includes exons 23 and 24, was lost in these individuals.

Interestingly, when Agata *et al.* (2006) screened the families using the P002 MLPA kit, they detected only a deletion of exon 23 in the two families. Using the P087 MLPA kit as confirmation they found that the deletion extends into exon 24. This is probably due to cross-hybridization of the P002 exon 24 probe to an unrelated sequence, which is located on 4p28 (as declared by the manufacturer). They therefore believe that this deletion more than likely includes both exons. de la Hoya *et al.* (2006) performed their initial screen using the P002 kit and then confirmed all variations using the P087 kit. No mention was made about discrepancies of results when using the two different probe mixes.

The deletion detected by de la Hoya *et al.* (2006), most probably involves the *Alu* Jb (intron 22) and *Alu* Y (intron 24) sequences, since unequal recombination between these two *Alu*s will result in the removal of ~7.6kb. No other *Alu* “combinations” in this region will result in a deletion of that size.

It is possible that the deletion detected here could be similar to that detected by de la Hoya *et al.* (2006). It would therefore be worthwhile to design primers positioned in intron 21 (forward) and one just downstream of the *Alu* Y (reverse) sequence in intron 24. Primers will also be designed downstream of the *Alu* Jb (reverse) sequence in intron 24 as well as the *Alu* Sx (reverse) sequence in the 3’UTR. Long-range PCR and sequencing with these primers should lead to characterization of this large deletion.

3.4 SUMMARY OF RESULTS

Of the fifty-eight breast/ovarian cancer families screened by MLPA for large genomic rearrangements within *BRCA1*, three were identified with reduced dosage quotients for one or more exons within this gene. Of these, two were identified as true deletions (IVS-38del510; Del23-24), while the third (c.5215G→A) falsely appeared as a deletion.

The reduced DQs that falsely indicated a deletion of exon 18 were detected in two sisters (BRC119.1 and BRC119.2) diagnosed with ovarian cancer. Characterization of this suspected deletion led to the identification of a single nucleotide substitution at the 5' end of the long oligonucleotide. The G→A transition occurs 2bp away from the ligation site, thereby preventing complete hybridization of the long oligonucleotide and therefore absence of ligation. This R1699Q variant has been reported previously but its significance is uncertain.

The true deletions were indicated by a significant reduction of the DQs for exon 22 and both 23 and 24 in patients BRC100.1 and BRC158.1 respectively. Patient BRC100.1 is a recent immigrant to South Africa from Holland. Characterization of the suspected deletion of exon 22 by sequencing indicated that it was the founder Dutch deletion of 510bp, IVS-38del510, which is present in 6.39% of Dutch breast cancer families (Petrij-Bosch *et al.*, 1997). An attempt to characterize the suspected exon 23-24 deletion in patient BRC158.1 was unsuccessful since reverse primers downstream of the exon 24 MLPA probe were not available. Such primers will however be designed and the aberration characterized. Deletion of these two exons has been reported in two Italian families (Agata *et al.*, 2006) and a single Spanish family (de la Hoya *et al.*, 2006), although the breakpoints were not characterized.

The "false" deletion and deletion of exon 22 were detected with the P002 MLPA kit, while the deletion of exons 23-24 was detected with the P087 kit. The P087 kit is essentially a confirmation kit, and the manufacturer recommends using the P002 kit for initial screening purposes. The probes for the P087 kit are all located at least 20 nucleotides away from the P002 probes, except for the exon 24 probe, which is located in the 3'UTR, 530bp away from the P002 probe. Nine of these *BRCA1* probes' ligation sites are located within introns and the two probes for exon 1a and 1b are both located in the 5'UTR. It is therefore advisable to rather use the P002 kit for initial screening and the P087 kit only for confirmation. The P002 kit does however have some drawbacks since a number of probes have their ligation sites within or very near to polymorphisms, generating false positive results. Use of the P087 kit as confirmation should

however verify the validity of aberrant DQs. The single base pair change detected in this study would not have been detected as a deletion using the P087 kit. The manufacturer has however recently developed a new P002 kit (P002B), where only the exon 24 probe has been changed to that of the P087 kit. This is due to the fact that DQs from the old probe were reduced by only 25% in samples that had an exon 24 deletion. The manufacturer explains this as the result of the formation of 393bp product caused by six-nucleotide homology between the probe and the MLPA forward primer and not as the result of cross-hybridization of the probe to an unrelated sequence on 4p28 as was suggested by de la Hoya *et al*. (2006). It was therefore fortunate that the P087 kit was used to screen sample BRC158.1, otherwise the aberration may only have appeared as a deletion of exon 23, making characterization extremely difficult.

The detection of a single large genomic rearrangement in 57 South African breast/ovarian cancer families (Dutch immigrant excluded) indicates that such alterations play a small role (1.75%) in the disease in South Africa. The family identified with this mutation is South African of Greek ancestry. No rearrangements were detected within the 40 Afrikaner families, indicating that such mutations play no role in breast/ovarian cancer in this population. The Afrikaners are mainly descended from Dutch, German and to a lesser extent, French immigrants to the Cape. The founding Afrikaner population consisted of approximately 90 families by 1687. Since the Afrikaner population has French, Dutch and German ancestral links, it was expected that the mutations found within these population groups would be responsible for a portion of the familial breast cancer burden within South Africa. This result is however not surprising, since the search for small mutations within *BRCA1* and *BRCA2* in the South African population did not reveal any of the founder mutations present in the Dutch, German and French populations (Reeves *et al*., 2004; Schlebusch, 2004). In terms of disease-causing BRCA mutations, the South African population seems more similar to the Finnish population, where familial breast cancer mutations are distinct and large genomic rearrangements are absent (Huusko *et al*., 1998; Lahti-Domenici *et al*. 2001; Laurila *et al*., 2005).

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CHAPTER 4

CONCLUSIONS

Large genomic rearrangements within *BRCA1* have been reported with increased frequency over the last few years, where several different techniques have been utilized (Table 1.2). Although the first reported intragenic rearrangement in *BRCA1* was detected by Southern blotting (Puget *et al.*, 1997), this technique has a number of disadvantages, which include the generation of ambiguous results, requirement for large quantities of genomic DNA, occurrence of high levels of background when using non-radioactive labeled probes (as shown in this study), and the difficulty to optimize. A newer technique, Multiplex Ligation-dependent Probe Amplification (MLPA) has now "revolutionized" the laborious technical approaches that severely hampered the identification of large rearrangements, and has relatively recently allowed such studies to become almost routine. To date, MLPA has been used to detect 53 of the sixty-nine reported large rearrangements in *BRCA1*. The fact that MLPA hybridization occurs in solution and includes a ligation step makes it a rapid and sensitive method. The precision of the ligation step means that filter capture and high stringency washes are not needed. One draw-back of MLPA however is that single base pair changes at the ligation site will erroneously indicate the occurrence of a deletion. If all suspected deletions are however confirmed by long-range PCR and sequenced, this can in fact be advantageous and lead to the detection of other small, possible disease-causing mutations, as was shown in this study. Since the MLPA probes do not hybridize to entire exons, but rather portions of them, it is possible that false negatives could be obtained, should the deletions/duplications involve only a section of the exon (especially if a large portion of the intron and splice site and a small section of the exon is involved). Another problem with this technique is that it will be unable to detect genomic inversions. Although this methodology is not 100% sensitive, it will detect each of the large rearrangements previously reported using other techniques, as the kit was designed in this manner. Since this methodology has become by far the most popular, it may explain why no inversions have thus far been reported.

