Screening of young and/or familial African breast cancer patients for the presence of *BRCA* mutations

By

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DECLARATION

I certify that the dissertation hereby submitted for the degree *M.Med.Sc* at the University of the Free State is in my independent effort and had not previously been submitted for a degree at another University/Faculty. I furthermore waive copyright of the dissertation in favor of the University of the Free State.

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N Peter

DEDICATION

I dedicate my thesis to my parents whom God utilized as vehicles to bring me into existence. Mpevu and maMgxongo I thank you profusely for the values you instilled in me. To my siblings thank you for your support, unselfish and nobel actions throughout my studies over the years. My beloved husband, for your prayers and unconditional love, for supporting my career and studies, thank you Jola.

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Last but not least God almighty, all things are possible through Christ who gives us strength.

Table of contents

List of Figures

2036 (lane 4), CAM 2037 (lane 5) and CAM 2038 (lane 6). The HA band shift for CAM 2035 is indicated by the arrow. **B.** DNA sequencing results for *BRCA2* exon 27B for CAM 2035 indicating the single base pair change, namely C>T (*BRCA2* c.9649-62C>T).

List of Tables

number, the nucleotide involved, the base change involved, the amino acid change if any, the new HGVS name, the number of entries onto the BIC, the mutation type, the ethnicity group in which the change has been detected and the rs number if available.

List of Abbreviations and Symbols

%	Percentage
≥	Greater than
-	Negative
+	Positive
°C	Degrees Celsius
®	Registered trademark
3'	Three prime end
5'	Five prime end
5'UTR	Five prime untranslated region
A	Adenine (in DNA sequence)
$AgNO_3$	Silver nitrate
Asn	Asparagine (amino acid)
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and RAD3 related
BC	Breast cancer
BC	Before Christ
BIC	Breast Cancer Information Core
bp	Base pair
BRC	*BRCA2* repeat motif
BRCA1	Breast Cancer Gene number one
BRCA2	Breast Caner Gene number two

BRCT	*BRCA1* C-terminus
BSE	Breast Self-Examination
C	Cytosine (in DNA sequence)
ca	Cancer
CBE	Clinical Breast Examination
CC	Cervical cancer
del	Deletion
dH_2O	Distilled water
DI	Deionized water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	1, 4-Dithiothreitol
dx	Age at onset
EDTA	Ethylenediamine tetra-acetic acid:$C_{10}H_{16}N_2O_8$
ER	Estrogen
et al.	Et alia (Latin abbreviation for 'for example")
EtBr	Ethidium bromide (2,7 –diamino-10-ethyl-9-phenyl-phenantridium bromide):$CH_3H_{20}BrN_3$
EtOH	Ethanol:CH_3CH_2OH
ETOVS	Ethics committee University of the Free State
Fig	Figure
g	Gram
G	Guanine (in DNA sequence)

Glycerol	$C_3H_5(OH)_3$
h	Hour
HA	Heteroduplex
HCl	Hydrogen Chloride
HER2nue	Human epidermal growth factor receptor 2
HGVS	Human Genome Variation Society
HIV	Human immunodeficiency virus
HR	Homologous recombination
HRM	High resolution melting
IHC	Immuno-Histo Chemical
Ile	Isoleucine (amino acid)
in vitro	Latin abbreviation for "in a test tube"
IVS	Intervening sequence
kb	Kilo base pair
KCl	Potassium Chloride
kD	Kilo Dalton
KOAc	Potassium acetate
LOVD	Leiden Open Variation Database
Lys	Lysine (amino acid)
MAF	Minor alle frequency
M	Missence mutation
M	Molar (moles per liter)
mg/ml	Milligrams per milliliter

$MgCl_2$	Magnesium Chloride
min	Minutes
ml	Milliliters
MLPA	Multiplex ligation-dependent probe amplification
mM	Milli molar
mm	Millimeter
Mre11	Meiotic recombination 11
NaCl	Sodium chloride
$NaCO_3$	Sodium Carbonate
ng	Nanogram
ng. μl^{-1}	Nanogram per microlitre
NHLS	National Health Laboratory Services
Nbs1	Nijmegen breakage syndrome
NHEJ	Non-homologous recombination
NLS	Nuclear localization sequence
NGS	Next generation sequence
PCR	Polymerase chain reaction
pH	Potential of Hydrogen
Phe	Phenylalanine (amino acid)
pmol	Pico molar
PALB2	Partner and localizer of *BRCA2* gene
PR	Progesterone
PTT	Protein truncation test

RAD51	Homolog of RecA of E.coli
RB	Retinoblastoma protein
Rs	Reference SNP ID number (RefSNP)
SA	South Africa
SANCA	South African National Cancer Registry
SDS	Sodium dodecly sulphate: $C_{12}H_{25}NaSO_4$
sec	Seconds
Ser	Serine (amino acid)
SES	Socio-Economic Status
SET	Sodium chloride EDTA- Tris HCl
SSCP	Single strand conformation polymorphism
Syn	Synonymous
T	Thymine (in DNA sequence)
T_a	Annealing temperature
Taq	DNA polymerase (EC 2.7.7.7) isolate from *Thermus aquaticus*
TB	Tuberculosis
TBE	Tris borate-EDTA buffer (5X TBE: 0.089M Tris, 0.089M boric acid, 0.002M EDTA)
TEMED	N,N,N' ,N' –tetrametylethylenediamine
Tris	2-Amino-2-(hydroxymethyl)-1,3-propanediol:$C_4H_{11}NO_3$
™	Trademark
UK	United Kingdom
USA	United States of America

UV	Ultraviolet
V	Volts
v/v	Volume per volume
w/v	Weight per volume
WHO	World Health Organization
µ	Micro
$\mu g.ml^{-1}$	Micrograms per milliliter
µl	Micro liters
µM	Micromolar

Chapter 1

Introduction

Cancer is a global problem. Increasing cancer research is a collaborative international endeavor. Studying breast cancer (BC) in women of African descent can make an important contribution to our understanding of the global dimensions of this problem in Black women around the world. It will help define many of the relevant research questions and methodological issues that must be addressed if Black women are to fully benefit from the many scientific advances that are being made against BC.

The importance and relevance of race for the genetics and biology of the disease are controversial and poorly understood. However, it does appear that some of the disparity in outcome can be attributed to lack of access to high quality medical care, both for screening and early detection and for treatment. Less access to, or utilization of, screening services, such as mammograms, may explain in part the tendency toward more advanced disease at presentation that contributes to a higher mortality rate in Black women. Black women are distributed throughout the Diaspora; cultural issues may differ significantly from community to community. The impact of cultural beliefs and traditions on treatment and course of BC cannot be ignored.

To date, very little information exists regarding the prevalence of BC gene mutations in other South African (SA) population groups such as the Black Africans and Coloureds (van der Merwe *et al.,* 2012). One in 36 SA women is at risk to develop BC with a lifetime risk of one in 81 for Black woman, one in 63 for Coloured women and one in 13 for Caucasian female population (Vorobiof *et al.,* 2001; Reeves *et al.,* 2004). Since 1993, BC has become the most common cancer found in SA women with an incidence of 16.6% (Sitas *et al.,* 1998; Vorobiof *et al.,* 2001). The majority of BC cases are sporadic with only 5%-15% being attributed to an inherited predisposition (Boyd, 1995; Liu and West, 2002;

Thompson and Easton, 2002; Sigurdson *et al.,* 2004; Yoshida and Miki, 2004; Henderson, 2005).

The breast cancer susceptibility gene 1 (*BRCA1*) and the breast cancer susceptibility gene 2 (*BRCA2)* are both relatively large genes and several mutations spanning the entire coding sequence of both have been reported (Breast Cancer Information Core database http://research.nhgri.nih.gov/bic). Mutation frequencies vary among different ethnic and geographically distinct populations. Few *BRCA1* and *BRCA2* studies have been done on African populations. Even less comprehensive molecular-genetic studies have been completed for the various SA populations. To be able to implement predictive genetic testing for this disease successfully in SA, the genes have to be characterized in our BC families. The mutation spectrum has to be to determined and possible founder mutations identified. The aim of the study is to screen young/familial Black African BC patients for the presence of *BRCA* mutations. This study will help to define the diagnostic settings in which genetic testing will become an indispensable tool to improve clinical outcomes within SA.

Chapter 2

Literature Review

2.1 Breast cancer

Breast cancer is a malignant (cancerous) growth caused by the abnormal and uncontrolled growth and division of breast cells. It originates in breast epithelium and is associated with progressive molecular and morphological changes (Dooley *et al.,* 2001). The cancer cells can invade and destroy surrounding tissue and spread throughout the body via blood or lymph fluid to metastasize at new sites. Breast cancer mainly affects women, although less commonly, men can also develop BC (Wei, 2007). It is caused by mutations in genes responsible for regulating both the growth and well-being of cells. Breast cancer is clinically a heterogeneous disease (Cianfrocca and Gradishar, 2009). The two most common types of breast cancer are named after the parts of the breast in which they start; namely lobular and ductal carcinoma (http://www.cansa.org.za). The cancer types may be classified into *in-situ* and invasive carcinomas. Lobular and ductal patterns are seen in both *in-situ* and invasive categories, with lobular carcinoma occurring in about 8% of cases and ductal carcinoma in 85 to 90% of all cases. The other subtypes of invasive BC are less common (Wei, 2007).

When abnormal cells grow in an uncontrolled manner, they form a tissue mass called a tumour which can be benign or malignant. Benign tumours are not cancerous, do not spread to other parts of the body, are surgically removable and do not recur (www.cancer.gov). Malignant tumour cells on the other hand are cancerous.

Until recently, BC was treated as a single deadly disease for which extreme treatments were necessary. In 1600 B.C., Egyptian physicians recorded the use of cauterization to treat breast cancers, while extensive surgery removing the breast and all the surrounding muscle and bone was used during the Renaissance

(Singletary and Connolly, 2006). Clinicians became aware that not all BC affected patients share identical prognoses or require the same treatment. Attempts were made to describe characteristics that could consistently differentiate tumours that required aggressive treatment from those that did not. In 1904, the German physician Steinthal suggested the division of BC into three prognostic stages: small tumours that appeared to be localized to the breast (Stage I), larger tumours that involved the axillary lymph nodes (Stage II) and tumours that have invaded tissues around the breast (Stage III) (Singletary and Connolly, 2006). Later in 1925, Greenough introduced stage (IV) representing disease that had metastasized throughout the body (Greenough, 1925).

Well known risk factors of BC include the presence of a positive family history (Silvera *et al.,* 2005) and various epidemiological factors. These include factors such as a high breast tissue density, hormone related factors (early onset of menstruation, late menopause, late first-term pregnancy, nulliparity, no breast-feeding, more than four years use of hormone replacement therapy, postmenopausal obesity), alcohol consumption, exposure to cigarette smoke and radiation (American Cancer Society, 2005; Tam *et al.,* 2010). There is conflicting data regarding the use of oral contraceptives and BC prognosis (Williams *et al.*, 2006).

2.2 *BRCA* genes

BRCA1 is localized to chromosome 17q21 (Hall *et al.*, 1990, Miki *et al.,* 1994) and contains 24 exons of which 22 are coding. The *BRCA1* mRNA of 7.8 kb encodes a full-length protein of 1863 amino acids (Deng, 2006). Wooster (1995) and Tavtigian (1996) localized the second breast cancer susceptibility gene, *BRCA2* on chromosome 13q12-13. The *BRCA2* gene is larger consisting of 27 exons of which 26 are coding for a 3418 amino acid nuclear protein (Wooster *et al.*, 1995; Tavtigian *et al.*, 1996). Distinct variants of *BRCA1* and *BRCA2* have been identified across all population groups, of which 710 *BRCA1* and 872 *BRCA2* have been identified as deleterious (www.research.nhgri.nih.gov/projects/bic). Of these pathogenic variants, only 24 *BRCA1* and 18 *BRCA2* mutations were reported to be present in populations of African descent (Williams *et al.*, 2006).

Both *BRCA1* and *BRCA2* are involved in maintaining genome integrity at least in part by engaging in DNA repair, cell cycle checkpoint control and even the regulation of key mitotic or cell division steps (O'Donovan and Livingston, 2010). Due to their function in cell cycle regulation and the cellular damage response, mutations in these genes are expected to lead to susceptibility for deregulation and cancer in more than one tissue type. It is not completely understood why mutations in these two genes are mainly involved in malignancies of the breast and ovaries, but it is thought that the interaction with estrogen and progesterone could play a role (Schlebusch *et al.*, 2010).

There appears to be differences in age of onset of BC and also in the type of cancers that develop even within families with the founder mutation. Neuhausen (2000) suggested that there are both genetic and lifestyle factors that modify the penetrance of *BRCA1* and *BRCA2.* Penetrance is the probability that a person who inherits the mutation, will manifest signs of the associated condition (Loubser *et al.,* 2008). Penetrance of individual mutations is influenced by non-genetic factors. This means BC can be prevented by early intervention if treatment is targeted at the combination of contributing genetic and lifestyle risk factors (Kotze *et al.,* 2005).

BRCA1 and *BRCA2* mutations are without doubt important determinants of risk for breast and/or ovarian cancers, but they are not the only genes involved in familial BC. One other major breast cancer susceptibility gene is proposed to exist (De Jong *et al.,* 2002). Women with a family history of breast and/or ovarian cancer that do not have a *BRCA1* or *BRCA2* mutation may have a mutation in an as yet undiscovered gene (Neuhausen, 2000). After accounting for *BRCA1* and *BRCA2,* Peto *et al.* (1999) suggested that there are several other genes, possibly of lower risk, that account for a proportion of non-*BRCA* breast cancers.

The phenotype of breast cancers in women carrying *BRCA1* mutations differs from that of women carrying *BRCA2* mutations and sporadic cases (Williams *et al.,* 2006). *BRCA1* mutations tend to be of higher histological grade, have a higher proportion of tubular differentiation, all of which are poor prognostic features (Williams *et al.,* 2006). *BRCA2* associated tumours are more similar to sporadic breast tumors, because they are more often than not of intermediate grade, are normally hormone receptor positive, and occur at later ages compared to *BRCA1* associated tumours (Williams *et al.,* 2006).

2.3 Familial (hereditary) breast cancer

Familial BC exhibits an autosomal dominant mode of inheritance (www.cancer.gov) (Figs. 2.1 and 2.2). According to the Mendelian mode of inheritance, every affected individual (or mutation carrier) has at least one affected parent (or mutation carrier), but two affected parents or mutation carriers may have unaffected children or children not carrying the mutation (Sitas *et al.,* 1998). Hereditary BC accounts for 5 – 10% of all familial breast cancers with 90-95% accounting for sporadic cases. The difference between familial and sporadic cancer is in the majority of cases determined by early age of onset, excess bilaterality, specific tumour associations, vertical transmission and improved survival (Phipps and Perry, 1988).

An early onset of BC has been associated with a greater incidence of unfavorable prognostic features such as hormone receptor negativity, advanced stage distribution and worse outcome (Newman *et al.,* 2002). Most BC in women with a *BRCA1* mutation are diagnosed at a young age (between ages 30 and 50 years) and are triple negative for their Immuno-Histo Chemical (IHC) status (Estrogen (ER), Progesterone (PR) and Human epidermal growth factor receptor 2 (HER2nue). To a large extent, the natural history of cancer in women carrying *BRCA* mutations is similar to that of young women with triple-negative BC (Narod, 2010). The most significant features of triple-negative cancers include a susceptibility to a high risk of early recurrence (one to four years after diagnosis) of breast cancer and a reduced association between tumor size, lymph-node status and survival (Dent *et al.,* 2007).