In different populations, the reported frequency of intragenic *BRCA1* rearrangements varies between 0% in the Finnish and French-Canadian populations to 36% of all *BRCA1* mutations in the Dutch population (Lahti-Domenici *et al.* 2001; Laurila *et al.*, 2005; Moisan *et al.*, 2006; Petrj-Bosch *et al.*, 1997). The contribution of *BRCA1* rearrangements to the burden of breast cancer risk in South Africa was unknown. This is therefore the first study of large genomic rearrangements within *BRCA1* in South Africa. Seventy-four patients from 58 *BRCA1/2* small

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mutation negative South African breast / ovarian cancer families were screened for large rearrangements within this gene to determine this proportion.

At the start of this study, Southern blot with DIG-dUTP labeled probes was utilized to detect large genomic rearrangements within *BRCA1*. This methodology proved laborious and was unsuccessful due to the occurrence large quantities of background. While this technique was being optimized, MLPA was developed and made commercially available. This method was then utilized and proved to be simple, efficient, sensitive and cost effective. Of the fifty-four families screened, 3 were identified with DQ aberrations. Two of these were found to be deletions (IVS21-36del510 and DelEx23-24) while the third was a missense mutation falsely identified as a deletion (R1699Q).

The individual found to harbor the exon 22 deletion (IVS21-36del510) is Dutch and recently immigrated to South Africa from Holland. It is therefore not surprising that she presented with one of the two common Dutch founder mutations. This particular mutation occurs in 6.39% of Dutch breast cancer families (Petrij-Bosch *et al*., 1997). The deletion of exons 23 and 24 was detected in a South African of Greek ancestry. The breakpoints of this deletion were not characterized due to the absence of reverse primers located downstream of the 3'UTR. Such primers will be designed and the mutation characterized shortly. The simultaneous deletion of these two exons has been reported in two Italian (Agata *et al*., 2006) and one Spanish family (de la Hoya *et al*., 2006) where the breakpoints could not be determined. The missense mutation (c.5215G→A / R1699Q) detected is located 2 base pairs away from the ligation site at the 5' end of the long oligonucleotide. This variant was detected in two individuals from a single family, both affected with ovarian cancer. The pathological significance of this mutation is unconfirmed.

Since only one deletion was detected in a South African family (Dutch Immigrant excluded), large genomic rearrangements account for 1.75% (1/57) of South African breast/ovarian cancer families. No rearrangements were detected in the forty Afrikaner families. Previous studies of *BRCA1* and *BRCA2* in mixed South African breast/ovarian cancer families have shown that these two genes are responsible for 20% and 39% of inherited susceptibility respectively (Reeves *et al*., 2004; Schlebusch, 2004). In the Afrikaner population, small *BRCA1* and *BRCA2* mutations account for 18.79% (31/165) and 44.24% (73/165) of familial breast/ovarian cancer risk respectively (unpublished data). Founder effects are also observed within the Afrikaner population, where two *BRCA1* founders and one *BRCA2* founder mutation occur. Interestingly, these founder mutations are specific to the South African Afrikaner where the two *BRCA1* mutations have not been previously reported, while the *BRCA2* founder has been reported only once previously to the BIC (Reeves *et al*., 2004; Schlebusch, 2004), indicating that the mutation

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YUNIBESITHI YA PRETORIA

spectrum of this population is unique. The fact that no rearrangements were detected in the Afrikaner families again confirms that the *BRCA1* mutation status in the Afrikaner population is distinct from that of its ancestors (Reeves *et al*., 2004), the Dutch, French and German populations. The mutations present in the Afrikaner population therefore appear to have arisen independently, or alternatively are rare in the countries of origin (Reeves *et al*., 2004). Instead of showing similarities to the Dutch, German and French populations, the mutation status within the Afrikaner population rather seems similar to that of the Finnish population, where founder *BRCA1* and *BRCA2* mutations are not shared with other populations and large genomic rearrangements are absent (Huusko *et al*., 1998; Lahti-Domenici *et al*. 2001; Laurila *et al*., 2005). Furthermore, as opposed to other countries, the frequency of *BRCA2* small mutations in Finland and the Afrikaner population is higher than that in *BRCA1* (Schlebush, 2004; Huusko *et al*., 1998). Despite these similarities, the contribution of *BRCA1* and *BRCA2* mutations to Finnish familial breast/ovarian cancer (7% *BRCA1*; 14% *BRCA2*) is much smaller than in the Afrikaner population (18.79% *BRCA1*; 44.24% *BRCA2*). For other populations (eg. Dutch and Italian), it has been suggested to use MLPA as an initial detection method in familial breast cancer. This will be neither cost nor time efficient in the South African population, due to the low frequency of these mutations. It would however be advantageous to first screen samples by means of SSCP/HA and PTT, and following a negative report, screen using MLPA. This will be particularly important when families have ancestral links with the Mediterranean countries as well as the Dutch and German, as a result of the large contribution rearrangements make to breast/ovarian cancer risk in these countries (de la Hoya *et al*., 2006; Hartmann *et al*., 2004; Hogervorst *et al*., 2003; Montagna *et al*., 2003).

Due to this distinct mutation structure in South Africa, breast cancer risk could possibly be explained by large rearrangements within *BRCA2*. Although this gene has substantially fewer *Alu* repeats, at least 13 large rearrangements have been reported within this gene so far (Agata *et al*., 2005; Bunyan *et al*., 2004; Nordling *et al*., 1998; Tournier *et al*., 2004; Walsh *et al*., 2005; Woodward *et al*., 2005; Wang *et al*., 2001). Of course, since SSCP and PTT would detect ~85% of small mutations, it is possible that some may have been missed using these techniques. Another possibility may be that inversions occurred within this population group that could not be identified using MLPA. Other unknown breast cancer susceptibility gene(s) or low penetrance genes may also be responsible for a proportion of breast/ovarian cancer predisposition in South Africa. Screening genes such as *p53* and *CHEK2* for mutations in high risk families and determination of the occurrence of large genomic rearrangements in *BRCA2* will mark our next quest into determining the etiology of breast and / or ovarian cancer in South Africa.

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Electronic-Database Information

Online Mendelian Inheritance in Man (OMIM): http://www.ncbi.nlm.nih.gov/Omim/
Breast Cancer Information Core (BIC): http://research.nhgri.nih.gov/bic
MRC-Holland: www.mrc-holland.com

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Appendix A

Copy of Ethics Approval

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PROVINSIALE ADMINISTRASIE

GAUTENG

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0001

Date: 25-02-98

NUMBER : **Nommer** : 18/98

TITEL : Molecular Genetic Analysis of Inherited Predisposition to Breast/ Ovarian Cancer: The Frequency and Nature of BRCA1 and BRCA2 Mutations in South African Breast / Ovarian Cancer Families.

APPLICANT : Prof E J van Rensburg; Dept Human Genetics; Pretoria Academic Hospital;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

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Appendix B

Clinical details of patients

Appendix B: Clinical details of patients

Patient	Ancestry	Number of Breast Cancers (Bilateral)	BC Dx ≤ 50	BC Dx > 50	BC Age at dx unknown	Average age at diagnosis (Breast)	Number of Ovarian Cancers (Bilateral)	OC Dx ≤ 50	OC Dx > 50	OC Age at dx unknown	Average age at diagnosis (Ovarian)	Other cancers
BRC 4.1	Afrikaner	2	1	1	-	50.5	2	1	1	-	44	-
BRC 6.1	Afrikaner	3	3	-	-	36.7	-	-	-	-	-	Lung, stomach
BRC 9.3 BRC 9.4	Afrikaner	4 (1)	3	1	-	52.3	1	-	1	-	56	Lymphoma Cancer of female parts
*BRC 11.2 *BRC 11.3	Afrikaner	8 (2)	6	2	-	35.6	2	1	1	-	49	Stomach, squamous cell carcinoma of neck
BRC 14.1	Afrikaner	3	2	1	-	44.7	-	-	-	-	-	Leukemia
BRC 16.1	Afrikaner	2, one of which male	1	1	-	Male: 63 Female:43	-	-	-	-	-	Prostate
BRC 19.1	Afrikaner	3	2	1	-	47.7	1	-	1	-	61	Colon, skin, thyroid
BRC 23.1	Ashkenazi Jewish	4 (2)	1	3	-	57.5	-	-	-	-	-	-
BRC 26.1 BRC 26.2	Afrikaner / Dutch	1	1	-	-	43	2 (1)	-	2	-	68	Colon, stomach
BRC 28.1	Afrikaner / English	3	2	1	-	52.3	-	-	-	-	-	-
BRC 30.1	Afrikaner / Lebanese	3 (1)	2	1	-	50.3	-	-	-	-	-	Uterus?
BRC 32.1	Afrikaner	2	2	-	-	40	1	1	-	-	52	-
BRC 40.1	English (UK)	4 (1)	2	2	-	48	-	-	-	-	-	-
BRC 41.1 BRC 41.2 BRC 41.3	Afrikaner	5	3	1	1	48.8	-	-	-	-	-	-
BRC 45.1	Afrikaner	5 (2)	3	2	-	50.8	-	-	-	-	-	-
Patient	**Ancestry**	**Number of Breast**	**BC Dx ≤ 50**	**BC Dx > 50**	**BC Age at dx**	**Average age at**	**Number of Ovarian**	**OC Dx ≤ 50**	**OC Dx > 50**	**OC Age at dx**	**Average age at**	**Other cancers**

		Cancers (Bilateral)			unknown	diagnosis (Breast)	Cancers (Bilateral)			unknown	diagnosis (Ovarian)	
BRC 46.2	Afrikaner	7	3	3	1	48.7	-	-	-	-	-	Stomach, prostate, lung, brain
BRC 50.1	Portuguese	4	2	2	-	58.8	1	1	-	-	49	Primary unknown - metastases to liver. Prostate
BRC 54.1	Afrikaner	5 (1)	3	2	-	53.4	-	-	-	-	-	Sarcoma: upper leg
BRC 56.2	Belgian	5	4	1	-	43.4						Prostate, female parts
BRC 59.1	English	3, of which one is male	2	1	-	Male: 85 Female:48	-	-	-	-	-	-
BRC 60.1 BRC 60.2	Afrikaner	4	2	2	-	56.8	-	-	-	-	-	Throat / oesophagus
BRC 61.1	Afrikaner / English	5 (2)	3	2	-	52.6	-	-	-	-	-	-
BRC 66.1 BRC 66.3	Afrikaner/ German/ British	2	2	-	-	41.5	-	-	-	-	-	Pancreas, colon
BRC 67.1	Dutch	5	3	1	1	38.8	-	-	-	-	-	Brain, stomach, colon
BRC 68.1 BRC 68.2	Afrikaner	2	2	-	-	45	1	-	1	-	55	Stomach
BRC 69.1	Afrikaner	4 (1)	3	-	1	45	-	-	-	-	-	-
BRC 71.1 BRC 71.2	Dutch	4	4	-	-	36.8	-	-	-	-	-	-
BRC 73.1 BRC 73.2	Afrikaner / English	4	3	-	1	41.3	-	-	-	-	-	Prostate, melanoma, lymphoma, endometrial
BRC 79.1	Afrikaner	3	2	-	1	43	-	-	-	-	-	-
BRC 80.1	Afrikaner	5	2	-	3	43.5	3	-	3	-	72	Stomach? Colon
BRC 84.1	Ashkenazi Jewish	3	1	2	-	57	-	-	-	-	-	Gastric, prostate

Patient	Ancestry	Number of Breast Cancers (Bilateral)	BC Dx ≤ 50	BC Dx > 50	BC Age at dx unknown	Average age at diagnosis (Breast)	Number of Ovarian Cancers (Bilateral)	OC Dx ≤ 50	OC Dx > 50	OC Age at dx unknown	Average age at diagnosis (Ovarian)	Other cancers
BRC 86.1	Ashkenazi Jewish	4, one of which is male	3	1	-	Male: 30 Female: 52.7	-	-	-	-	-	-
BRC 88.1	Afrikaner / Norwegian	2, one of which is male	2	-	-	Male: 29 Female: 40	-	-	-	-	-	Pancreas
BRC 92.1 BRC 92.2 BRC 92.3	Afrikaner	6	1	5	-	59.8	-	-	-	-	-	Kidney, prostate
BRC 93.1	Afrikaner	4	1	2	1	55.3	-	-	-	-	-	-
BRC 94.1 BRC 94.2	Afrikaner	5	2	3	-	51.4	-	-	-	-	-	Hodgkins Lymphoma, liver
BRC 95.2 BRC 95.3	Afrikaner	8 (1), one of which male	5	2	1 (male)	44.4	-	-	-	-	-	Liver, stomach, fibroadenoma
BRC 96.1	German	2	1	1	-	48	1	-	1	-	66	Bladder, small intestine
BRC 98.1	Afrikaner	6 (2)	4	2	-	51.2	-	-	-	-	-	Oesophagus
BRC 100.1	Dutch	7 (2)	5	2	-	39.7	4	1	3	-	56.5	-
BRC 101.1	English / Australian	3	2	1	-	50.3	-	-	-	-	-	-
BRC 114.1	Afrikaner	4 (1)	2	-	2	43	-	-	-	-	-	Melanoma, stomach
BRC 117.1	Afrikaner / English	8 (2)	6	1	1	46.7	-	-	-	-	-	-
BRC 119.1 BRC 119.2	Afrikaner	-	-	-	-	-	2	-	2	-	66	Leukemia
BRC 121.1	Scottish / English	3	1	2	-	54.7	-	-	-	-	-	Melanoma, gastric
BRC 122.1	Polish	3	3	-	-	41	-	-	-	-	-	Prostate
BRC 127.1	English	-	-	-	-	-	3	-	3	-	57	-
BRC 129.1	English	5 (1)	4	1	-	42.8	-	-	-	-	-	Prostate, bladder