2.4 Founder Mutations

Founder populations have proven to be a powerful resource to localize additional breast cancer susceptibility loci, because of the reduction in locus heterogeneity. Founder mutations normally occur within ethnic isolates such as the Dutch and French–Canadian population groups. A common set of founder mutations is present in Eastern Europe encompassing Poland, Russia, Belarus and the Baltic states, reflecting their common Slavic ancestry (Narod, 2011). Founder mutations

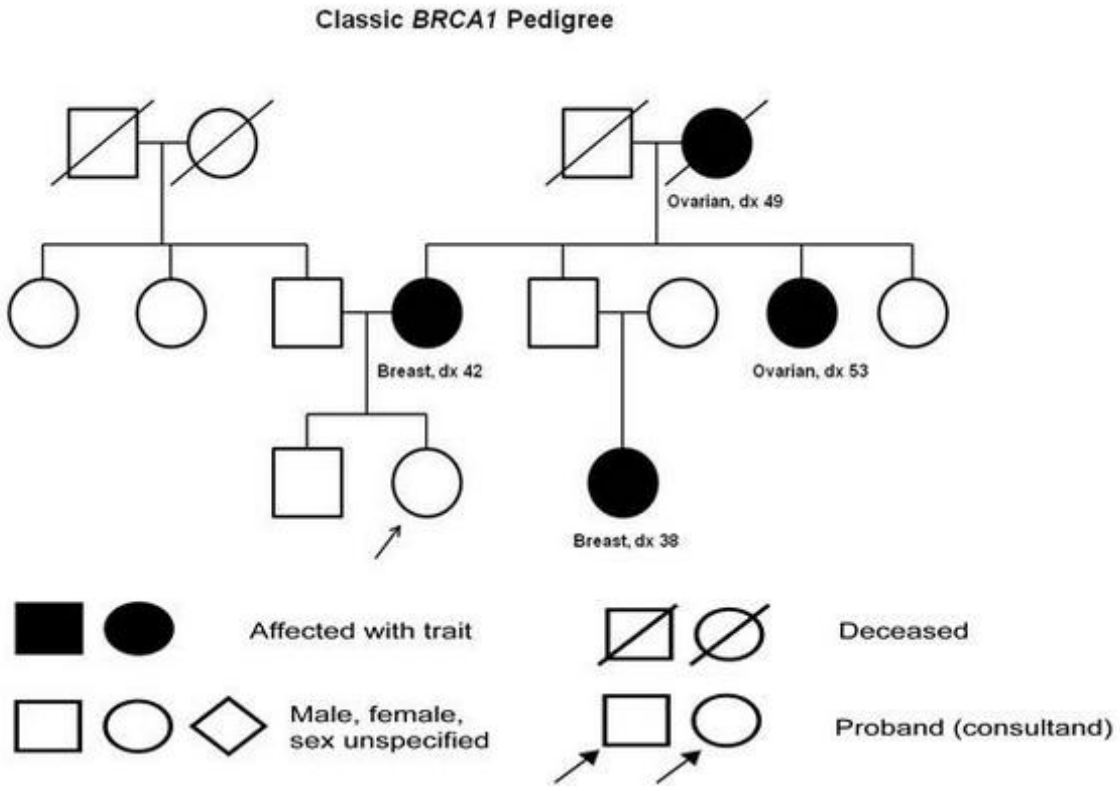


Figure 2.1 A classical pedigree showing the features of a family with a deleterious *BRCA1* mutation across three generations, including affected family members with breast or ovarian cancer and a young age at diagnosis (dx). *BRCA1* families may exhibit some or all of these features. As an autosomal dominant syndrome, transmission can occur through maternal or paternal lineages, as illustrated in the figure (source www.cancer.gov).

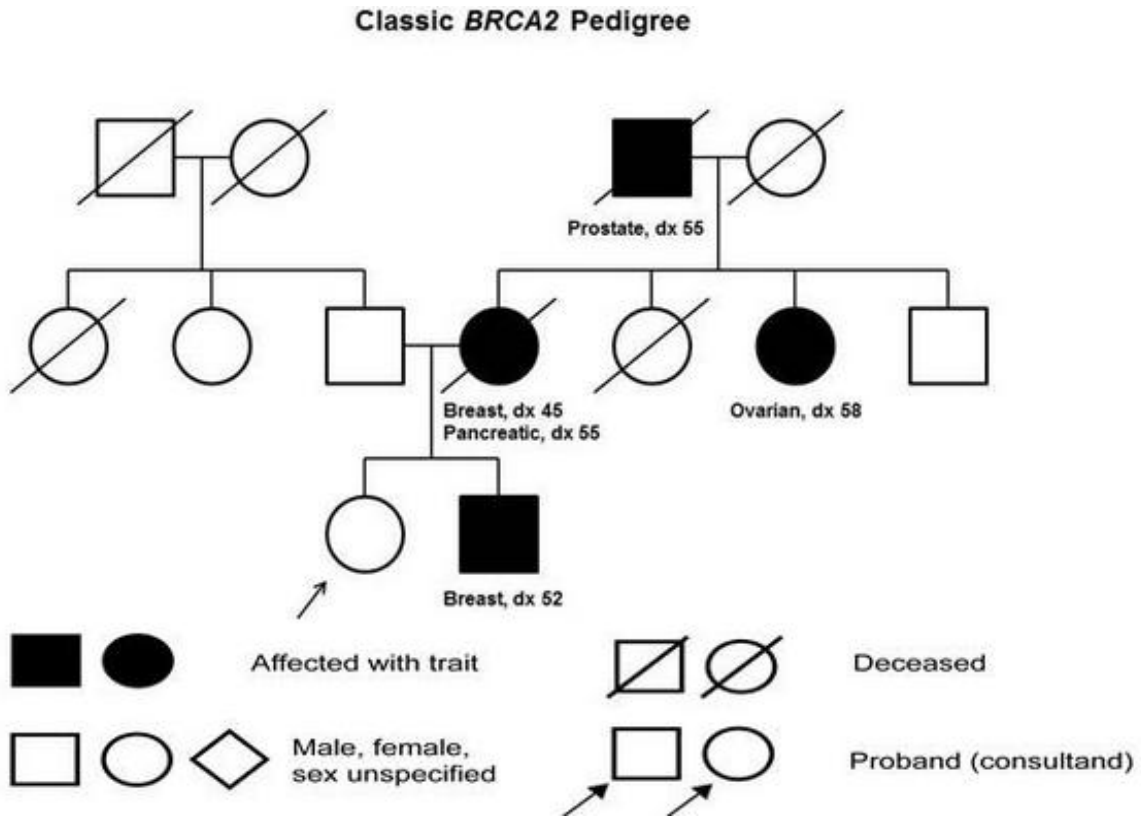


Figure 2.2 A classical pedigree showing features of a family with a deleterious *BRCA2* mutation across three generations, including affected family members with breast (including male breast cancer), ovarian, pancreatic or prostate cancers and relatively young age at diagnosis (dx). *BRCA2* families may exhibit some or all of these features. As an autosomal dominant syndrome, transmission can occur through maternal or parental lineages, as depicted in the figure (source www.cancer.gov).

have also been seen in island populations such as Iceland (Tulinius *et al.,* 2002), Greenland (Harboe *et al.,* 2009), Cyprus (Loizidou *et al.,* 2008) and the Bahamas (Donenberg *et al.,* 2011).

Founder mutations seem to be less common in the United Kingdom, Western and Southern Europe, Asia and Africa (Narod, 2010). BC has a genetic basis of which expression varies in different population groups (Vona-Davis and Rose, 2009). Ethnic differences determine cancer risk and potential preventive measures and treatment (Wiencke, 2004).

The best-known example of a founder effect is present within the Ashkenazi Jewish population. The term Ashkenazi is used to describe Jews who have ancestors from Eastern and Central Europe, such as Germany, Poland, Lithuania, Ukraine and Russia. Hereditary BC occurs at a much higher percentage within this group, as an individual of Ashkenazi Jewish descent often carry one of their three founder mutations. Firstly, the *BRCA1* 185delAG (c.68_69del, p.Glu23ValfsX17) mutation is found in 1% of the Jews and contributes to 16–20% of BC diagnosed before age 42 (Peto *et al.*, 1999). A second founder mutation in the *BRCA1* gene, 5382insC (c.5266dup, p.Gln1756ProfsX74) is found in 0.13% of this population. The third founder mutation, 6174delT (c.5946del, p.Ser1982ArgfsX22) in the *BRCA2* gene, has a frequency of 1.52% in Ashkenazi Jews. Eight percent of Ashkenazi women with BC diagnosed before age 42 and 7% of those with BC at age 42–50 with a strong family history of breast cancer or ovarian cancer are carriers of this mutation (Ferla, 2007).

Founder mutations with the *BRCA* genes have been previously reported for multiple populations worldwide (van der Merwe *et al.*, 2012). A study of 90 Afrikaner breast and ovarian cancer families containing three or more affected individuals identified four exon 11 *BRCA1* mutations within the South African Afrikaner population (Reeves *et al.*, 2004). These included a previously reported 1127sinA, the novel E881X (2760 G-T), 1493delC and S451X (1471C-A) mutations. Haplotype analysis revealed that each of these mutations originated from a single mutational event and is internationally unique to SA.

Identification of founder mutations in the various ethnic groups is extremely important towards the development of genetic counseling since it makes it possible to use a more specific approach to molecular testing that would also be cheaper and quicker. A less expensive mutation detection strategy might allow

the extension of genetic counseling and testing to families with a low hereditary history such as the Black African. The high frequency of founder mutations allows for the analysis of large families that might provide more accurate information regarding their penetrance. Evidence of differences in susceptibility and in age onset of cancer among carriers of a specific mutation could make it possible to define the role and importance of risk-modifying factors with the resulting improved disease management (Ferla, 2007).

2.5 Geographical distribution of South African Ethnic groups

South Africa is a diverse country of over 51 million people divided into 79.2% of Black African origin, 8.9% of White origin, 8.9% of Mixed-race origin, 2.5% of Asian/Indian origin and 0.5% making up for other (Census, 2011). Although they represent approximately 80% of the population, the Black African group is neither culturally, linguistically nor genetically homogenous. The Black South African population is reported to have migrated from central Africa in three main groups, namely the Ngunis (Xhosa, Tembu, Swazi and Zulu) along the east coast, the Sothos (South Sotho, North Sotho/Pedi, West Sotho/Tswana) who settled further west on the Transvaal highveld, and the Vendas living in the then northern Transvaal area (Du Toit *et al.*, 1987) (Fig. 2.3).

In African populations founder mutations are difficult to identify, most probably due to the diversity in the African diaspora (Fackenthal *et al.,* 2005). As Black African family structures are quite complicated, it tends to be extremely difficult to follow heritable diseases.

2.6 Biology of BC in Africa

The large number of resource poor nations in Africa makes it extremely difficult to have accurate estimates of the number of diagnosed BC cases. There is therefore a lack of data on the genetics of BC in the African Black female. The fact that Black women develop BC at a younger age compared to White women, suggests that risk factors for early onset BC may be different for Black women as a whole. Unique genetic features of racial groups combined with environmental

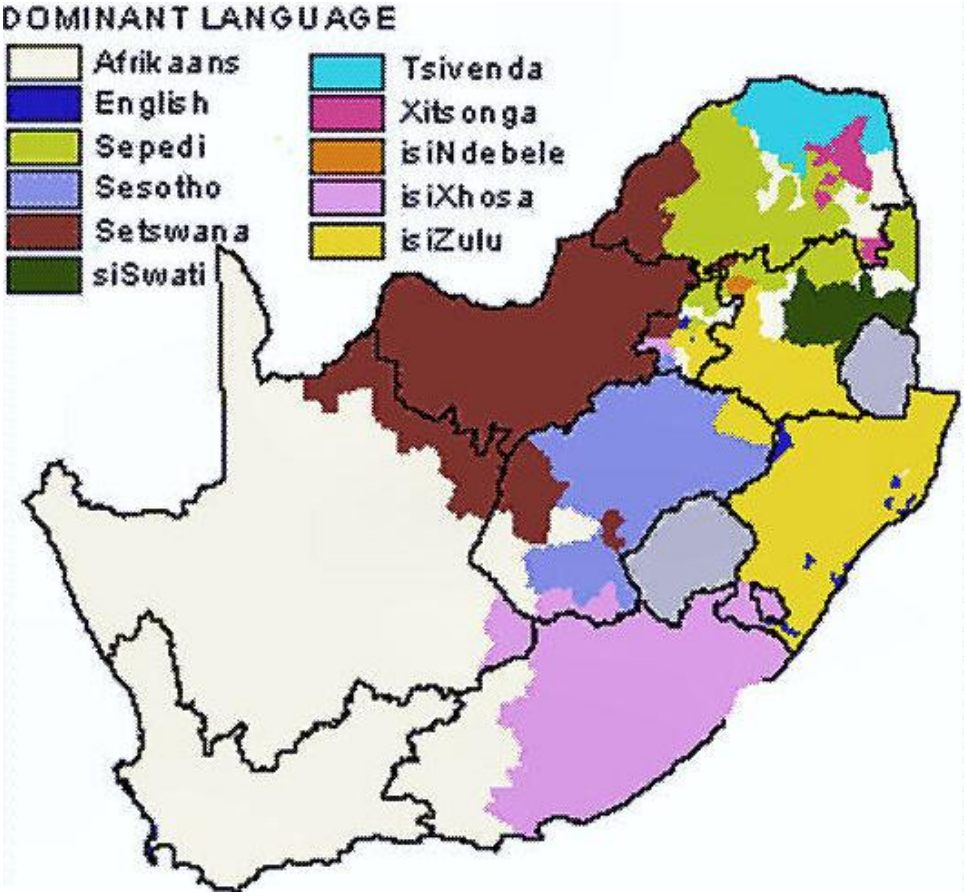


Figure 2.3 Population groups map of South Africa. http://www.theoccidentalobserver.net/authors/Kurtagic-Deconstruction.html

factors can influence carcinogenic mechanisms leading to biological differences in molecular profile of a tumour (Taioli *et al.,* 2010).

In general, African BC patients are diagnosed at a young age and are triple negative to the IHC status (Vona-Davis and Rose, 2009). African patients have more aggressive, larger tumours and multiple nodal involvements (Easton, 2005; Fregene and Newman, 2005). They are also characterised by advanced stages of disease and poor clinical and pathological prognostic factors (Adebamowo *et al.,* 2003).

2.7 The burden of BC in Africa

Just about all the current investment in Africa's health systems is focused towards communicable diseases such as malaria, cholera and HIV (Munemo, 2010). This is caused mainly by the fact that cancer is regarded a Western disease (Munemo, 2010). In Africa and other developing countries, the BC burden is increasing and poor reporting and data availability may underestimate the exact statistics (Kruger and Apffelstaedt, 2007). The influence of ethnic origin on BC prognosis has been a controversial matter (Dansey *et al.,* 1988).

BC is considered the most common cancer related to deaths amongst women worldwide. While the incidence of BC is higher in White women compared to Black African women, the mortality pattern is the opposite. Many factors contribute to these disparities including inequalities in access to healthcare, poverty and lower education levels (Bradley *et al.,* 2002).

Literature predicts that by 2020, there will be 70% more cancer cases in developing countries. The majority of these cancers occurring in females will be of the breast (Kruger and Apffelstaedt, 2007). In Africa, the cancer epidemic is not only related to the underestimation of the incidence of tumours but mostly to the low level of cancer education and awareness. The ignorance of the general population has led to devastating results as seen in the high mortality rate (Munemo, 2010).

In 2010, the World Health Organization (WHO) stated that cancer was the leading cause of death with a mortality rate above that of HIV, malaria and TB combined. Unless some intervention takes place to halt this problem, cancer will

become another of Africa's burdens, adding to poverty and starvation (Munemo, 2010).

In the first study of its kind, the entire coding regions and intron/exon boundaries of *BRCA1* and *BRCA2* were screened for mutations in 70 African BC patients younger than 40 years (Gao *et al.,* 2000). These patients were identified at the University of Ibadan College of Medicine, Nigeria, and were randomly selected. In this study, two *BRCA1* truncation mutations, four *BRCA1* missense mutations, one *BRCA2* truncation mutation and nine different *BRCA2* missense variations were identified. The protein truncation *BRCA1* mutations, Q1090X and 1742insG, were unique to this cohort while the amino acid substitutions had been described in other populations. The *BRCA2* truncating mutation, 3034del4 had been identified as a mutational hotspot. One missense variation was unique to the African cohort, while other alles had been previously reported as unclassified variants.

A study by Fackenthal *et al.* (2005) analyzed the frequency and mutational spectra of *BRCA1* and *BRCA2* germline mutations in 39 Nigerian breast cancer patients. From this cohort, 29 of the 39 carried at least one *BRCA1/2* genetic variation with 69% having sequence variations in *BRCA2.* Only one truncation allele was detected, namely *BRCA2* 3034del4. No founder mutation was identified from this analysis. Five of the 13 different detected variants (38%) were rare non-protein truncation *BRCA2* alleles that were not detected in a population of 74 unaffected Nigerian control subjects. Eleven different *BRCA1/2* alleles were shown to be potentially deleterious, suggesting that the significant level of genetic variation in *BRCA1* and *BRCA2* may contribute to breast cancer risk in populations of African ancestry.