Patient	Ancestry	Number of Breast Cancers (Bilateral)	BC Dx ≤ 50	BC Dx > 50	BC Age at dx unknown	Average age at diagnosis (Breast)	Number of Ovarian Cancers (Bilateral)	OC Dx ≤ 50	OC Dx > 50	OC Age at dx unknown	Average age at diagnosis (Ovarian)	Other cancers
BRC 132.1	English / Welsh	4	2	2	-	48.8	-	-	-	-	-	-
BRC 139.1 BRC 139.2	Afrikaner	6 (2)	4	2	-	45.8	-	-	-	-	-	Womb, lymphoma
BRC 142.1	Dutch / Afrikaner	3	1	2	-	44	-	-	-	-	-	Bladder, colon, prostate
BRC 145.1	Afrikaner	4	1	2	1	58	-	-	-	-	-	-
BRC 148.1	Afrikaner	3	-	1	-	43	-	-	-	-	-	Unknown cancer
BRC 150.1	French / Afrikaner	5	2	3	-	51.2	-	-	-	-	-	-
BRC 155.1	Afrikaner	6	5	-	1	44.2	-	-	-	-	-	Stomach, liver, retinablastoma
BRC 158.1	Greek	3	3	-	-	38.7	-	-	-	-	-	-
OV 2.1	Afrikaner	-	-	-	-	-	4 (2)	2	2	-	59	Liver, stomach

BC: Breast cancer
OC: Ovarian cancer
Columns 3 and 8 represent the total number of breast (column 3) or ovarian (column 8) cancers that occur within each family. Each breast or ovarian cancer case scores one, while a bilateral case scores 2. The number of individuals affected with bilateral cancer is indicated in brackets.
Columns 4, 5, 9 and 10 represent the whether the specific cancer was diagnosed before or at the age of 50 (column 4 and 9), or whether later than the age of 50 years (columns 5 and 10).
Cases where the age at diagnosis of the specific cancer was unknown are indicated in columns 6 (for breast cancer) and 11 (for ovarian cancer).
The average age at which the diagnoses were made for each family are indicated in columns 7 (for breast cancer) and 12 (for ovarian cancer).
Nature of other cancers that occur in families are indicated in column 13.

Appendix C

Ligation Sequences of BRCA1-MLPA probes, position of the ligation sites, and product sizes

Ligation Sequences and Length of Probes in Mix P002

BRCA1 Exon	Sequence of probe at ligation site (5' – 3'; short – long oligo))	Ligation site L78833	Probe length
1A	CAGGAGGCCT-TCACCCTCTG	3442-3443	148
1B	GGGGCACTGA-GTGTCCGTGG	3672 – 3673	157
2	TTTATCTGCTC-TTCGCGTTGA	4654-4655	166
3	AAGGAACCTG-TCTCCACAAA	12977-12978	175
5	TTCTCAACCA-GAAGAAAGGG	22227-22228	184
6	AGATTTAGTC-AACTTGTTGA	23806-23807	208
7	CCGTGCCAAA-AGACTTCTAC	24576-74577	217
8	TGGAACTGTGAGAACTCTGA	28897-28898	226
9	TTAATAAGGC-AACTTATTGC	31476-31477	235
10	GTTACAAATC-ACCCCTCAAG	32837-32838	244
11	GCGTGCAGCT-GAGAGGCATC	33963-33964	268
11	CTAGCCCTTT-CACCCATACA	36779-36780	277
12	CTGAAGACTG-CTCAGGGCTA	37748-37749	286
13	GTGACTCTTC-TGCCCTTGAG	46280-46281	295
14	AGAAGGCCTT-TCTGCTGACA	52176-52177	304
15	CTGGGAGTCT-TCAGAATAGA	54276-54277	328
16	CTGGAATCAG-CCTCTTCTCT	57524-57525	337
17	GCCAGAAAAC-ACCACATCAC	61065-61066	346
18	TGTGTGTGAA-CGGACACTGA	64801-64802	355
19	CCAGTCTATT-AAAGAAAGAA	65376-65377	364
20	GTCAATGGAA-GAAACCACCA	71634-71635	388
21	AATCTGTTGC-TATGGGCCCT	77646-77647	397
22	CTGTGGTGAA-GGAGCTTTCA	79588-79589	406
23	CACCCAATTG-TGGTTGTGCA	81049-81050	415
24	CCGAGAGTGG-GTGTTGGACA	82979-82980	424

Ligation site according to Genbank accession number L7833

Ligation Sequences and Length of Probes in Mix P087

BRCA1 Exon	Sequence of probe at ligation site (5' – 3'; short – long oligo))	Ligation site L78833	Probe length
Promotor region	ATCCGGGGGC-AGACTGGGTG	3184-3185	157
5'UTR	GGTGGAACTA-CGAGTGCGCA	2547-2548	148
5'UTR	TGGCAACGGA-AAAGCGCGGG	3318-3319	436
2	GAAAATCTTA-GAGTGTCCCA	4704-4705	166
3	ACCACATATT-TTGCAAGTAA	13002-13003	175
5	ACCAAAAGGT-ATATAATTTGG	22280-22281	346
6	CTTTTCAGCT-TGACACAGGT	23849-23850	208
7	TATCATCCAA-AGTATGGGCT	24549-24550	217
8	TCTTTACCAT-ACTGTTTAGC	28843-28844	226
9	GGTGAGTCAA-AGAGAACCTT	31497-31498	235
10	TGAAATCAGT-TTGGATTCTG	32867-32868	355
11	CTCTCAGAGT-GACATTTTAA	35207-35208	277
12	TTAAAATGTC-ACTCTGAGAG	37770-37771	286
13	TGGCTGAACTAGAAGCTGTG	46211-46212	244
13	AAGTGACTCT-TGCCCTTGAG	46278-46279	295
14	TGGAAAGGTA-AGAAACATCAA	52247-52248	265
15	ACAGCTGGAA-GAGTCTGGGC	54346-54347	328
16	AGAGTCAGCT-CGTGTTGGCA	57582-57583	337
17	TAAAGGTTCT-TGGTATACCT	61133-61134	406
18	AAAATGGGTA-GTTAGCTATT	64846-64847	184
Intron 18	AAAAGAGCAC-GTTCTTCTGC	65315-65316	364
Intron 19	TTCTCTTATCC-TGATGGGTTG	71570-71571	388
Intron 21	TTTGTCTTAC-ATAGTGGAGT	77723-77724	397
22	GCTTTCATCA-TTCACCCTTG	79600-79601	199
Intron 23	GCATGTACCT-GTGCTATATG	81115-81116	415
(3'UTR)	AATGGAAGGA-GAGTGCTTGG	83510-83511	424

Ligation site according to Genbank accession number L7833

Appendix D

Dosage Quotients of MLPA analysis

The Schouten analysis generates a single DQ value, which is indicated in the table. With the Wallace method, a series of DQs are generated for each ligation product. The mean of each ligation product is given for these samples