In 2012, Fackenthal and colleagues completed a sequence analysis of *BRCA1* and *BRCA2* exons and intron-exon boundaries for 434 Nigerian breast cancer patients from the University College Hospital in Ibadan, Nigeria. Contrary to previous suggestions that *BRCA1/2* mutation frequencies are low or undetectable in African American populations, this study found that Nigerian breast cancer patients have a high frequency of *BRCA1* and *BRCA2* mutations (7.1 and 3.9% respectively). Sixteen different *BRCA1* mutations were detected, of which seven were never reported previously. Thirteen different *BRCA2* mutations

were identified, six of which were previously unreported. The data supported enrichment for genetic risk factors in this relatively young cohort.

A study by Boder *et al.* (2011) evaluated the incidence of BC in Libya where they described the clinical-pathological and demographic features of the disease. These features were compared with corresponding data from patients from Nigeria and Finland. The study consisted of 234 breast carcinoma patients admitted at the African Oncology Institute in Sabratha, Libya over a 5-year period (2002-2006). The study concluded that in Libya and other African countries, premenopausal BC was more common than postmenopausal breast cancer, while the opposite was true for Europe.

In Egypt, the majority of BC tumours are advanced at presentation, and BC is largely responsible for the 8.2% cancer-related deaths amongst females (Boder *et al*., 2011). Due to late diagnosis, BC in Tunisia is associated with a poor survival rate. Approximately 55% of the BC patients presenting at the Tunisian Oncology Institute of Salah Aziiz were found to be characterized by rapid disease progression, inflammation and edema (Boder *et al*., 2011).

A descriptive study by Elgaili *et al.* (2010) shed light on the type, stage and age distribution of BC at diagnosis of women living in central Sudan. The study comprised of 1255 women from central Sudan diagnosed with breast cancer. These women were referred and treated at the Institute of Nuclear Medicine, Molecular Biology and Oncology from January 1999 to December 2006. The study found that 74% of the women were premenopausal (aged 50 and younger) and 26% postmenopausal, indicating early onset of the disease. This observation was consistent with literature as was reported in several other studies conducted in sub-Saharan Africa (Elgaili *et al.,* 2010).

2.8 Screening and early detection in women in Africa

The cornerstones for BC screening are breast self-examination (BSE), clinical breast examination (CBE) and mammography (Baines *et al.,* 1989; Nyström *et al.,* 2002). Historically, BC was diagnosed when a woman seeks medical attention for a breast symptom such as a palpable mass or soreness. A biopsy is obtained by surgically removing part of the palpable mass or by surgically excising an

abnormal area identified by a mammogram with a surgical needle. BC is then diagnosed by pathologic review of the breast tissue specimen.

Reduction of breast cancer mortality is the primary and fundamental objective of breast cancer screening. Screening should have the potential to reduce BC morbidity through less extensive surgical procedures. However, even when Black and White women are compared stage for stage, mortality in Black women is higher. The reasons for this are unknown (Ries *et al.,* 1999).

No organized BC screening programs exist in Africa. In advocating screening programs as part of early detection, it is important to avoid imposing first world technology on countries that lack infrastructure and resources to use it appropriately. Before embarking on general screening programs, it is critical to recognize the roles of socio-economic status and racial/ethnic factors, the access to health care and compliance with recommended protocols, all of which influence disease prognosis. Finally, the lack of adequate health insurance is a problem for women of poor low-income and women in reproductive age.

2.9 Impact of culture, education and socio-economic status

Cultural factors concerning beliefs and expectations about BC vary dramatically by ethnic group and geographic location. The relevance of these cultural factors is increasingly recognized, since culture influences all spheres of human life. BC survivors of African descent, similar to survivors of other chronic illnesses, also seek causes for their illness. Their explanatory models of illness and attributions of cancer are in conflict with Western biomedical concepts of illness and evidence based medicine.

The stigma associated with cancer has not decreased. Because of this mentality, BC survivors perceive the disease as the disapproval of ancestral spirits. Additional reasons could include jealousy, supernatural powers over others, bad blood, crossing an evil line, fate and the devil. This cultural variation is also mirrored in the meanings associated with the word cancer across the different cultural groups. Of the nine ethnic Black languages in South Africa only three (Zulu, Swazi and Xhosa) have words for cancer (Williams *et al.,* 2006). The reaction of patients to these words may not include any concept of a disease that may spread to other sites of the body. In rural communities in SA, indigenous

healers are often perceived to be the only legitimate and successful healers of cancer.

Cultural variation has been observed in terms of disclosure of the disease, as diagnosis of BC can cause great suffering to the patient and family. In SA, patients with BC particularly in the rural areas are not necessarily the key decision-makers with regard to the different therapeutic available choices. Care and help seeking involves family members and sometimes elders of the community. Given the role these individuals play in decision making, patients are often encouraged to abscond from Western treatment and rather visit the traditional healer. In comparison, Black patients living in urban communities who have exposure to Western medical care, have the necessary freedom of choice and action to obtain medical attention (Williams *et al.,* 2006).

Survival after BC remains significantly poor for women of African descent. This has been partially attributed to both competing cultural and cognitive models that adversely affect cancer detection and prevention outcomes, as well as actual knowledge gaps about risk, screening and prevention. These translate into the lack of awareness about cancer detection and a failure of cancer prevention efforts. Health education in general and BC specific literacy were shown to be inadequate in women of African descent. Good education and acceptable societal values of a community do not necessarily exclude age old cultural beliefs.

In SA, two groups of randomly selected women in rural and urban area were interviewed using a structured questionnaire (Pillay, 2002). The questionnaire assessed their knowledge and attitude regarding breast and cervical cancer and screening options. The age range was 21-59 with a mean of 35.23 years. Almost one-fifth of the women had not heard of cancer or was aware of BSE techniques. Generally lower awareness levels were found in older and rural women. The author credited the findings to the effects of oppression and deprivation experienced by South Africans of African descent and the persistence of its effect in the post-apartheid SA.

Low socio-economic status (SES) constantly translates into factors that limit the access of the individual to good education, improved literacy, adequate health literacy and good communication ability. Culturally tailored written material about BC screening and its risk for women of African descent with low literacy skills are not available. Disparities in rates of later-stage disease and death may be related

to lower screening rates due to behavioral and structural barriers on low SES women (Young *et al.,* 2002). The lack of information regarding risk, early signs and symptoms of BC and knowledge of self-examination adversely influences BC survival rates in women of African descent. These disparities exist across all SES and geographic backgrounds in women of African descent, being more apparent in rural areas (Pillay, 2002; Santora *et al.,* 2003).

The influence or importance of cultural and lifestyle factors that influence the beliefs, expectations and practices in terms of BC in women of African descent cannot be determined. Nevertheless, women of African descent, particularly young women, who are less educated, uninsured or underinsured, and women who reported not having family history, are less likely to have adequate information regarding detection and prevention (Strzelczyk and Dignan, 2002).

2.10 BC overview in South Africa

Good quality mortality data is only available for three of the Sub-Saharan African countries, namely Benin, Botswana and Mauritius. It has been submitted to the WHO in 1996. In an attempt to collect data and report on cancer incidence and mortality, the South African National Cancer Registry (SANCA) was established in 1986. The National Cancer Registry collects information of cancer patients diagnosed by histology, cytology and hematology from laboratories all around the country. As a result, an annual average of 3785 new BC cases was diagnosed in SA between 1993 and 1995. However, it is believed the data is still an underestimation of the true cancer incidence (Voroboif *et al*., 2001).

BC is the most common cancer diagnosed in SA women. Figures published in 2005 by SANCA indicated BC accounted for 19.4% of all cancers in women, compared to 10% worldwide. The incidence of breast cancer is increasing in sub-Saharan Africa, including South Africa. This increase could be due of the adoption of a more western lifestyle, including women being physically less active, having fewer children with the first child born at a later age, and a shorter duration of breast feeding (Loubser *et al.,* 2008; van der Merwe *et al.,* 2012). Many factors account for the difference in incidence between developed countries and a developing country such as South Africa and amongst the different races within South Africa.

Chapter 3

Material and Methods

3.1 Patients

Thirty five breast cancer patients who attended the Breast and Oncology clinics at the Universitas and National Hospitals in Bloemfontein were selected for inclusion in this study (Table 3.1). The selection criteria were based on ethnicity, the age at diagnosis (preferably breast cancer diagnosed before 45 years), presence of a positive family history and bilaterality of the disease. Ethnicity was determined during an interview conducted by the medical scientist. Each patient was informed in their native language, during which the project was explained. Discussions during these interviews focused mainly on the age of the patient at diagnosis, the presence of a family history and the importance of individual genetic testing. To make sure that the patient understood what was conversed, they were asked to repeat according to their understanding what was told to them. Only then was informed consent signed and blood samples collected with the assistance of the ward doctors and nurses. Although all of the screened patients were affected, only a few reported a positive family history (Table 3.1). Each patient was given a unique sample number to ensure confidentiality. Patients with a positive family history, together with single case patients were included in the study. The histopathological characteristics are listed in Table 3.1.

3.2 Ethics

This study was initially approved by the Ethics Committee of the Faculty of Health Sciences, University of the Free State in Bloemfontein during 2008 (ETOVS 65/08). An addendum was submitted during 2010, indicating the details of the specific student that was allocated to the project (Addendum 65/08) (Appendix A), together with the consent forms necessary for the collection of blood samples

Table 3.1 Compilation of patients included in this study, indicating the allocated laboratory number, the presence of a cancer (ca) family history, the patient's age at diagnosis and the tumor's immunohistochemical (IHC) status.

Patient number	Laboratory number	Family history	Age at diagnosis	Immunohistochemical (IHC) characteristics		
				ER status	PR status	Her2/Nue
1	1971	No	22	No report	No report	No report
2	1989	Yes (Father, prostate ca)	26	No report	No report	No report
3	2027	No	35	MNF116-	CK/AE13-	BETA+
4	2251	Yes (Not indicated)	36	ER-	PR-	HER-
5	2028	No	37	ER+	PR-	HER-
6	2073	No	41	No report	No report	No report
7	2252	Yes (Not indicated)	41	ER-	PR-	HER-
8	2123	No	42	ER+	PR+	HER+
9	2038	No	44	No report	No report	No report
10	2279	Yes (Not indicated)	44	ER-	PR-	HER-
11	1979	Yes (Maternal grandmother uterus ca)	46	No report	No report	No report
12	2034	No	46	ER+	PR-	HER+
13	2250	Yes (Mother, uterus ca)	48	ER-	PR-	HER-
14	1976	Yes (Mother, breast ca)	49	No report	No report	No report
15	2204	No	49	No report	No report	No report
16	2209	No	49	No report	No report	No report
17	2074	No	51	No report	No report	No report
18	2124	No	51	No report	No report	No report
19	1978	Yes (Maternal grandmother & sister, breast ca)	52	No report	No report	No report
20	2075	No	52	ER+	PR-	HER+

21	2122	No	52	No report	No report	No report
22	2037	Yes (Mother, uterus ca)	53	No report	No report	No report
23	2125	No	54	No report	No report	No report
24	1973	Not known	55	ER+	PR-	HER-
25	2036	Not known	57	No report	No report	No report
26	2084	No	57	ER+	PR+	HER+
27	2035	Yes (Nephew, prostate ca)	58	No report	No report	No report
28	2033	Yes (Maternal aunt, breast ca)	58	ER+	PR-	HER-
29	2080	No	58	No report	No report	No report
30	1980	Yes (Maternal grandmother, breast ca)	62	No report	No report	No report
31	1974	No	64	No report	No report	No report
32	1972	Not known	65	No report	No report	No report
33	2253	Yes (Maternal grandmother, mother, brother, two nieces, breast ca)	73	ER-	PR-	HER-
34	1975	No	77	No report	No report	No report
35	1977	Yes (Maternal grandmother, mother, granddaughter, breast ca)	79	No report	No report	No report

Permission was obtained from the Head of Clinical Services of the Universitas Hospital and the Business Manager of the National Health Laboratory Services (NHLS) to approach patients attending clinics or being admitted to the respective institutions (Appendix B and C). Permission was also obtained from the Head of the Human Genetics Division and the Business Manager of NHLS to use the space and equipment of the Molecular Genetics Laboratory for this project (Appendix D and E).

3.3 Methodology

3.3.1 DNA extraction

Two DNA extraction methods were used during the study. While the phenol: chloroform method was initially used, due to problems experienced with toxic waste disposal, the salting out method was subsequently used.

3.3.1.1 Phenol: chloroform method

Ten to twenty milliliter of blood was collected in ethylenediaminetetraacetic acid (EDTA) tubes. The blood was transferred to two labeled Nunc tubes and stored at -20°C. DNA was extracted from lymphocytes according to an adapted phenol: chloroform procedure (Sambrook *et al.,* 1989). Once thawed, the red cells were ruptured using 45 ml cold lysis buffer [0.3 M sucrose, 10 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) pH 7.8, 5 mM $MgCl_2$, 1% (v/v) t-octylphenoxypolyethoxyethanol (Trixton X-100)]. The solution was centrifuged for 20 min at 15 000 *g* at 4°C, where after the pellet was resuspended in 1x SET buffer (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA) containing 10 $\mu g.\mu l^{-1}$ proteinase K and 1% (w/v) sodium dodecyl sulphate (SDS). The solution was incubated overnight at 37°C.

Equal volumes of phenol pH 8 (USB Corporation) and chloroform:isoamyl alcohol (24:1) was added to the solution and mixed thoroughly on an orbital shaker for 1 h at room temperature. Centrifugation for 10 min at 15 000 *g* at 4°C followed, where after the supernatant was transferred to a new tube. An equal volume of chloroform:isoamyl alcohol (24:1) was added, mixed thoroughly and centrifuged as described above. The DNA was precipitated from the supernatant with 2 volumes ice-cold 100% (v/v) ethanol and sodium acetate (pH 5.4) to a final

concentration of 0.3 M. After 10 min at -20°C, the precipitated DNA was scooped from the solution, transferred to an Eppendorf tube and washed with 70% (v/v) ethanol. The air dried DNA was dissolved in $T._1E$ buffer (10 mM Tris-HCl pH 7.6, 0.1 mM EDTA). If no DNA was visible during precipitation, the tubes were incubated at -20°C overnight. The tubes were then centrifuged for 20 min at 15 000 *g* at 4°C to pellet the DNA, the pellet washed with 70% (v/v) ethanol, air dried and dissolved in $T._1E$ buffer.

3.3.1.2 Salting out method

Peripheral blood was transferred from the EDTA tubes into Nunc tubes and stored at -20°C. Once thawed, the cells were ruptured using the lysis buffer as described (3.3.1.1). After centrifugation, the pellet was resuspended in 1x SET buffer (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA) containing 10 $\mu g.\mu l^{-1}$ proteinase K and 1% (w/v) SDS and incubated overnight at 37°C. A volume of 1.4 ml saturated NaCl was added and the tubes shaken vigorously for 15 sec. After centrifugation for 15 min at 15 000 *g* at 4°C, the supernatant was transferred to a new tube. Precipitated DNA was either scooped from the solution or recovered as described in 3.3.1.1.

3.3.2 DNA concentration determination

The DNA concentration and purity were determined using spectrophotometry (NanoDrop® ND-100 Spectrophotometer v3.01, NanoDrop Technologies Inc) according to the manufacturer's instructions. The DNA concentration was expressed as $ng.\mu l^{-1}$. The samples were diluted to 50 $ng.\mu l^{-1}$ for use during the conventional polymerase chain reactions (PCR).

3.3.3 Molecular analysis using PCR

Since the screening of patients for the two familial breast cancer genes are performed routinely within the laboratory, no optimization of the PCR conditions was required. Lists of all the primer sets used during the mutational analyses are presented in Tables 3.2 and 3.3. The primer sequences were obtained from the Breast Cancer Information Core (http://research.nhgri.nih.gov/bic/). The primers,

Table 3.2 Oligonucleotides used for screening the *BRCA1* gene. Indicated are exon numbers, the sequences of both forward and reverse primers and the optimized annealing temperature for each specific primer set. The final column indicates the percentage that the particular exon represents of the entire coding region of the gene.

Exon	Forward primer cited in the 5′→3′ direction	Reverse primer cited in the 5′→3′ direction	Annealing temperature (°C)	% of gene
2	GACGTTGTCATTAGTTCTTTGG	GGTCAATTCTGTTCATTTGC	56	1.77
3	AACGAACTTGAGGCCTTATG	TTGGATTTTCGTTCTCACTT	56	0.96
5	CTCTTAAGGGCAGTTGTGAG	ATGGTTTTATAGGAACGCTATG	58	1.39
6	CTTATTTTAGTGTCCTTAAAAGG	TTTCATGGACATCACTTGAGTG	52	1.59
7	GGTTTCTCTTGGTTTCTTTG	AGGAGCTGCTTCCTAGCCTC	50	2.50
8	TGTTAGCTGACTGATGATGGT	AACCCAGCAATTATTATTAAATAC	56	1.90
9	CCACAGTAGATGCTCAGTAAATA	TAGGAAAATACCAGCTTCATAGA	56	0.82
10	GATCTTGGTCATTTGACAGTTC	CCCAAATGGTCTTCAGAATA	58	1.38
12	GCGTTTATAGTCTGCTTTTACA	TGTCAGCAAACCTAAGAATGT	56	1.59
13	AATGGAAAGCTTCTCAAAGTA	ATGTTGGAGCTAGGTCCTTAC	56	3.08
14	CTAACCTGAATTATCACTATCA	GTGTATAAATGCCTGTATGCA	56	2.27
15	CAGACTTCTAGGCTGTCTTGC	GTGTTTGTTCCAATACAGCAG	56	3.42
16	AATTCTTAACAGAGACCAGAAC	AAAACTCTTTCCAGAATGTTGT	56	5.56
17	AGCTGTGTGCTAGAGGTAACTC	GTGGTTTTATGCAGCAGATG	52	1.57
18	GGCTCTTTAGCTTCTTAGGAC	CTCAGACTCAAGCATCAGC	52	1.27
19	CTGTCAATCTTCCTGTGCTC	CATTGTTAAGGAAAGTGGTGC	56	0.73
20	ATATGACGTGTCTGCTCCAC	AGTCTTACAAAATGAAGCGG	56	1.50
21	AAGCTCTTCCTTTTTGAAAGTC	GTAGAGAAATAGAATAGCCTCT	56	0.98
22	TCCCATTGAGAGGTCTTGCT	GAGAAGACTTCTGAGGCTAC	56	1.32
23	CAGAGCAAGACCCTGTCTC	ACTGTGCTACTCAAGCACCA	56	1.09
24	ATGAATTGACACTAATCTCTGC	GTAGCCAGGACAGTAGAAGGA	58	5.00

Table 3.3 Oligonucleotides used for screening the *BRCA2* gene. Indicated are exon numbers, the sequences of both forward and reverse primers and the optimized annealing temperature for each specific primer set. The final column indicates the percentage that the particular exon represents of the entire coding region of the gene.

Exon	Forward primer cited in the 5′→3′ direction	Reverse primer cited in the 5′→3′ direction	Annealing temperature (°C)	% of gene
2	CCAGGAGATGGGACTGAATTAG	CTGTGACGTACTGGGTTTTTAGC	57	1.0
3	GATCTTTAACTGTTCTGGGTCACA	CCCAGCATGACACAATTAATGA	56	2.4
4	AGAATGCAAATTTATAATCCAGAGTA	AATCAGATTCATCTTTATAGAACAAA	50	1.1
5/6	TGTGTTGGCATTTTAAACATCA	CAGGGCAAAGGTATAACGCT	56	0.9
7	CCTTAATGATCAGGGCATTTC	CAACCTCATCTGCTCTTTCTTG	54	1.1
8	GCCATATCTTACCACCTTGTGA	AGGTTTAGAGACTTTCTCAAAGGC	58	0.5
9	CTAGTGATTTTAAACTATAATTTTTG	GTTCAACTAAACAGAGGACT	50	1.1
12	AGTGGTGTTTTAAAGTGGTCAAAA	GGATCCACCTGAGGTCAGAATA	54	1.0
13	GCATCCGTTACATTCACTGAAA	ACGGGAAGTGTTAACTTCTTAACG	56	0.7
14A	ACCATGTAGCAAATGAGGGTCT	GCTTTTGTCTGTTTTCCTCCAA	58	4.2
14B	CACAGAGTTGAACAGTGTGTTAGG	GGGCTTTAAAATTACCACCACC	58	4.2
15	GGCCAGGGGTTGTGCTTTTT	AGGATACTAGTTAATGAAATA	50	1.8
16	TTTGGTAAATTCAGTTTTGGTTTG	AGCCAACTTTTTAGTTCGAGAG	54	1.8
17	CAGAGAATAGTTGTAGTTGTTGAA	AGAAACCTTAACCCATACTGC	46	1.7
18A	GATCCACTATTTGGGGATTGC	GATCTAACTGGGCCTTAACAGC	54	3.5
18B	GCAGATACCCAAAAAGTGGC	TCTGGACCTCCCAAAAACTG	56	3.5
19	AAGTGAATATTTTTAAGGCAGTT	TATATGGTAAGTTTCAAGAAT	50	1.5
20	CACTGTGCCTGGCCTGATAC	ATGTTAAATTCAAAGTCTCTA	50	1.4
21	GGGTGTTTTATGCTTGGTTCT	CATTTCAACATATTCCTTCCTG	47	1.2
22	AACCACACCCTTAAGATGAGC	GGGCATTAGTAGTGGATTTTGC	54	1.9
23	ACTTCTTCCATTGCATCTTTCTCA	AAAACAAAACAAAAATTCAACATA	54	1.6
24	GCAGCGACAAAAAAAACTCA	ATTTGCCAACTGGTAGCTCC	56	1.4
25	GCTTTCGCCAAATTCAGCTA	TACCAAAATGTGTGGTGATGC	54	3.0
26	GTCCCAAACTTTTCATTTCTGC	GGAGCCACATAACAACCACA	54	1.4
27A	CTGTGTGTAATATTTGCGTGCT	GCAAGTTCTTCGTCAGCTATTG	58	4.8
27B	GAATTCTCCTCAGATGACTCCA	TCTTTGCTCATTGTGCAACA	52	4.8

deoxyribonucleotide triphosphates (dNTPs) and *Taq* DNA polymerase were obtained from Roche Diagnostics (Roche diagnostics GmbH Mannheim, Germany). PCR was performed using the MyCycler™ (Bio-Rad Laboratories Inc., Hercules). All PCR reactions had a final volume of 50 µl. A no template control containing all PCR reagents except DNA was included with every run to detect possible DNA contamination of reagents.

3.3.4 Mutation analysis

The coding regions of both *BRCA* genes were screened using a combination of techniques, namely single strand conformational polymorphism (SSCP), heteroduplex analysis (HA), protein truncation test (PTT) and multiplex ligation dependent probe amplification (MLPA). The *BRCA1* analysis excluded exons 1 and 4, since exon 1 is non-coding and exon 4 is only a splicing variant. All other exons and exon-intron boundaries were screened for all the patients using SSCP and HA. *BRCA1* exon 11 and *BRCA2* exons 10 and 11 were screened using PTT which detects changes on DNA level that result in the premature truncation of the protein product. It is an ideal method to screen large genomic regions (60% of gene) as it only detects disease-causing mutations that results in a frame shift.

Sanger sequencing was always considered the gold-standard (Sanger *et al.*, 1977). This method powered the Human Genome Project and still continues today to generate highly accurate, reliable sequencing data. As the field of mutation screening is rapidly evolving, Sanger sequencing is on the brink of being replaced by new paradigms such as Next Generation Sequencing (NGS), which implies an increased workflow, speed and enhanced sensitivity (McCourt *et al.*, 2013). Although these technologies are nationally and internationally available, it is costly and difficult to analyze. Mutation screening of the familial breast cancer genes was therefore performed with apparatus what was to our disposal which delivered reliable results. Due to these factors, mutation screening was performed making use of a combined SSCP/HA analysis, followed by Sanger sequencing.

3.3.4.1 Combined SSCP/HA analysis

Each 50 µl PCR reaction contained 300 ng template, 250 µM dNTPs, 20 pmol of each primer, 100 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl and 1 U *Taq*

DNA polymerase. The amplification regime was as follows: one cycle at 95°C for 1 min, followed by 32 cycles at 94°C for 45 sec, optimal annealing temperature for 1 min and 72°C for 45 sec, with a final elongation step at 72°C for 10 min.

To each amplification reaction, 10 µl loading buffer [95% (v/v) formamide, 1 mM EDTA pH 8.0, 0.05% (w/v) xylene cyanol FF, 0.05% (w/v) bromophenol blue] was added. Samples were denatured at 94°C for 5 min, snap cooled on ice for 5 min and separated on a 10% (w/v) polyacrylamide gel (37.5 acrylamide: 1 bis-acrylamide, 2.7% cross linking) containing 1x TBE (0.089 M Tris-HCl pH 8.0, 0.089 M boric acid, 2 mM EDTA). The gels were run on a SE600 vertical electrophoresis system (Hoefer Pharmacia Biotech Inc) connected to a circulating low temperature water bath. The temperature was recorded and documented before and after each run to ensure that the optimum running temperature was maintained. The gels were run using 1x TBE as running buffer at a constant temperature (10° - 16°C depending on the fragment) for 14 - 20 h at 260 – 280 V depending on the length of the gel plates.

3.3.4.2 Silver staining

The gels were lifted from the glass plates and incubated for 10 min in 10% (v/v) ethanol, 0.5% (v/v) acetic acid after which the gels were rinsed twice for 5 min in deionised water (DI). The gels were then incubated in 1 M dithiothreitol (DTT) in 0.5 M KOAc (pH 4.5) for 10 min followed by a brief rinse for 30 sec in DI. After a 10 min incubation in 0.1% (w/v) silver nitrate and a rinse with DI, the gels were developed with 1.5% (w/v) $NaCO_3$, 0.155% (v/v) formaldehyde. Once clear bands were visible, the reaction was stopped with incubation in 0.01 M citric acid for 10 min. The gels were finally rinsed with tap water where after the image was digitally captured using the Bio-Rad Gel documentation system (Bio-Rad Laboratories Inc., Hercules).

3.3.4.3 Protein truncation test

The PTT was used to screen for protein-truncating mutations present within *BRCA1* exon 11 and *BRCA2* exons 10 and 11. All forward primers contained a T7 promoter sequence and eukaryotic translation initiation sequence (Tables 3.4 and 3.5). The sections of genomic DNA were amplified in three (*BRCA1*) and five (*BRCA2*) overlapping fragments, respectively (Fig. 3.1).

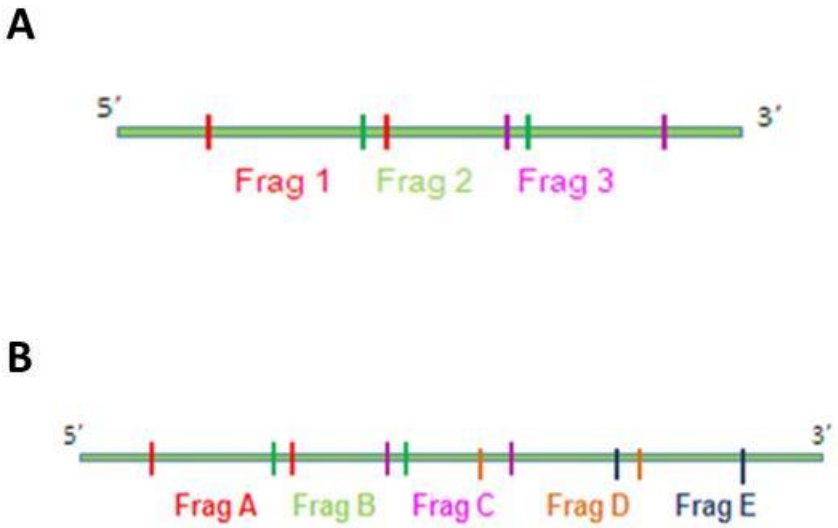


Figure 3.1 Illustration of the design of the overlapping PTT fragments for *BRCA1* and *BRCA2*. **A** Schematic diagram illustrating sections of genomic DNA of approximately 1400 bp amplified in three *BRCA1* overlapping fragments. **B** Schematic diagram illustrating sections of genomic DNA of approximately 1400 bp amplified in five *BRCA2* overlapping fragments.

Table 3.4 Primer sequences for amplification of *BRCA1* exon 11. Indicated are fragment numbers of forward and reverse primer sets and the annealing temperature for that specific set.

Exon	Forward primer cited in the 5′→3′ direction	Reverse primer cited in the 5′→3′ direction	Annealing temperature
BRCA1 exon 11 Fragment 1	CGCTAATACGACTCACTATAGGAACAGACCACCA TGGCTTGTGAATTTTCTGAGACGG	CAGGAAACAGCTATGACATGAGTTGTAGG TTTCTGCTGTG	55.5°C
BRCA1 exon 11 Fragment 2	CGCTAATACGACTCACTATAGGAACAGACCACCA TGGACAATTCAAAAGCACCTAAAAAG	CAGGAAACAGCTATGACAACCCCTAATCT AAGCATAGCATTC	55.5°C
BRCA1 exon 11 Fragment 3	CGCTAATACGACTCACTATAGGAACAGACCACCA TGGCACCACTTTTTCCCATCAAGTC	CAGGAAACAGCTATGACATTATTTTCTTCC AAGCCCGTTCC	55.5°C

Table 3.5 Primer sequences for amplification of *BRCA2* exons 10 and 11. Indicated are fragment numbers of forward and reverse primer sets and the annealing temperature for that specific set.

Exon	Forward primer cited in the 5′→3′ direction	Reverse primer cited in the 5′→3′ direction	Annealing temperature
BRCA2 exon 10 Fragment E	GGATCCTAATACGACTCACTATAGGAACAGACCACCATG ATTGGAAAGTCAATGCCAAA	AAACACAGAAGGAATCGTCATA	55.5°C
BRCA2 exon 11 Fragment A	CGCTAATACGACTCACTATAGGAACAGACCACCATGGTG CATTCTTCTGTGAAAAGAAGC	GCACTTCAAATGTACTCTTCTGC	55.5°C
BRCA2 exon 11 Fragment B	CGCTAATACGACTCACTATAGGAACAGACCACCATGGTA AAGCAGCATATAAAAATGACTC	GATCTTTTCATCACGTTCGG	55.5°C
BRCA2 exon 11 Fragment C	CGCTAATACGACTCACTATAGGAACAGACCACCATGGTT GATGGCAGTGATTCAAG	TGCTACATTCATCATTATCTAGAGAG	55.5°C
BRCA2 exon 11 Fragment D	CGCTAATACGACTCACTATAGGAACAGACCACCATGGAT GACAAAAATCATCTCTCCG	AACTGACTACACAAAAATGGCTG	55.5°C

Each 25 µl reaction contained 50 ng genomic DNA, 250 µM dNTPs, 4 µM of each primer, 100 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl and 1 U of Takara *Ex Taq* (Otsu, Japan). The amplification regime was as follows: 95°C for 1 min followed by 35 cycles of 93°C for 4 min, 55°C for 1 min and 72°C for 3 min and a final extension at 72°C for 5 min. Ten microliters of the amplification product were subsequently separated on a 2% (w/v) agarose gel (Hogervorst *et al.,* 1995) prepared with 1x TBE containing ethidium bromide to a final concentration of 0.5 $\mu g.ml^{-1}$. The gel was run at 120 V using 1x TBE as running buffer.

Protein products were synthesized in a coupled *in vitro* transcription/ translation reaction using the TNT® T7 Quick Coupled Transcription/ Translation System (Promega, Madison, WI) with minor adaptations and analyzed by gel electrophoresis (Sambrook *et al*., 1989). Following PCR amplification, 5 µl PCR product was added to a master mix containing 8 µl TNT® T7 Quick Master mix, 0.5 µl TNT® T7 Enhancer and 0.6 µl 10 $mCi.ml^{-1}$ L-[^{35}S] methionine (PerkinElmer Inc, Boston, USA). Samples were incubated at 30°C for 90 min. A 1: 4 dilution of PTT product to Laemmli sample buffer [0.05% (w/v) bromophenol blue, 1 M DTT, 10% (w/v) SDS, 0.05 M Tris-HCl pH 6.8] was prepared and denatured at 95°C for 5 min.

The samples were separated on a 12% (w/v) SDS-PAGE gel (0.38 M Tris-HCl pH 8.8, 0.0625% (w/v) SDS) with a 4% (w/v) stacking gel (0.13 M Tris-HCl pH 6.8, 0.005% (w/v) SDS) together with the Benchmark™ Prestained Protein Ladder (GibcoBRL Life Technologies, California, USA). The gels were run using Tris and Glycine (25mM Tris, 192mM glycine, 0.1%SDS) as running buffer (Bio-Rad Laboratories Inc., Hercules) at 120 V for 15 min to compress the sample, where after it was run for approximately 90 min at 220 V or until the dye front reached the bottom. The gels were fixed in 65% (v/v) isopropanol, 10% (v/v) acetic acid, briefly rinsed in tap water, shaken for 15 min in Amplify (GE Healthcare, UK) for sensitization and briefly rinsed. There after the gels were dried under vacuum for a minimum of 2 h and exposed to an X-ray film. Once developed, the X-ray image was captured using the Bio-Rad gel documentation system. Interpretation of results was done by scoring products according to size.