Sample Info	c.5q31	c.6p21	brca1-1a	brca1-1b	brca1-2	brca1-3	brca1-5	c.15q21	brca1-6	brca1-7	brca1-8	brca1-9	brca1-10	c.2q14	brca1-11.1	brca1-11.2	brca1-12	brca1-13	brca1-14
BRC4.1	0.98	0.91	0.98	1.00	0.95	0.88	1.00	1.05	1.12	1.01	0.97	1.00	1.03	0.96	1.03	0.93	0.99	1.14	0.99
BRC6.1	0.97	1.04	0.96	0.97	0.94	1.01	1.00	1.00	0.91	1.06	1.00	0.95	1.00	0.96	0.95	1.03	1.03	1.06	1.08
BRC9.3	0.98	1.05	1.05	0.99	1.00	1.09	0.99	1.03	1.04	1.01	1.03	1.04	0.97	0.96	0.98	1.01	1.01	0.99	1.00
BRC9.4	0.96	0.98	0.95	1.01	0.99	1.03	0.98	1.03	1.02	1.00	0.98	1.00	0.99	1.02	0.97	0.99	0.96	1.02	1.03
BRC11.2	0.97	0.98	1.03	1.00	1.04	1.06	1.03	0.99	1.02	1.05	1.04	1.02	1.01	0.92	1.02	1.03	1.03	1.09	0.97
BRC11.3	0.99	1.08	1.01	1.05	1.00	0.99	0.99	1.02	0.96	1.06	0.97	0.95	1.00	1.01	0.99	1.05	1.10	1.07	0.98
BRC14.1	0.99	1.04	1.03	0.95	1.02	0.96	0.99	1.09	0.98	0.96	0.91	0.96	0.94	1.07	1.04	1.03	0.90	0.93	1.01
BRC16.1	0.98	1.02	0.99	0.98	1.02	0.93	0.98	1.02	0.97	0.81	0.99	1.01	0.94	0.99	0.93	1.08	1.10	1.06	1.01
BRC19.1	0.98	1.09	1.16	1.12	1.14	1.14	1.17	0.98	1.11	1.12	1.12	1.10	1.09	0.92	1.07	1.06	1.11	1.04	1.07
BRC23.1	1.00	0.95	0.98	1.02	1.08	0.93	0.98	1.02	0.90	0.85	0.99	0.99	0.93	0.96	0.91	1.06	1.14	1.01	0.98
BRC26.1	1.13	1.10	1.05	1.03	1.07	1.03	1.07	1.12	1.03	0.93	1.06	1.01	0.94	1.01	0.98	0.95	0.96	0.98	0.97
BRC26.2	1.06	1.09	1.02	1.01	1.04	1.04	1.06	1.06	1.03	1.09	1.00	1.02	1.02	1.03	0.98	1.05	1.06	1.02	0.94
BRC28.1	0.97	0.99	0.96	1.02	1.06	0.96	0.99	1.01	0.96	0.86	0.97	1.01	0.94	0.99	0.91	0.95	1.04	0.93	1.00
BRC30.1	0.99	0.96	0.98	1.01	1.02	0.91	0.99	1.00	0.94	0.92	0.98	1.02	0.96	0.97	0.89	0.99	1.03	0.94	1.03
BRC32.1	0.86	0.94	0.97	1.04	0.98	1.00	0.96	0.94	0.98	0.91	0.94	0.98	0.95	1.04	0.95	0.99	1.03	0.98	1.04
BRC40.1	0.98	0.96	0.96	0.93	0.94	0.98	0.97	0.95	0.98	0.94	1.05	1.02	0.99	1.02	1.07	1.05	1.00	1.01	1.07
BRC41.1	1.02	1.02	0.98	0.94	1.02	0.92	1.02	0.98	0.95	0.87	1.08	1.01	0.98	1.09	0.94	0.92	1.00	0.95	0.95
BRC41.2	0.97	0.94	0.96	0.89	0.88	0.99	0.97	0.95	1.00	1.11	0.97	0.95	1.05	1.01	1.06	1.11	0.98	1.03	0.99
BRC41.3	0.94	0.93	0.98	0.93	0.96	0.95	1.02	0.98	0.98	0.96	1.03	1.03	1.03	0.97	0.96	0.99	1.04	0.96	1.08
BRC45.1	1.06	1.00	0.95	1.04	0.96	1.03	0.99	1.04	0.97	1.02	0.94	1.03	0.95	1.08	0.92	1.01	1.01	1.03	1.02
BRC46.2	1.02	1.03	1.02	0.99	1.07	0.99	1.08	1.03	1.00	0.89	1.08	1.05	0.96	0.99	0.99	0.98	1.07	1.04	1.02
BRC50.1	1.02	1.04	1.06	1.04	1.04	0.99	0.95	1.01	1.02	1.09	1.04	0.96	1.00	1.00	1.00	0.99	0.98	1.05	1.02
BRC54.1	1.09	1.09	1.01	1.02	1.01	0.96	1.02	1.14	0.96	0.99	0.99	1.02	0.96	1.04	0.95	1.02	1.04	0.99	0.94
BRC56.2	0.96	0.94	0.96	0.92	1.00	0.90	1.01	0.96	0.95	0.92	0.95	0.99	0.96	0.99	0.93	0.98	1.02	1.02	1.02
BRC59.1	1.04	1.03	1.01	0.95	0.98	1.03	0.93	0.98	1.01	0.90	1.02	0.94	0.94	0.95	1.00	0.95	0.98	0.93	1.02
BRC60.1	0.95	0.90	0.97	0.93	0.93	0.96	0.97	1.01	0.97	0.91	0.97	1.04	0.96	1.02	0.95	1.09	1.07	0.92	1.05
BRC60.2	0.94	0.99	0.96	0.90	0.95	1.00	0.99	1.02	1.05	1.04	0.98	0.97	0.98	1.05	0.98	0.98	1.02	1.01	1.03
BRC61.1	1.04	0.99	1.03	0.99	0.96	1.04	0.98	1.03	1.00	1.08	1.02	1.01	1.01	0.96	0.96	1.01	0.99	1.13	0.98
BRC66.1	0.90	0.91	0.96	0.97	0.94	1.00	0.99	0.93	1.03	1.14	1.00	0.96	1.08	1.00	1.07	1.02	0.95	1.05	1.06
BRC66.3	0.96	0.94	0.95	0.88	0.87	0.86	0.88	0.92	0.97	1.05	0.97	0.91	1.02	1.04	0.98	1.00	0.89	0.95	0.97
BRC67.1	0.97	0.96	0.97	0.92	0.93	1.02	1.01	0.97	1.00	1.03	1.01	0.95	1.00	1.05	1.03	1.03	0.98	0.99	1.00
BRC68.1	1.01	0.92	0.97	0.95	0.94	1.00	0.96	1.03	0.97	1.13	0.99	0.97	1.00	1.06	1.02	1.00	0.95	1.00	1.02
BRC68.2	0.94	0.96	0.97	0.97	0.92	0.94	0.96	0.94	0.98	1.04	1.00	0.96	1.02	1.03	1.03	1.01	0.98	1.02	1.08
BRC69.1	0.92	0.91	0.93	0.89	0.92	1.03	0.97	0.94	1.03	1.01	0.98	0.95	0.96	1.02	1.03	1.01	0.97	0.99	1.04
BRC71.1	0.90	0.94	0.96	0.91	0.88	1.03	1.01	0.94	1.02	0.97	0.98	0.97	1.03	1.01	1.06	1.06	1.00	1.02	1.01
BRC71.2	1.02	0.99	1.01	1.00	0.96	1.07	1.02	1.00	1.02	1.10	0.99	0.98	0.95	0.98	0.98	1.03	0.99	1.00	1.03
BRC73.1	0.95	0.89	0.98	1.03	0.95	1.02	1.03	0.94	1.00	1.03	0.92	1.06	0.96	0.95	0.98	1.06	1.01	1.02	1.06
BRC73.2	0.97	0.93	0.97	0.98	0.95	0.99	0.97	0.95	1.00	1.01	1.03	0.98	1.00	1.05	1.02	1.00	0.98	1.00	1.01