For PTT fragments indicating a truncated fragment, the area of the mutation was more closely defined using various SSCP primer sets. The SSCP PCR

fragments representing a specific PTT section were analysed using SSCP/HA analysis, followed by silver staining as described in sections 3.3.4.1 and 3.3.4.2. Once the appropriate SSCP fragment was identified, the amplification process for that specific SSCP fragment was repeated, where after it was subjected to vertical electrophoresis. Before electrophoresis, the PCR products were covered in paraffin oil, denatured for 5 min at 95°C using a Techne dri block (E-C Apparatus Corporation), where after it was slowly cooled to 37°C over a period of 2 h. To each amplification reaction, 10 µl loading buffer [95% (v/v) formamide, 1 mM EDTA pH 8.0, 0.05% (w/v) xylene cyanol FF, 0.05% (w/v) bromophenol blue] was added. The samples were separated using a 10% (w/v) polyacrylamide gel (37.5 acrylamide: 1 bis-acrylamide, 2.7% cross linking), containing 1x TBE and 15% (w/v) urea. Depending on the size of the fragments, the gels were run for approximately 16 h at 260-380 V. After the run, gels were gently rinsed in 500 ml 1x TBE buffer containing 0.05 $\mu g.ml^{-1}$ ethidium bromide for 10 min. The banding patterns were visualized by exposure to UV light, where after each of the heteroduplex bands were excised separately and transferred to an Eppendorf tube. The fragments were briefly rinsed in dH_2O, where after the DNA was eluted overnight from the gel in 50 µl $T._1E$ at 4°C. The following day, the samples were centrifuged for 10 min, where after the supernatant was used as template for Sanger sequencing.

3.3.4.4 Sequencing

DNA sequencing was performed for selected samples to confirm the possible mutation observed with SSCP banding patterns. Each sample was re-amplified and purified using SigmaSpin Post–Reaction clean-up columns (Sigma–Aldrich, St Louis, USA) according to the manufacturer's instructions. DNA fragments were bi-directionally sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Each sequencing reaction contained 2 µl template, 1 µl terminator ready reaction mix, 3.2 pmol primer and 2 µl BigDye® sequencing buffer. The sequencing regime was as follows: one cycle at 96°C for 1 min, followed by 25 cycles at 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min, with a final holding temperature of 4°C. The sequenced products were precipitated by adding 5 µl 125 mM EDTA and 60 µl 100% (v/v) ethanol, followed by 30 min incubation at room temperature in the dark. After centrifugation at 14 000 *g* for 30

min, the supernatant was aspirated, the pellet washed once with 60 µl 70% (v/v) ethanol and finally air dried in the dark.

Hi-Di™ formamide (Applied Biosystems, US) buffer (30 µl) was added to the pellet, where after it was denatured for 5 min at 96°C and the tubes snap cooled in a cold block. The pellets were resuspended by pipetting and loaded on a microtiter plate for analysis on the ABI 3130 Genetic Analyzer (Applied Biosystems, US). The electropherograms were analyzed by visual inspection using proprietary sequence analysis software (Chromas version 2.31, www.technelysium.com.au). The sequences were aligned to the reference sequences (NM_005905.3 for *BRCA1* and NM_00059.3 for *BRCA2*) with LALIGN (www.ch.embnet.org/software/LALIGN) and translation was performed using the Expasy translate tool (http://au.expasy.org/tools/dna.html).

3.3.4.5 Multiplex ligation-dependent probe amplification (MLPA)

Multiplex ligation-dependent probe amplification was performed by the Research Institute of the Departments of Oncology and Human Genetics at the McGill University Health Centre, Montréal, Canada. They made use of the SALSA MLPA probe mix P002-C2 for *BRCA1*, and SALSA MLPA probe mix P090-A4 for *BRCA2* (MRC Holland, the Netherlands). The genomic DNA was purified using the Magnapure (Roche) and Millipore columns (Sigma-Aldrich, St Louis, USA).

Chapter 4

Results

4.1 *BRCA* patients

The selection criteria for inclusion in the study were based on ethnicity, the age at diagnosis (preferably BC diagnosed before 45 years), the presence of a positive family history and/or bilaterality of the disease. Thirty-five Sotho/Tswana patients, diagnosed with breast and/or ovarian cancer, were screened for potential mutations within the *BRCA* genes using PTT and SSCP/HA analysis. Fourteen patients (40%) reported a positive family BC history ranging from very low to moderate risk pedigrees. Eighteen patients (51.5%) did not have a family history, whereas 3 (8.5%) had no history re-collection. The age at BC diagnosis varied from 22 to 79 years, with an average of 45.3 years. Of the 35 patients, five were triple negative for the IHC status ER, PR and HER2nue. This implied that the growth of the cancer is not supported by oestrogen, progesterone or by the presence of HER2nue receptors.

4.2 *BRCA1* results

4.2.1 Screening of exon 11 using the PTT

BRCA1 exon 11 was amplified in three overlapping fragments using forward primers that contained the T7 promoter and a transcription/translation initiation sequence (Hogervorst *et al.,* 1995). To confirm successful amplification, the PCR products were separated on a 2% agarose gel (Fig. 4.1). The amplification of these fragments was dependent on the quality of the DNA, as some samples did not initially amplify (Figs. 4.1A and B). These samples needed an additional DNA clean-up to remove possible impurities. The DNA concentration of these samples

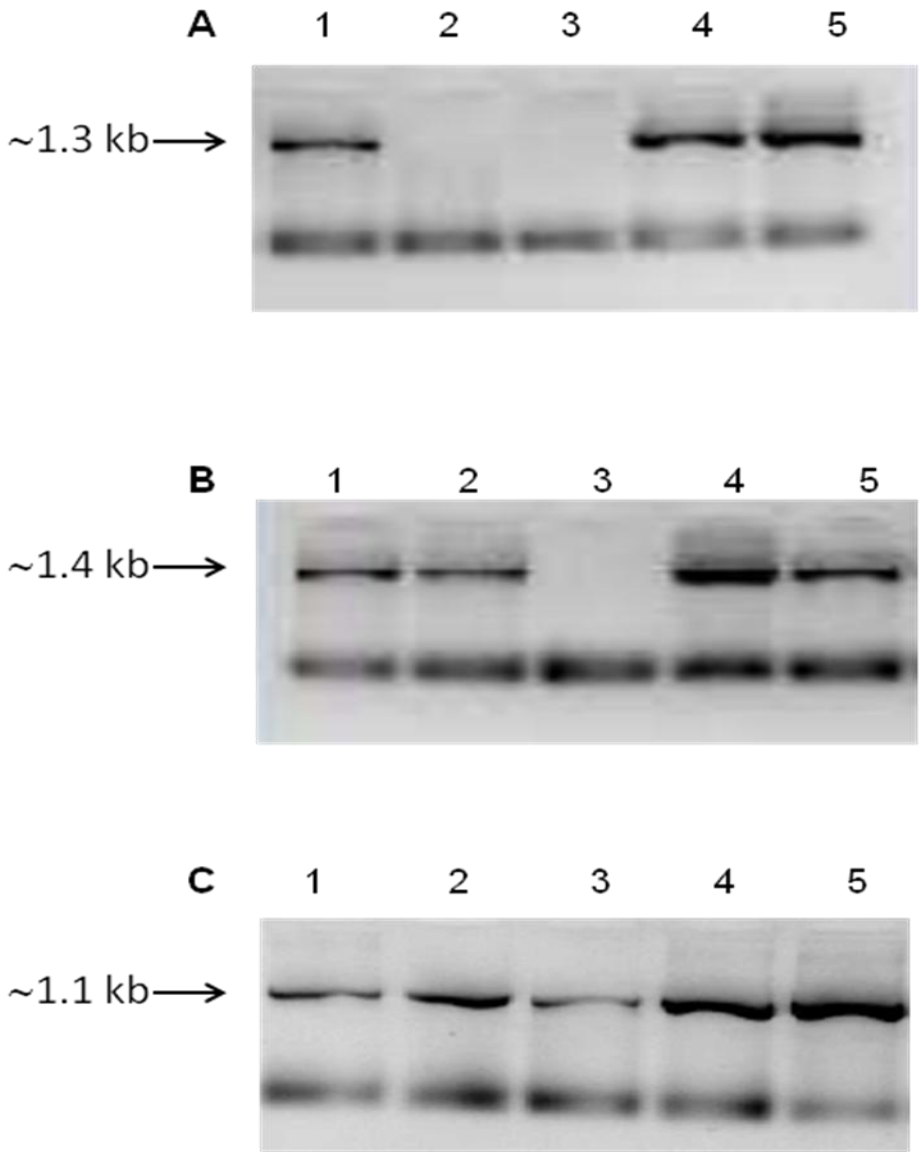


Figure 4.1 Amplification of *BRCA1* exon 11. The products were separated using a 2% agarose gel and visualized by ethidium bromide staining. **A.** Amplification products of ~1.3 kb for *BRCA1* fragment 1. **B.** Amplification products of ~1.4 kb for *BRCA1* fragment 2. **C.** Amplification products of ~1.1 kb for *BRCA1* fragment 3. The fragments represent the amplified products for CAM 2028 (lane 1), CAM 2033 (lane 2), CAM 2034 (lane 3), CAM 2035 (lane 4) and CAM 2036 (lane 5) in each figure.

was again determined after the clean-up, where after the fragments were re-amplified. Although some of the fragments exhibited primer dimers, the dimers do not interfere with separation of amplicons on the agarose gels (Fig. 4.1). The majority of amplified fragments produced wild type polypeptides ranging between ~62 kD and ~52 kD (Fig. 4.2). Only one PTT variant was identified in *BRCA1* fragment 2 of exon 11 for patient CAM 1973 (Fig. 4.3). As the truncated polypeptide was small (approximately 10 kD), the mutation must have occurred towards the 5' end of the amplified DNA fragment.

Four smaller SSCP/HA primer sets that span the 5' end of exon 11 were selected to determine the position and the type of mutation present. Three unaffected patients were included as controls. All four patients (CAM 1971, CAM 1972, CAM 1974 and CAM 1973) were analyzed for each of the four SSCP fragments (Fig. 4.4). SSCP fragment H indicated a distinctly different banding pattern for patient CAM 1973 compared to the controls.

The observed heteroduplex bands (Fig. 4.5) were excised from the gel and the recovered DNA fragments bi-directionally sequenced. Sequencing results indicated a four base pair deletion within *BRCA1* exon 11 for patient CAM 1973 (Fig. 4.6). According to the latest HGVS nomenclature, the mutation is classified as c.2069delAAAG. This deletion causes the changing of a Lysine to Serine amino acid at codon 653 resulting in a STOP codon at codon 699 and a subsequent truncated polypeptide. This mutation has never been reported in the BIC database.

4.2.2 SSCP and HA results

The remainder of the exons and splice site boundaries of *BRCA1* were amplified with previously optimized primers. The samples were analyzed using a combination of the SSCP and HA techniques. Although old technology, these techniques allowed for clear banding patterns which could unambiguously be compared and scored in the majority of cases. For the majority of exons, no variations were detected in the SSCP/HA banding patterns, suggesting that there were no nucleotide changes that occurred within those specific fragments (Fig. 4.7). The samples included in the group of 35 served as normal controls for each other as the likelihood of identifying the same DNA change in all these patients are extremely small.

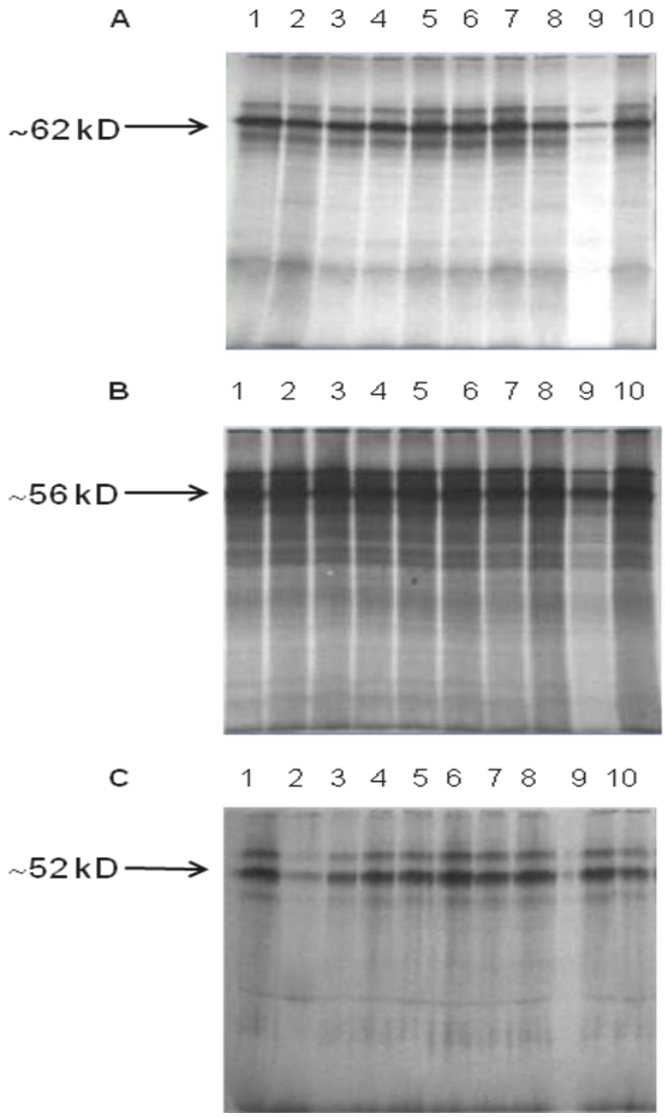


Figure 4.2 Polypeptides generated for sections of *BRCA1* exon 11. The translated polypeptides were separated on a 12% poly-acrylamide gel and exposed overnight to an X-ray. **A.** Polypeptides of ~62 kD representing fragment 1 of *BRCA1* exon 11. **B.** Polypeptides of ~56 kD representing fragment 2 of *BRCA1* exon 11. **C.** Polypeptides of ~52 kD representing fragment 3 of *BRCA1* exon 11. Lanes 1 to 10 within each figure represent amplified DNA fragments for CAM 1979 (lane 1), CAM 1976 (lane 2), CAM 1978 (lane 3), CAM 1989 (lane 4) CAM 2037 (lane 5), CAM 2038 (lane 6), CAM 2027 (lane 7), CAM 2028 (lane 8), CAM 2034 (lane 9) and CAM 2035 (lane 10).