Sample Info	c.12p12	brca1-15	brca1-16	brca1-17	brca1-18	brca1-19	c.4q26	brca1-20	brca1-21	brca1-22	brca1-23	brca1-24	c.11p13	c.12p13	c.3p21
BRC4.1	0.99	1.05	1.02	0.98	0.99	1.03	1.01	1.04	0.93	1.06	0.94	0.97	1.03	0.93	1.08
BRC6.1	1.01	1.06	1.03	1.03	1.03	1.02	0.95	0.97	1.08	1.15	0.96	0.99	1.02	1.03	1.08
BRC9.3	0.95	1.01	1.00	1.02	1.00	1.05	1.01	1.03	1.02	1.03	1.05	1.03	1.02	1.03	0.99
BRC9.4	1.02	1.06	1.13	1.05	0.98	0.97	0.93	1.01	1.01	1.09	1.01	1.02	1.00	0.98	0.99
BRC11.2	0.94	1.05	1.00	1.00	1.02	0.96	0.94	0.98	1.01	1.00	1.02	0.99	0.92	0.93	0.85
BRC11.3	1.04	0.95	0.98	0.97	0.98	0.95	0.96	0.93	0.97	0.90	1.02	0.97	0.95	1.00	0.98
BRC14.1	1.09	1.02	0.96	0.94	1.01	1.05	1.02	1.07	0.99	0.96	1.02	1.00	1.08	0.97	1.09
BRC16.1	1.04	1.00	0.99	1.01	1.01	0.99	1.12	0.99	1.06	0.96	0.94	1.00	1.09	1.00	1.02
BRC19.1	0.99	1.05	1.11	1.11	1.15	1.16	1.07	1.11	1.12	1.08	1.18	1.19	0.99	1.07	0.95
BRC23.1	0.97	0.96	1.07	0.95	1.06	1.01	1.12	1.00	1.03	1.08	1.02	1.02	1.02	1.07	1.07
BRC26.1	0.99	0.97	0.98	0.94	0.98	0.97	1.01	0.83	0.95	0.99	0.85	0.85	0.85	0.92	0.89
BRC26.2	0.95	1.03	0.96	0.95	0.91	0.99	0.99	0.89	0.90	0.98	0.91	0.93	0.83	0.90	0.91
BRC28.1	1.02	1.00	0.98	0.99	1.04	1.02	1.08	1.05	1.03	1.14	1.04	1.02	1.07	1.05	1.07
BRC30.1	0.99	1.04	1.02	0.95	1.06	1.04	1.06	1.09	1.05	1.07	1.04	1.03	1.06	1.05	1.11
BRC32.1	1.02	0.97	1.00	0.99	1.07	1.05	1.00	1.09	1.08	1.11	1.10	1.12	1.10	1.10	1.10
BRC40.1	0.96	0.97	1.04	1.08	1.06	1.05	1.02	1.00	1.07	1.01	0.99	1.09	1.03	1.05	1.05
BRC41.1	1.03	0.99	1.01	0.92	1.05	1.05	1.15	1.02	1.04	1.01	0.91	1.01	1.15	0.95	1.09
BRC41.2	1.07	1.02	1.09	1.10	1.06	1.00	0.99	1.08	0.99	1.06	1.06	1.10	1.05	1.05	1.03
BRC41.3	0.98	0.99	1.04	1.03	1.03	1.10	1.05	1.04	1.04	1.04	1.08	1.07	0.96	1.10	1.06
BRC45.1	0.93	0.98	0.96	0.97	0.99	0.98	1.01	1.05	0.91	1.11	1.01	0.98	0.96	1.09	1.05
BRC46.2	0.98	0.96	1.02	0.96	0.98	0.99	1.01	0.88	0.99	1.08	0.99	0.94	0.87	0.88	0.88
BRC50.1	1.08	0.96	0.99	0.92	0.98	0.89	0.96	0.98	0.94	0.91	1.01	1.04	0.96	0.89	0.93
BRC54.1	1.02	0.97	1.00	0.94	0.97	1.03	1.04	0.93	0.95	0.96	0.99	0.94	0.92	0.89	0.92
BRC56.2	1.03	1.04	1.03	1.10	1.06	1.07	1.12	0.99	1.06	1.16	1.06	1.08	1.09	1.14	1.11
BRC59.1	0.98	1.11	0.96	1.08	1.06	0.94	0.97	1.03	1.05	1.16	1.02	1.03	1.03	1.00	1.04
BRC60.1	1.00	1.04	0.98	1.04	1.00	1.07	1.05	1.06	1.03	1.15	1.07	1.16	1.06	1.12	1.10
BRC60.2	1.01	1.09	1.05	1.00	1.01	1.03	1.01	1.07	1.03	1.03	1.04	1.02	1.01	0.97	1.07
BRC61.1	1.01	1.00	1.00	1.01	0.99	0.96	1.02	0.98	0.94	1.04	0.97	0.97	0.94	0.92	0.95
BRC66.1	1.01	1.07	1.03	1.06	1.02	1.02	0.96	1.08	1.01	0.95	1.12	1.01	1.01	0.98	1.02
BRC66.3	1.04	0.96	0.97	1.00	0.94	0.97	0.99	0.93	0.95	0.93	1.00	0.98	1.07	0.97	1.13
BRC67.1	1.03	1.05	1.01	1.09	1.02	1.03	1.01	1.03	1.05	0.91	1.00	1.14	1.05	1.03	1.07
BRC68.1	1.02	1.01	1.02	1.08	1.04	1.00	0.99	1.02	0.96	0.97	1.05	1.07	1.07	1.02	1.09
BRC68.2	0.98	1.04	1.04	1.08	1.05	1.09	1.01	1.00	1.07	0.94	1.09	1.09	1.05	1.10	1.04
BRC69.1	1.06	1.06	1.04	1.13	1.02	1.03	1.10	1.02	1.08	0.99	1.05	1.15	1.15	1.17	1.14
BRC71.1	0.97	1.09	1.02	1.03	1.06	1.04	1.13	1.09	1.06	1.00	1.01	1.01	1.10	1.15	1.07
BRC71.2	1.05	1.05	0.96	1.07	1.04	0.91	0.95	0.94	0.96	0.87	0.97	1.12	0.98	1.04	0.96
BRC73.1	0.92	1.02	1.06	1.11	1.03	1.05	0.92	1.02	1.10	1.05	1.10	1.11	0.98	1.05	0.92
BRC73.2	1.05	1.01	1.03	1.00	1.02	1.01	1.04	1.06	1.05	0.93	1.06	1.05	1.07	1.04	1.07