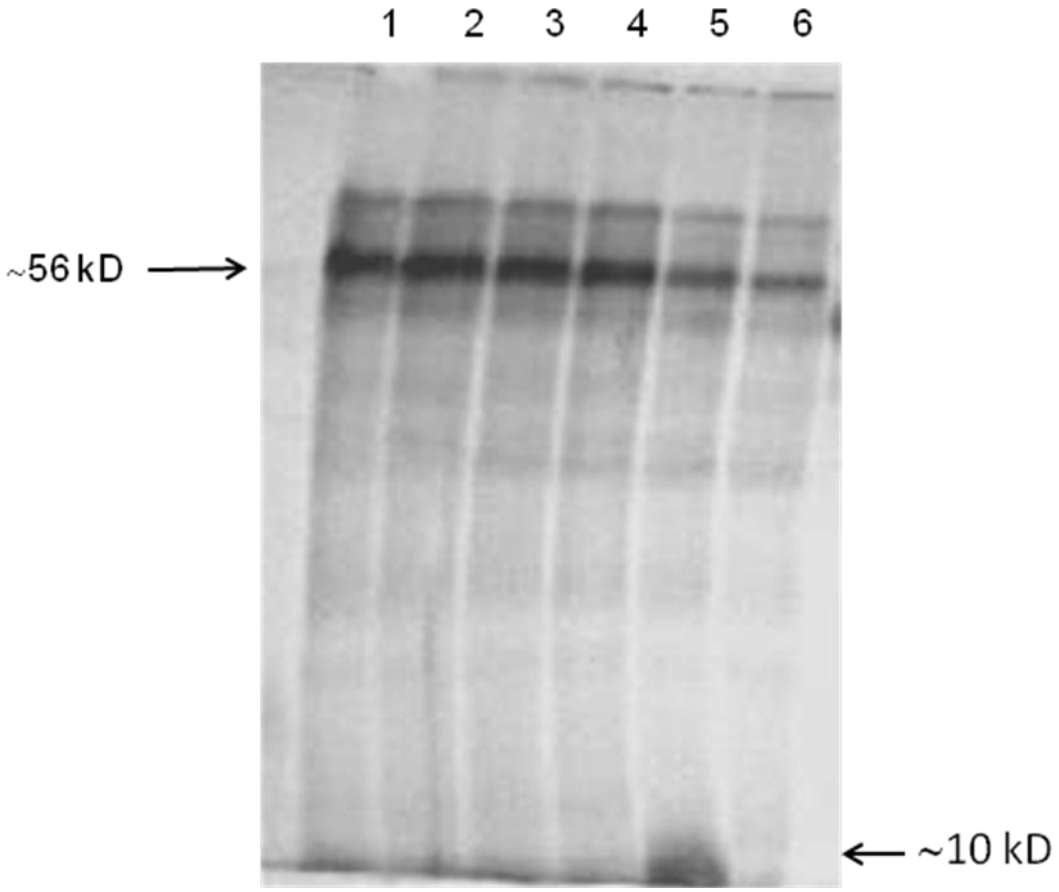


Figure 4.3 Polypeptides generated for fragment 2 of *BRCA1* exon 11. The fragments represent the amplified DNA fragments for CAM 1971 (lane 1), CAM 1972 (lane 2), CAM 1974 (lane 3), CAM 1975 (lane 4), CAM 1973 (lane 5) and CAM 1976 (lane 6). Translated polypeptides were separated on a 12% poly-acrylamide gel and exposed overnight to an X-ray. A premature truncated polypeptide of ~10 kD visible for CAM 1973 in lane 5 is indicated by the arrow.

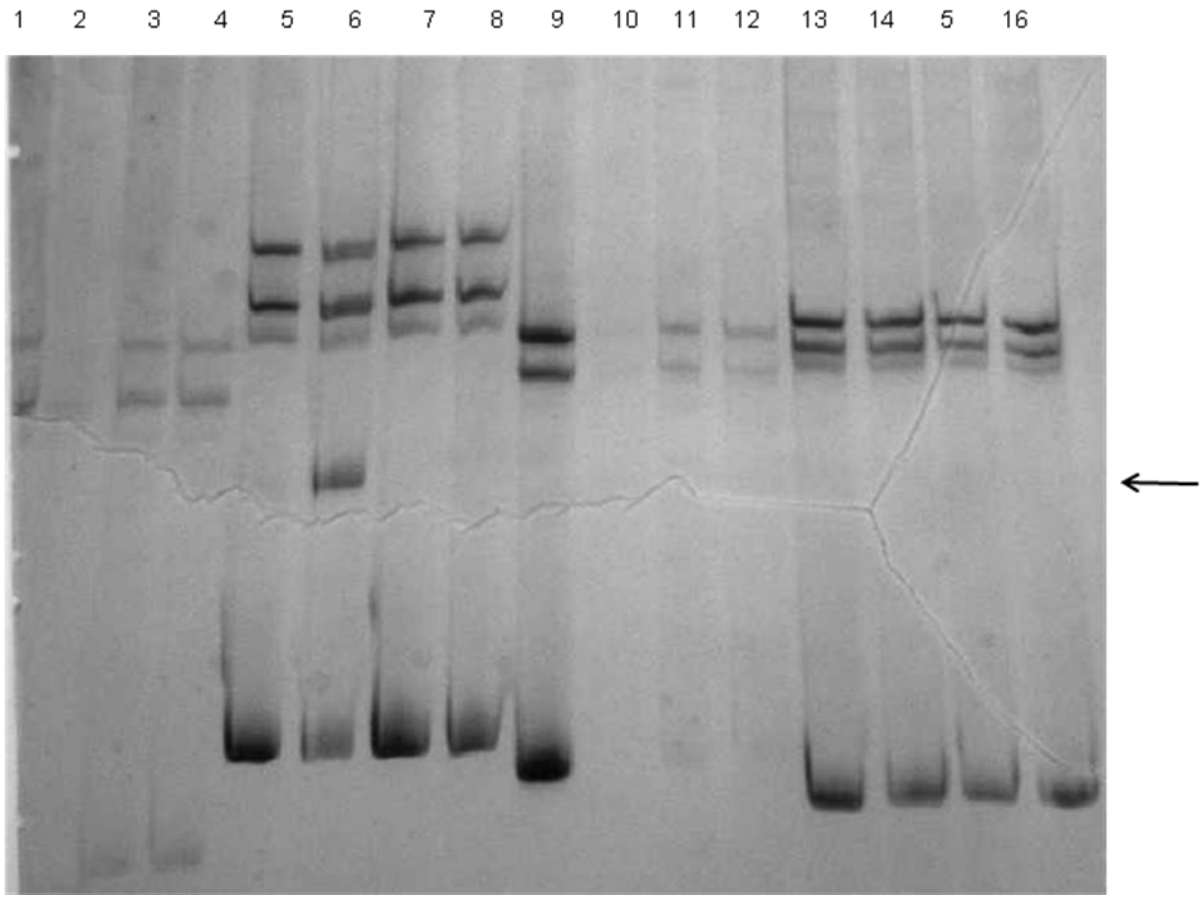


Figure 4.4 SSCP analysis of the primer sets spanning fragment 2 of *BRCA1* exon 11. Results for SSCP fragment G are presented in lanes 1 to 4 (representing CAM 1972, CAM 1973, CAM 1974 and CAM 1975 respectively), fragment H in lanes 5 to 8 (CAM 1972, CAM 1973, CAM 1974 and CAM 1975), fragment I in lanes 9 to 12 (CAM 1972, CAM 1973, CAM 1974 and CAM 1975) and fragment J in lanes 13 to 16 (CAM 1972, CAM 1973, CAM 1974 and CAM 1975). The different banding pattern visible for CAM 1973 for SSCP fragment H is indicated by the arrow.

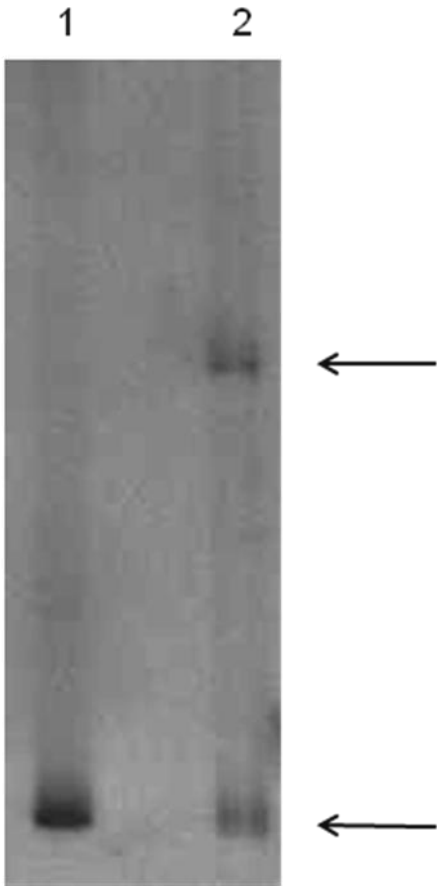


Figure 4.5 Heteroduplex analysis for fragment H of *BRCA1* exon 11. Lane 1 represents CAM 1971 (a normal control for *BRCA1* exon 11), with CAM 1973 exhibiting a truncation in *BRCA1* exon 11 in lane 2. The heteroduplex fragments are indicated by the arrows.

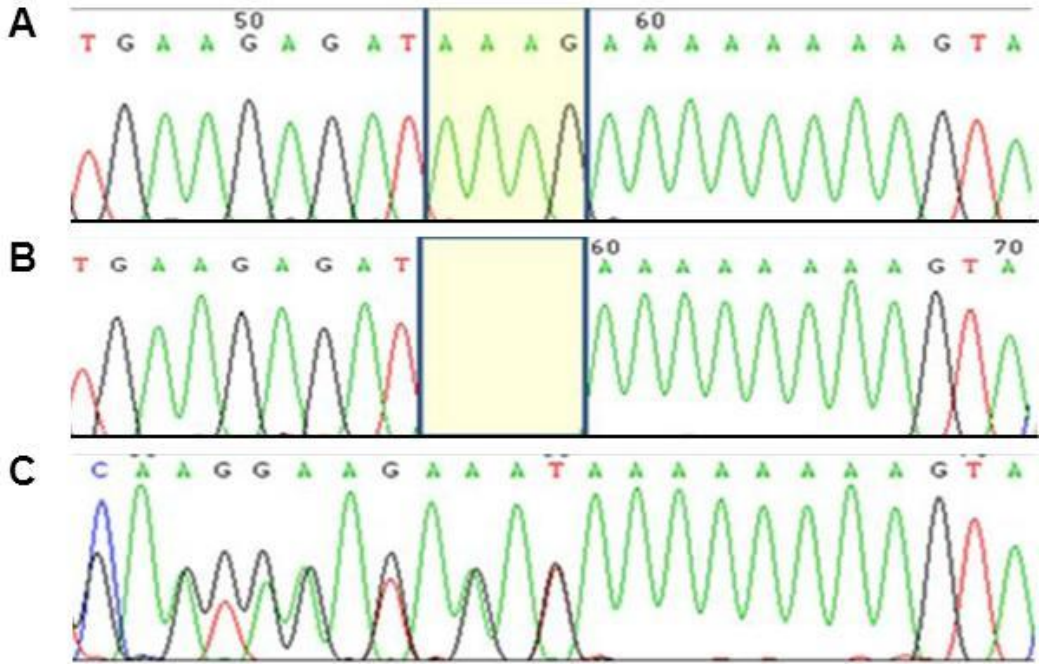


Figure 4.6 Electropherograms illustrating DNA sequencing results for fragment 2 of *BRCA1* exon 11. **A.** Electropherogram representing the first excised heteroduplex band for CAM 1973, indicating homozygosity for the normal wild type sequence. **B.** Electropherogram for CAM 1973 representing the second excised heteroduplex band indicating homozygosity for a four base pair deletion (highlighted by the yellow box). **C.** Electropherogram for CAM 1973 indicating heterozygosity for the deletion when compared to the wild type sequence.

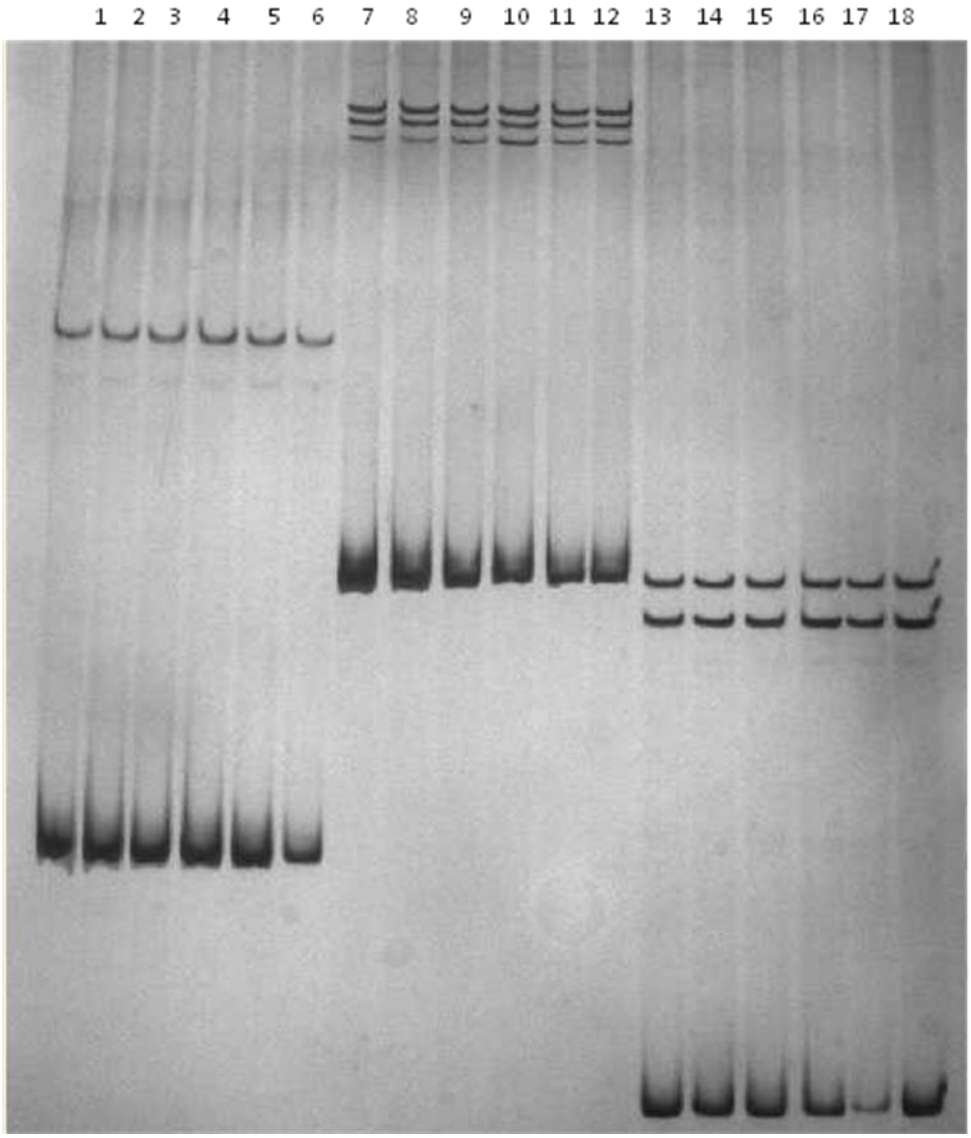


Figure 4.7 SSCP and HA analysis for *BRCA1* exons 14, 15 and 17. Presented in lanes 1 to 6 are SSCP/HA results for *BRCA1* exon 14 (representing CAM 2124, 2152, 2123, 2180, 2204, 2250), with the results for exon 15 in lanes 7 to 12 (CAM 2124, 2152, 2123, 2180, 2204, 2250). The SSCP/HA patterns for *BRCA1* exon 17 are represented in lanes 13 to 18 (CAM 2124, 2152, 2123, 2180, 2204, 2250).

For some exons such as *BRCA1* 14, a different running temperature (17°C instead of 12°C) was selected in order to obtain optimal separation of the SSCP bands (Fig. 4.8). Any fragment exhibiting a shift in either the SSCP or HA banding pattern was selected for DNA sequencing.

4.2.2.1 *BRCA1* coding mutations

Only two DNA changes were observed within the coding regions of *BRCA1* for the 35 patients screened (Table 4.1), namely *BRCA1* c.114G>A,p.Lys38= (rs1800062) and *BRCA1* c.4308T>C,p.Ser1436= (rs1060915) (Figs. 4.9 and 4.10). Both missense mutations did not result in a change in the amino acid sequence and are listed as synonymous mutations on the Breast Cancer Information Core (http://research.nhgri.nih.gov/projects/bic) (Table 4.1).

4.2.2.2 Intronic variants within *BRCA1*

A total of 10 intronic variants were identified (Table 4.1). Five of these were detected before as they are listed on the BIC. Although listed, their clinical significance is still indicated as unknown. The other five variants are novel changes as they have not been previously reported.

An intronic variant *BRCA1* IVS1-89delTTTAC (*BRCA1* c.-19-89delTTTAC) was detected for CAM 1975 (Fig. 4.11). This variant has no effect on the protein as the five base pair deletion occurs before the start codon of the gene. Although this variant has not been reported to the BIC or Leiden Open Variation Database (LOVD) before, it was once detected within the diagnostic laboratories of the NHLS in Bloemfontein for a patient from Kenya. A single base pair change (T>C) was also detected in intron 1 *BRCA1* IVS1-115T>C (*BRCA1* c.-19-115T>C) for CAM 2033 and CAM 2028 (Fig. 4.12). The altered banding pattern was observed on the SSCP/HA gel (Fig. 4.12A) and was confirmed with DNA sequencing (Fig. 4.12B). Although not reported on the LOVD, this variant was detected once on the BIC, but the ethnic group was not indicated.