Sample Info	c.5q31	c.6p21	brca1-1a	brca1-1b	brca1-2	brca1-3	brca1-5	c.15q21	brca1-6	brca1-7	brca1-8	brca1-9	brca1-10	c.2q14	brca1-11.1	brca1-11.2	brca1-12	brca1-13	brca1-14
BRC79.1	1.05	1.00	0.99	0.93	0.97	0.97	0.93	1.01	0.99	0.95	1.00	1.02	0.98	1.01	1.02	1.02	0.99	0.96	0.99
BRC80.1	0.97	1.04	0.99	0.97	0.99	0.95	0.93	1.06	0.97	0.98	0.97	0.93	0.97	0.99	0.97	1.05	0.97	0.90	0.99
BRC84.1	0.98	1.01	1.05	1.01	1.01	0.98	1.00	0.97	1.00	1.06	0.99	0.96	1.02	0.99	0.99	1.00	0.95	1.05	1.01
BRC85.1	1.01	1.03	1.11	1.01	0.97	1.01	0.91	0.99	1.02	0.97	0.92	0.97	0.97	1.00	1.01	1.02	0.96	1.01	1.00
BRC86.1	0.99	0.98	1.10	1.06	1.00	1.07	1.00	1.02	1.06	1.03	1.02	1.04	1.05	1.02	1.06	1.04	1.02	1.06	1.01
BRC88.1	0.89	0.88	1.05	0.94	0.93	0.99	1.00	0.95	1.04	1.01	1.02	1.05	1.07	1.03	1.11	1.03	1.01	0.94	1.04
BRC92.1	1.14	1.11	1.13	1.02	1.12	1.05	1.01	1.10	1.06	0.92	0.96	0.97	1.04	1.07	0.98	0.99	0.94	1.01	0.94
BRC92.2	1.00	1.01	0.93	0.97	1.00	0.98	1.02	0.94	0.94	0.98	1.03	0.98	0.96	1.04	0.98	1.00	1.00	1.05	1.00
BRC92.3	0.92	0.90	1.01	0.97	0.95	1.01	1.00	0.88	1.08	1.05	1.08	1.01	1.04	0.98	1.09	1.00	1.01	1.03	1.04
BRC93.1	0.96	0.90	0.93	0.86	0.93	0.95	0.97	0.96	1.00	1.01	1.02	0.99	1.04	1.00	1.04	1.04	0.96	1.07	1.04
BRC94.1	0.94	1.01	1.01	0.97	0.98	0.99	0.97	0.90	0.93	0.97	0.92	1.00	0.99	1.00	0.96	0.98	1.01	0.86	1.03
BRC94.2	1.03	0.99	1.02	1.02	1.04	1.01	0.98	0.98	0.95	1.06	0.97	1.04	1.04	1.00	1.04	1.01	1.04	0.99	0.98
BRC95.2	1.01	1.02	1.09	1.05	1.03	1.05	1.06	1.02	1.00	1.02	1.00	1.02	0.97	0.97	0.98	1.00	0.97	0.98	1.03
BRC95.3	1.04	0.99	1.02	1.00	0.99	1.04	0.91	0.96	1.07	1.00	0.99	1.00	1.00	1.00	1.06	1.02	0.99	1.01	0.97
BRC96.1	0.95	0.88	1.03	1.01	0.89	0.99	0.90	0.99	0.91	1.01	0.99	0.98	1.03	1.02	0.96	1.01	1.02	1.01	1.03
BRC98.1	1.01	1.07	0.99	0.97	0.97	0.96	0.96	0.99	0.98	0.97	1.02	0.96	0.97	1.06	0.95	0.98	1.02	1.01	1.02
BRC100.1	0.99	0.99	0.92	0.95	0.98	1.01	1.06	0.95	1.00	0.96	1.01	1.02	1.04	1.00	1.02	1.02	0.97	0.97	1.01
BRC101.1	0.93	0.98	0.95	0.96	0.93	0.97	0.97	0.94	0.97	1.17	0.97	1.00	1.05	0.99	1.09	1.00	0.95	1.01	1.01
BRC114.1	1.02	1.07	0.97	0.93	0.97	0.96	1.00	1.02	0.91	0.98	1.07	0.99	0.97	1.03	1.01	1.04	0.97	0.97	1.01
BRC117.1	0.95	0.93	0.97	0.98	0.95	0.95	0.94	1.01	1.03	0.99	1.04	0.99	1.01	0.97	1.00	0.95	0.97	1.03	1.04
BRC119.1	0.99	0.97	1.01	0.96	0.94	1.00	0.93	0.98	1.01	1.02	1.04	1.02	0.98	0.99	1.00	1.03	0.99	1.08	1.04
BRC119.2	1.03	1.10	1.00	1.00	1.02	1.02	1.03	0.98	1.05	1.01	1.03	0.97	1.04	0.98	1.02	1.03	0.98	1.07	1.00
BRC121.1	1.09	1.11	1.02	1.04	1.01	1.03	1.01	1.06	1.10	1.08	1.03	1.01	1.00	1.03	1.06	1.04	1.01	1.11	1.00
BRC122.1	1.02	0.99	1.02	1.05	1.05	1.05	1.05	1.01	1.03	1.02	1.00	1.02	1.01	0.97	1.03	1.00	0.99	1.04	0.94
BRC127.1	1.02	0.98	1.03	1.05	1.04	1.03	1.04	1.03	0.99	1.03	1.03	1.00	1.05	0.98	0.97	0.94	0.97	1.02	0.99
BRC129.1	1.04	1.07	1.00	1.06	1.17	1.03	1.15	1.06	1.07	0.98	0.97	1.04	0.99	0.98	1.01	1.01	1.05	1.01	0.95
BRC132.1	1.00	0.99	0.97	0.98	0.99	0.96	1.02	1.03	1.02	1.01	0.97	1.04	0.98	1.01	0.97	1.06	1.04	0.97	0.96
BRC139.1	0.94	0.93	0.93	0.94	0.93	0.96	1.04	0.96	0.98	0.96	0.98	0.97	1.03	1.02	0.98	1.00	1.03	1.00	1.00
BRC139.2	0.97	0.98	0.96	0.93	0.94	0.97	0.99	0.97	1.00	0.97	0.99	1.01	1.01	1.00	1.00	1.03	1.03	1.04	1.00
BRC142.1	1.01	1.04	1.05	0.99	0.98	0.97	0.98	1.07	1.06	1.05	1.08	1.02	0.97	1.02	1.00	1.04	0.99	1.09	1.01
BRC145.1	1.00	1.09	1.13	1.06	1.15	1.14	1.13	0.93	1.10	1.14	1.13	1.08	1.13	0.91	1.09	1.05	1.13	1.02	1.06
BRC148.1	1.01	1.00	0.98	0.96	1.00	1.04	1.02	1.02	0.91	0.98	1.03	1.03	0.96	1.05	1.01	1.01	0.99	1.07	1.00
BRC149.1	0.99	1.05	1.06	1.01	1.03	0.97	1.02	0.98	0.97	0.99	1.04	0.99	1.01	0.97	1.02	1.00	0.98	0.96	0.99
BRC150.1	0.95	0.91	1.01	1.06	1.03	1.01	1.03	0.97	1.02	0.99	1.01	1.01	1.02	1.01	0.94	0.95	1.00	1.00	1.02
BRC155.1	0.88	0.89	0.91	0.93	0.93	0.95	0.91	1.00	0.98	0.92	0.94	0.96	1.01	1.00	0.98	1.02	0.99	1.01	1.10
BRC158.1	0.98	1.06	1.03	0.97	0.98	1.02	0.96	0.95	1.00	1.00	1.01	0.99	0.98	1.00	1.03	1.01	0.98	0.98	1.02
OV2.1	1.02	0.90	0.93	0.94	0.88	0.88	0.80	1.15	0.85	0.86	0.79	0.88	0.90	1.00	0.80	0.87	0.89	0.90	0.92
EX13DEL	1.01	1.05	1.01	0.97	0.97	1.02	1.00	0.96	0.96	1.02	1.00	0.97	1.00	0.96	0.97	1.01	0.98	0.48	0.99