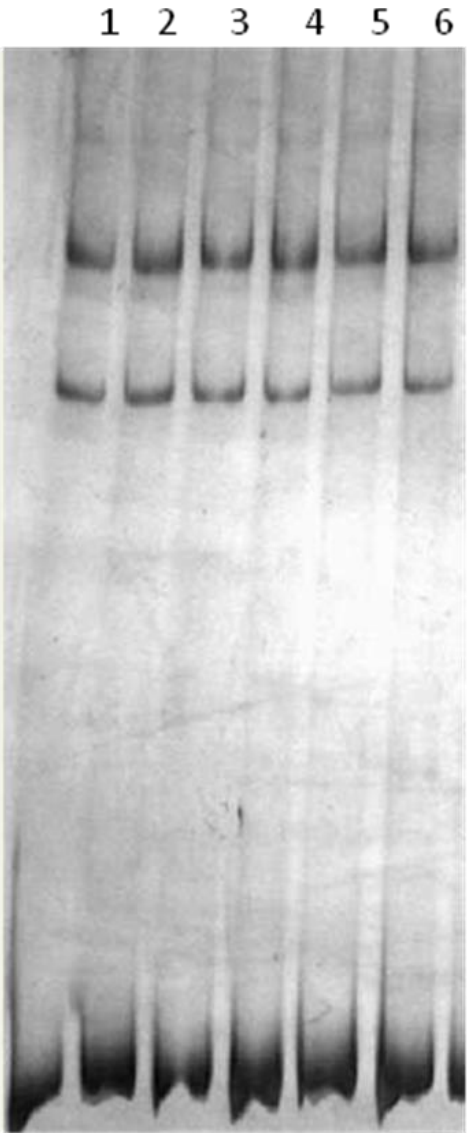


Figure 4.8 SSCP and HA analysis for *BRCA1* exon 14. The fragments were analyzed on a 10% poly-acrylamide gel, separated at 17°C for ≥ 16 hours, and visualized by silver staining. Presented in lanes 1 to 6 are SSCP and HA results for CAM 2038 (lane 1), CAM 2073 (lane 2), CAM 2074 (lane 3), CAM 2075 (lane 4), CAM 2084 (lane 5) and CAM 2122 (lane 6).

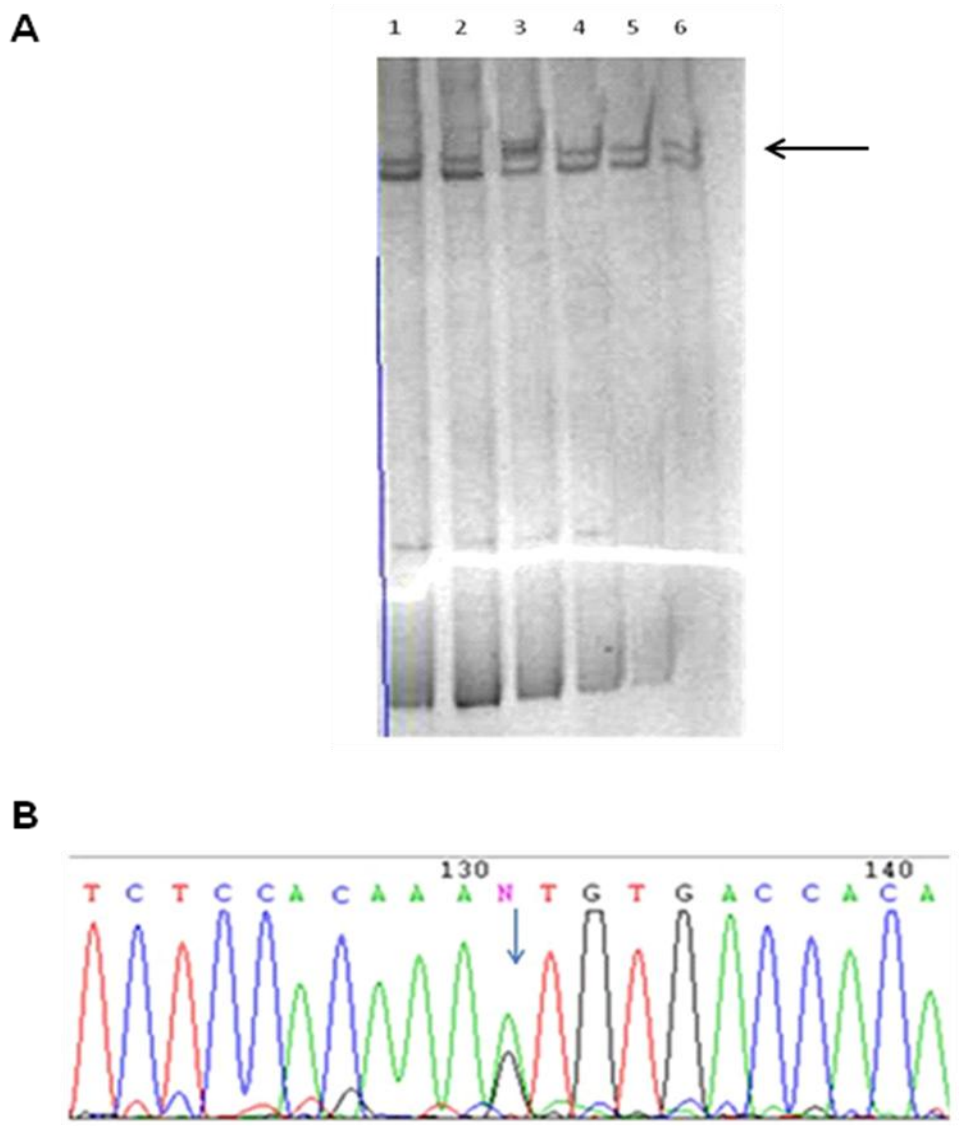


Figure 4.9 Mutation analysis of *BRCA1* exon 3 for patient CAM 1973. **A.** SSCP and HA analysis results. Presented in lanes 1 to 6 are results for CAM 2124 (lane 1), CAM 2152 (lane 2), CAM 1973 (lane 3), CAM 2123 (lane 4), CAM 2180 (lane 5) and CAM 2204 (lane 6). The SSCP band shift for CAM 1973 is indicated by the arrow. **B.** DNA sequencing results for *BRCA1* exon 3 for CAM 1973 indicating the single base pair change, namely a G>A (*BRCA1* c.114G>A,p.Lys38=).

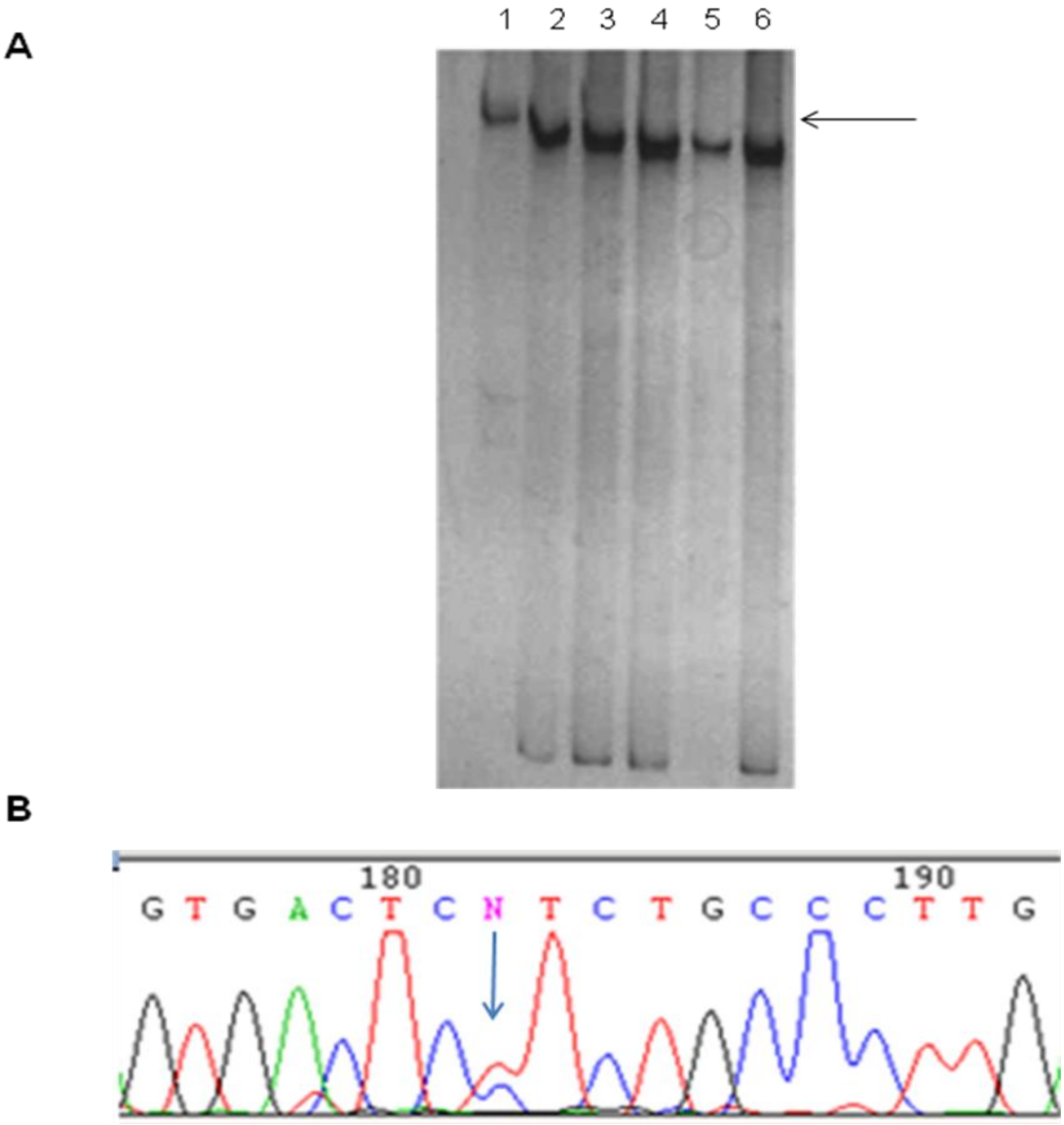


Figure 4.10 Mutation analysis of *BRCA1* exon 13 for patient CAM 1978. **A.** SSCP and HA analysis results. Presented in lanes 1 to 6 are SSCP and HA results for CAM 1978 (lane 1), CAM 1977 (lane 2), CAM 1979 (lane 3), CAM 1980 (lane 4), CAM 1989 (lane 5) and CAM 2027 (lane 6). The separation revealed a SSCP band shift for CAM 1978 as indicated by the arrow. **B.** DNA sequencing results for *BRCA1* exon 13 for CAM 1978 indicating the single base change, namely a T>C (*BRCA1* c.4308T>C,p.Ser1436=).

Table 4.1. Mutational variants detected within *BRCA1* for the 35 patients included in this study. Indicated are the exon or intron number, the nucleotide involved, the base change involved, the amino acid change if any, the new HGVS name, the number of entries onto the BIC, the mutation type, the ethnicity group in which the change has been detected and the rs number if available.

Exon/Intron	Nucleotide number	Codon	Base change	AA change	Designation	HGVS cDNA	HGVS protein	Entries on BIC	Mutation type	Ethnicity	rs number
3	233	38	G>A	Lys to Lys	233G/A	c.114G>A	p.Lys38=	17	Syn	Caucasian	rs1800062
13	4427	1436	T>C	Ser to Ser	4427T/C	c.4308T>C	p.Ser1436=	250	Syn	Caucasian	rs1060915
1	101-89		delTTTAC	Non coding	89delTTTAC	c.-19-89delTTTAC	-	Novel	IVS	-	-
1	101-115	-	T>C	Non coding	IVS1-115T>C	c.-19-115T>C	-	1	IVS	Not specified	-
2	200-218	-	C>G	Non-coding	IVS2-218C>G	c.81-218C>G	-	Novel	IVS	-	-
2	200-63	-	C>G	Non-coding	IVS2-63C>G	c.81-63C>G	-	Novel	IVS	-	-
2	200-12	-	C>G	Non-coding	IVS2-12C>G	c.81-12C>G	-	9	IVS	African	rs80358055
2	200-193	-	C>A	Non-coding	IVS2-193C>A	c.81-193C>A	-	Novel	IVS	-	-
7	561-34	-	C>T	Non-coding	IVS7-34C/T	c.442-34C>T	-	206	IVS	Chinese	rs799923
8	667-64	-	delT	Non-coding	IVS8-64delT	c.548-64delT	-	1	IVS	Turkish	-
18	5271+73	-	G>A	Non-coding	IVS18+73G>A	c.5152+73G>A	-	1	IVS	Not specified	-
18	5271+92	-	delT	Non-coding	IVS18+92delT	c.5152+92delT	-	Novel	IVS	-	-

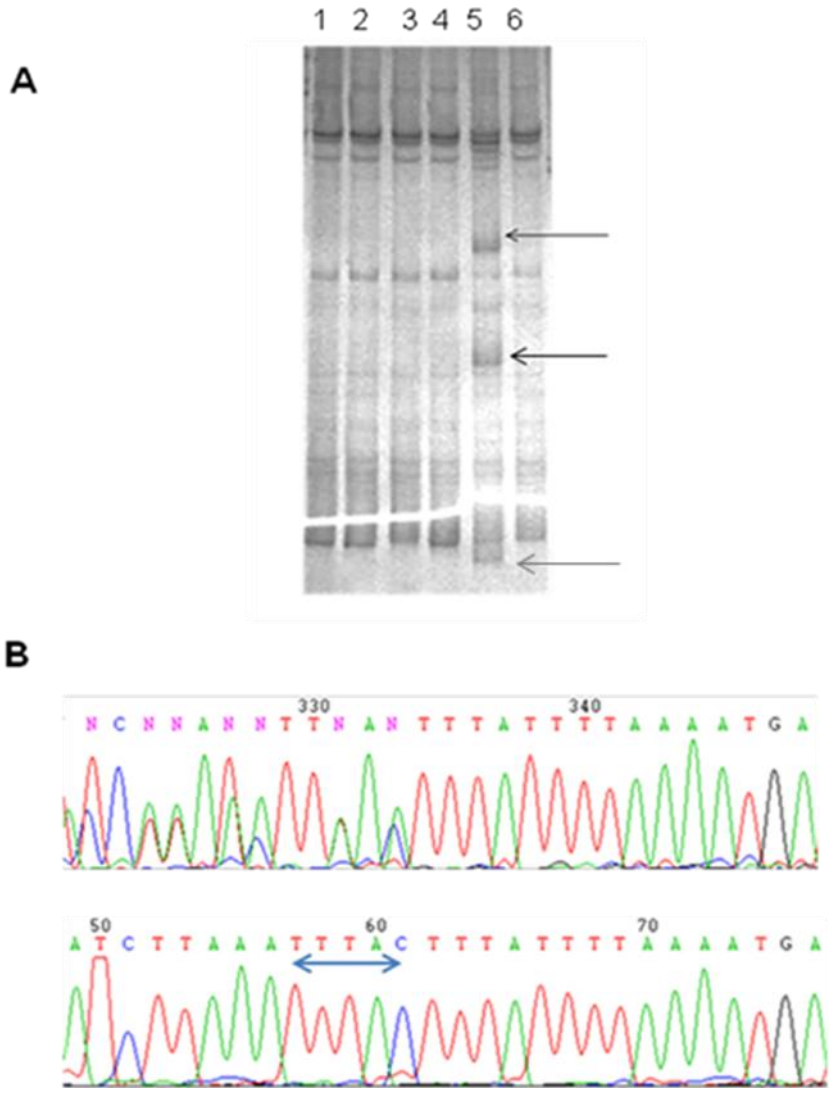


Figure 4.11 Mutation analysis of *BRCA1* intron 1 for CAM 1975. **A.** SSCP and HA analysis results. Presented in lanes 1 to 6 are SSCP and HA results for CAM 1971 (lane 1), CAM 1972 (lane 2), CAM 1973 (lane 3), CAM 1974 (lane 4), CAM 1975 (lane 5) and CAM 1976 (lane 6). The separation revealed various SSCP and HA band shifts for CAM 1975 as indicated by the arrows. **B.** DNA sequencing results for *BRCA1* intron 1 for CAM 1975 indicating a five base deletion, namely *BRCA1* IVS1-delTTTAC (*BRCA1* c.-19-89delTTTAC) with the double pointed arrow indicating the five base pair deletion.