Sample Info	c.12p12	brca1-15	brca1-16	brca1-17	brca1-18	brca1-19	c.4q26	brca1-20	brca1-21	brca1-22	brca1-23	brca1-24	c.11p13	c.12p13	c.3p21
BRC79.1	1.01	1.00	1.02	1.03	1.05	0.96	1.01	1.00	1.05	1.05	1.04	1.03	1.00	1.08	1.08
BRC80.1	1.06	0.96	1.02	1.00	1.01	1.02	0.97	0.98	1.18	0.98	1.07	1.01	1.17	1.06	1.12
BRC84.1	1.02	1.09	0.98	0.99	0.96	0.97	0.98	0.95	1.01	0.94	0.99	0.98	0.99	1.05	1.01
BRC85.1	0.95	1.10	1.00	1.00	1.03	0.93	0.99	1.02	0.96	1.05	1.08	1.04	0.97	1.06	1.01
BRC86.1	1.00	1.04	1.00	1.06	1.11	1.05	0.99	1.07	1.09	1.14	1.08	1.09	0.96	1.05	0.98
BRC88.1	1.07	1.06	1.08	1.02	1.05	1.10	1.00	1.19	1.12	1.08	1.10	1.07	1.09	1.15	1.17
BRC92.1	0.99	0.87	0.95	0.95	1.02	0.89	0.94	0.84	0.98	0.91	0.81	0.89	0.90	0.96	0.86
BRC92.2	1.05	1.00	1.01	1.02	0.99	1.06	1.06	0.94	1.06	1.12	1.02	1.04	1.09	1.06	0.98
BRC92.3	0.95	1.03	1.00	1.03	1.02	1.02	0.93	1.06	1.00	1.08	1.05	0.99	1.04	0.96	0.97
BRC93.1	1.04	1.05	0.98	1.04	1.03	1.05	0.97	1.17	1.04	1.07	1.06	1.09	1.16	1.02	1.16
BRC94.1	1.00	1.03	1.07	1.05	1.06	1.07	1.01	1.11	1.14	0.98	1.01	1.15	1.11	1.09	1.08
BRC94.2	0.97	0.98	0.95	0.96	1.04	0.99	0.90	0.99	1.05	1.06	0.96	0.96	0.96	0.96	0.96
BRC95.2	0.93	1.02	1.00	1.03	1.01	1.04	1.04	1.04	1.05	0.99	1.00	1.13	0.98	0.97	1.07
BRC95.3	0.94	1.03	1.00	0.98	0.98	0.95	0.94	1.06	0.95	1.03	0.97	1.04	1.00	0.96	0.95
BRC96.1	1.01	1.07	1.03	0.97	1.07	0.99	1.00	1.14	0.98	1.15	1.02	1.07	1.01	1.04	1.04
BRC98.1	1.02	0.99	1.02	1.01	1.00	1.01	1.07	1.02	0.95	0.98	1.01	1.10	0.99	1.04	1.03
BRC100.1	1.02	1.03	1.00	1.10	1.03	1.05	1.06	1.04	1.07	0.69	1.05	1.09	1.04	1.01	0.97
BRC101.1	1.01	1.02	1.00	1.02	0.92	1.06	1.03	1.14	1.10	0.99	1.05	1.04	1.13	1.04	1.10
BRC114.1	1.04	1.04	1.03	1.06	1.01	1.02	1.08	0.98	0.99	0.93	1.01	1.06	0.97	1.02	1.09
BRC117.1	1.02	1.02	0.98	1.04	1.09	1.00	1.07	1.05	1.05	1.08	0.99	0.99	1.03	1.03	1.05
BRC119.1	1.08	1.00	1.07	1.02	0.63	0.99	1.06	1.06	0.95	1.05	1.02	1.14	1.05	0.99	1.06
BRC119.2	0.96	0.99	1.04	1.04	0.58	1.01	1.01	0.97	0.97	0.94	0.99	1.09	1.00	0.97	1.00
BRC121.1	1.01	0.99	0.98	0.92	0.98	0.91	0.89	0.85	0.90	0.82	0.84	0.85	0.88	0.87	0.84
BRC122.1	0.96	0.91	0.97	1.00	1.04	0.96	0.98	0.99	0.99	1.01	0.97	0.92	0.89	0.93	0.93
BRC127.1	0.97	0.98	1.00	0.98	0.98	0.96	0.96	0.93	0.95	0.99	1.06	0.97	0.93	0.97	0.92
BRC129.1	0.95	0.88	0.87	1.04	0.99	0.97	0.97	0.83	1.00	0.97	0.94	0.83	0.90	0.91	0.89
BRC132.1	0.99	0.99	0.97	1.06	1.02	0.96	1.03	0.99	1.05	1.10	1.01	0.91	1.01	1.06	0.97
BRC139.1	0.99	1.05	1.03	1.04	1.07	1.06	1.05	1.07	1.10	1.17	1.08	1.01	1.00	1.11	1.04
BRC139.2	1.03	1.01	1.07	1.09	1.08	1.01	1.05	0.98	1.05	0.99	1.03	0.86	1.08	1.10	1.10
BRC142.1	1.03	0.96	0.98	0.97	0.96	0.96	1.04	0.86	0.87	0.86	0.88	1.01	0.96	0.94	1.05
BRC145.1	1.04	1.09	1.13	1.12	1.13	1.16	1.07	1.01	1.05	1.05	1.18	1.03	0.96	1.05	0.98
BRC148.1	1.02	0.98	1.01	1.03	0.99	1.05	1.05	0.98	0.98	1.04	1.02	0.86	1.00	1.06	1.02
BRC149.1	0.98	1.04	1.01	0.98	1.02	1.07	1.00	1.04	0.98	0.97	1.00	1.07	1.01	1.03	0.99
BRC150.1	0.97	0.99	0.95	1.03	0.97	1.00	0.99	1.00	1.13	1.03	0.97	0.97	1.07	1.04	0.99
BRC155.1	1.00	1.10	0.99	0.93	1.13	1.08	1.02	1.15	1.09	1.06	1.13	1.16	1.18	1.16	1.14
BRC158.1	1.02	1.05	1.00	1.02	1.02	1.02	1.03	0.95	1.02	0.99	0.54	0.55	0.94	1.01	1.03
OV2.1	0.96	0.90	0.82	0.86	0.93	0.87	1.02	0.94	0.91	0.91	0.88	0.91	1.00	0.89	1.12
EX13DEL	0.98	1.03	1.00	1.01	1.03	1.01	1.01	1.00	1.04	1.05	1.01	0.97	0.98	1.01	1.04