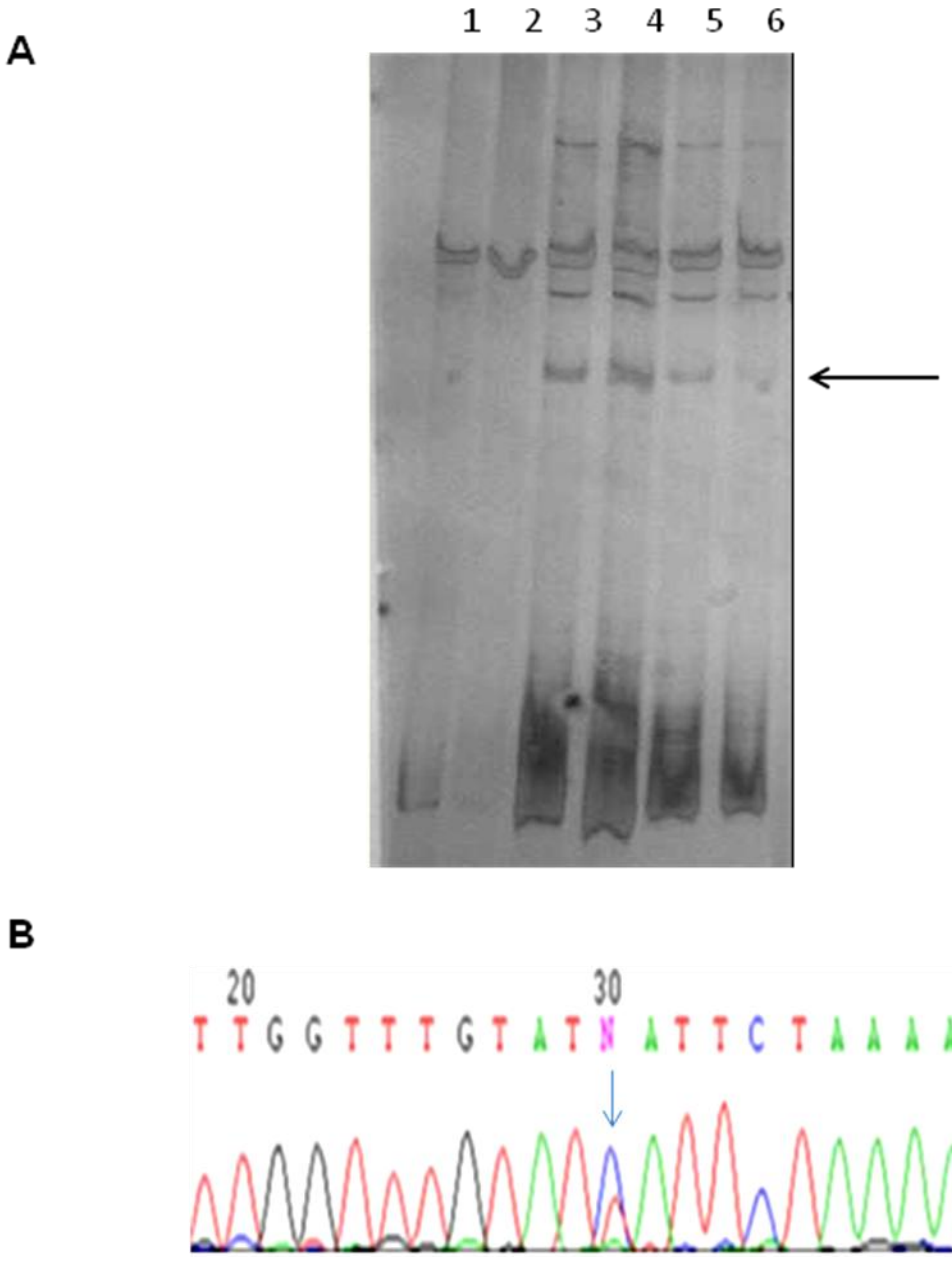


Figure 4.12 Mutation analysis of *BRCA1* intron 1 for CAM 2033 and CAM 2028. **A.** SSCP and HA results. Presented in lanes 1 to 6 are SSCP and HA results for CAM 2033 (lane 1), CAM 2028 (lane 2), CAM 2034 (lane 3), CAM 2035 (lane 4), CAM 2036 (lane 5) and CAM 2037 (lane 6). The separation revealed SSCP band shifts for CAM 2033 and CAM 2028 as indicated by the arrow. **B.** DNA sequencing results for *BRCA1* intron 1 indicating the single base pair change of T>C, namely *BRCA1* IVS1-115T>C (*BRCA1* c.-19-115T>C).

A total of four intron 2 variants were detected, namely *BRCA1* IVS2-218C>G (*BRCA1* c.81-218C>G), *BRCA1* IVS2-63C>G (*BRCA1* c.81-63C>G), *BRCA1* IVS2-193C>A (*BRCA1* c.81-193C>A) and *BRCA1* IVS2-12C>G (*BRCA1* c.81-12C>G) (Table 4.1). The first three are novel variants and have been detected once for CAM 1979, CAM 2028 and CAM 1974 respectively, whereas CAM 2084 carried rs80358055, namely IVS2-12C>G (c.81-12C>G). This variant was reported nine times to the BIC for patients representing an African heritage and also once on the LOVD.

BRCA1 IVS7-34C/T (*BRCA1* c.442-34C>T, rs799923) was detected in intron 7 for CAM 1978. This variant is common and has been reported 206 times to the BIC within the Austrian population and on the LOVD. Although common, it is still listed as a variant with unknown clinical significance. The *BRCA1* c.442-34C>T variant was also detected within the diagnostic laboratories of the NHLS in Bloemfontein for a patient from Kenya.

Two recurrent variants have been identified, one in intron 8 and another in intron 18. *BRCA1* IVS8-64delT (*BRCA1* c.548-64_548-64delT) was reported once to the BIC within a Turkish patient but not on the LOVD database. This variant was detected in three unrelated patients CAM 1978, CAM 2033 and CAM 2037. This variant was identified based on a HA band shift (Fig. 4.13A) and verified by DNA sequencing (Fig. 4.13B).

The second recurrent variant *BRCA1* IVS18+73G>A (*BRCA1* c.5152+73G>A) occurred within intron 18 (Fig. 4.14). The variant has been reported once before although the ethnicity was not specified (Table 4.1). This variant is not listed on the LOVD database. This variant was identified in three unrelated patients CAM 1978, CAM 1980 and CAM 2033. This variant was identified based on the presence of a SSCP band shift (Fig. 4.14A) and verified by DNA sequencing (Fig. 4.14B).

The last variant detected within *BRCA1* was also in intron 18. The variant *BRCA1* IVS18+92delT (*BRCA1* c.5152+92delT) was identified for CAM 2033. The variant is novel and has not been reported before in both BIC and LOVD database (Table 4.1).

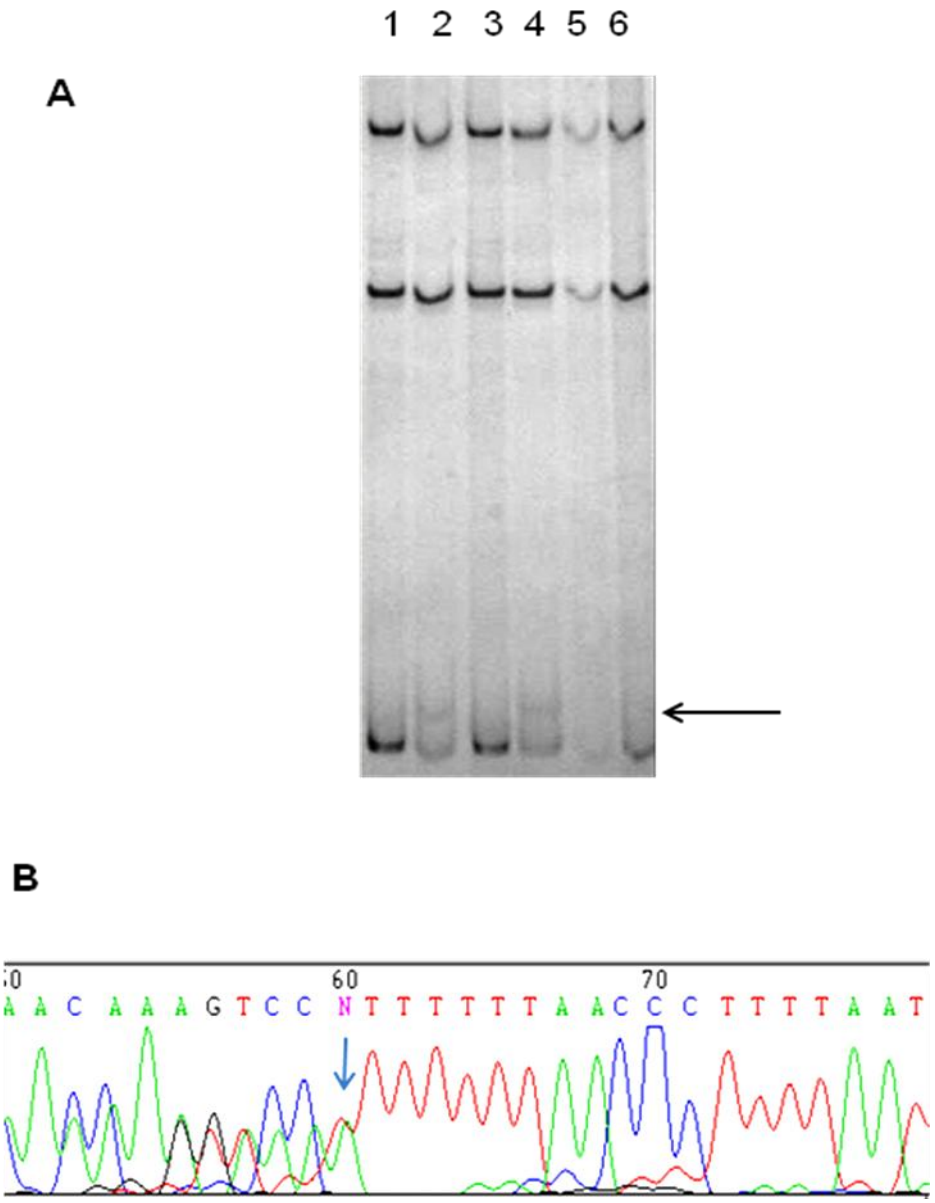


Figure 4.13 Mutation analysis of *BRCA1* intron 8 for CAM 1978 and CAM 2037. **A.** Presented in lanes 1 to 6 are SSCP/HA results for CAM 1977 (lane 1), CAM 1978 (lane 2), CAM 1979 (lane 3), CAM 2037 (lane 4), CAM 1980 (lane 5) and CAM 1989 (lane 6). The separation revealed a HA band shift for two patients as indicated by the arrow. **B.** DNA sequencing results for *BRCA1* intron 8 indicating the deletion of a single base pair (delT), namely *BRCA1* IVS8-64delT (*BRCA1* c.548-64_548-64delT).

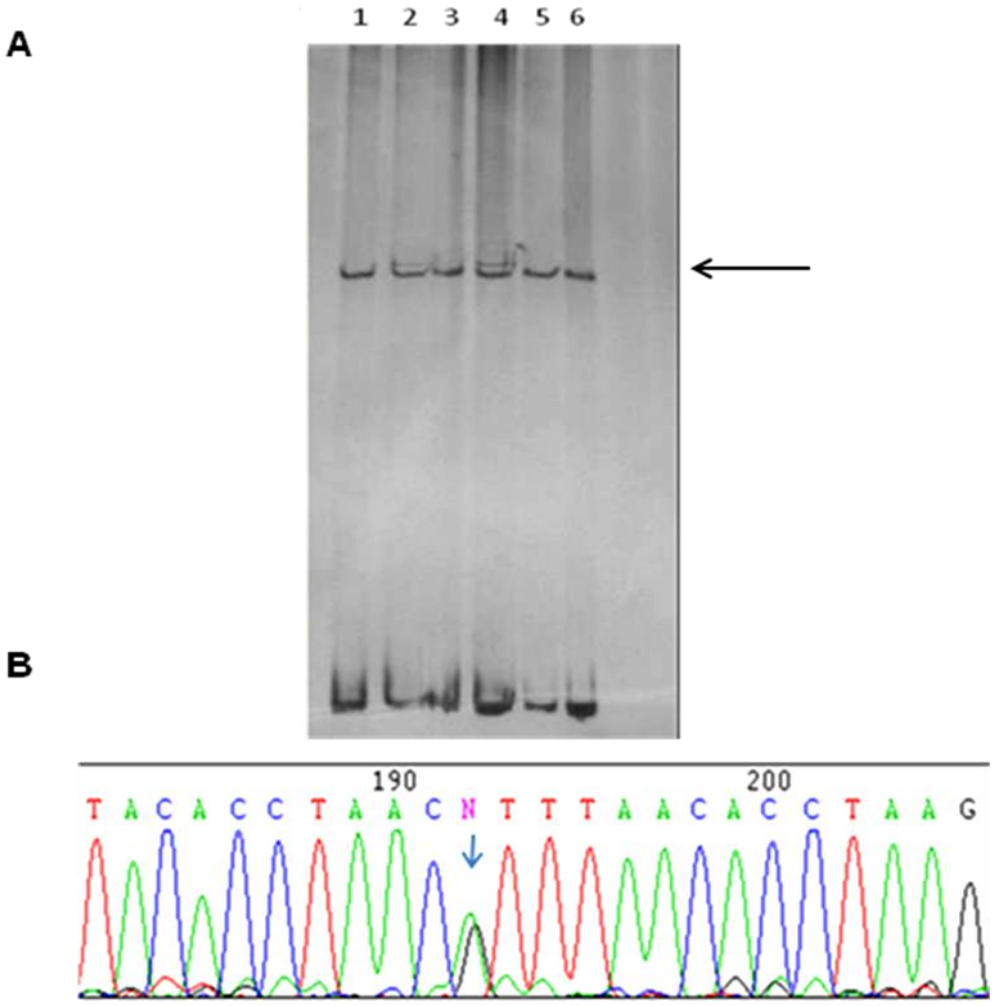


Figure 4.14 Mutation analysis of *BRCA1* exon 18. **A.** Presented in lanes 1 to 6 are SSCP/HA results for CAM 1977 (lane 1), CAM 1978 (lane 2), CAM 1989 (lane 3), CAM 1980 (lane 4), CAM 2084 (lane 5) and CAM 2037 (lane 6). The separation revealed a SSCP band shift as indicated by the arrow. **B.** DNA sequencing results for *BRCA1* exon 18 indicating a single base pair change, namely *BRCA1* IVS18+92delT (*BRCA1* c.5152+92delT).

4.2.3 MLPA

To detect large deletions or insertions, the *BRCA1* gene was screened using MLPA. Although all of the 35 samples were sent to Canada for analysis, results were only obtained for 12 patients. The DNA quantities of the other samples were to low to obtain any results. All 12 patients tested negative for the presence of major insertions or deletions within *BRCA1*. The patients included CAM 1971, CAM 1973, CAM 1978, CAM 2027, CAM 2033, CAM 2034, CAM 2074, CAM 2075, CAM 2084, CAM 2122, CAM 2252 and CAM 2253.

4.3 *BRCA2* results

4.3.1 Screening of exon 10 and 11 using PTT

BRCA2 exons 10 and 11 were amplified in five overlapping fragments. All amplicons were resolved on a 2% agarose gel to verify amplification before proceeding with *in vitro* transcription and translation (Fig. 4.15). The majority of translated polypeptides did not exhibit any prematurely truncated polypeptides (Fig. 4.16).

A single truncation was detected for CAM 2279 in *BRCA2* exon 11 in fragment D (Fig. 4.17). The appropriate SSCP/HA primer sets that span this region were selected for analysis to determine the position and the type of mutation that occurred. Two patients exhibiting polypeptides of the normal size were included as controls for the analysis. All three patient samples were analyzed for each of the five smaller SSCP fragments (primers for SSCP fragment S, fragment T, fragment U, fragment W and fragment X) (Fig. 4.18), where after the SSCP/HA banding patterns for each of these fragments were compared. Various major SSCP and HA band shifts were identified for CAM 2279 within SSCP fragment U.

Fragment U was re-amplified and separated on a 12% poly-acrylamide gel containing 6% urea. Both HA bands (Fig. 4.19) were excised separately, eluted overnight where after the supernatant was used as DNA template for Sanger sequencing. Bi-directional DNA sequencing revealed the presence of a single base pair deletion within *BRCA2* exon 11 (Fig. 4.20). According to the latest HGVS nomenclature, the mutation is classified as *BRCA2* c.6455_6455delT

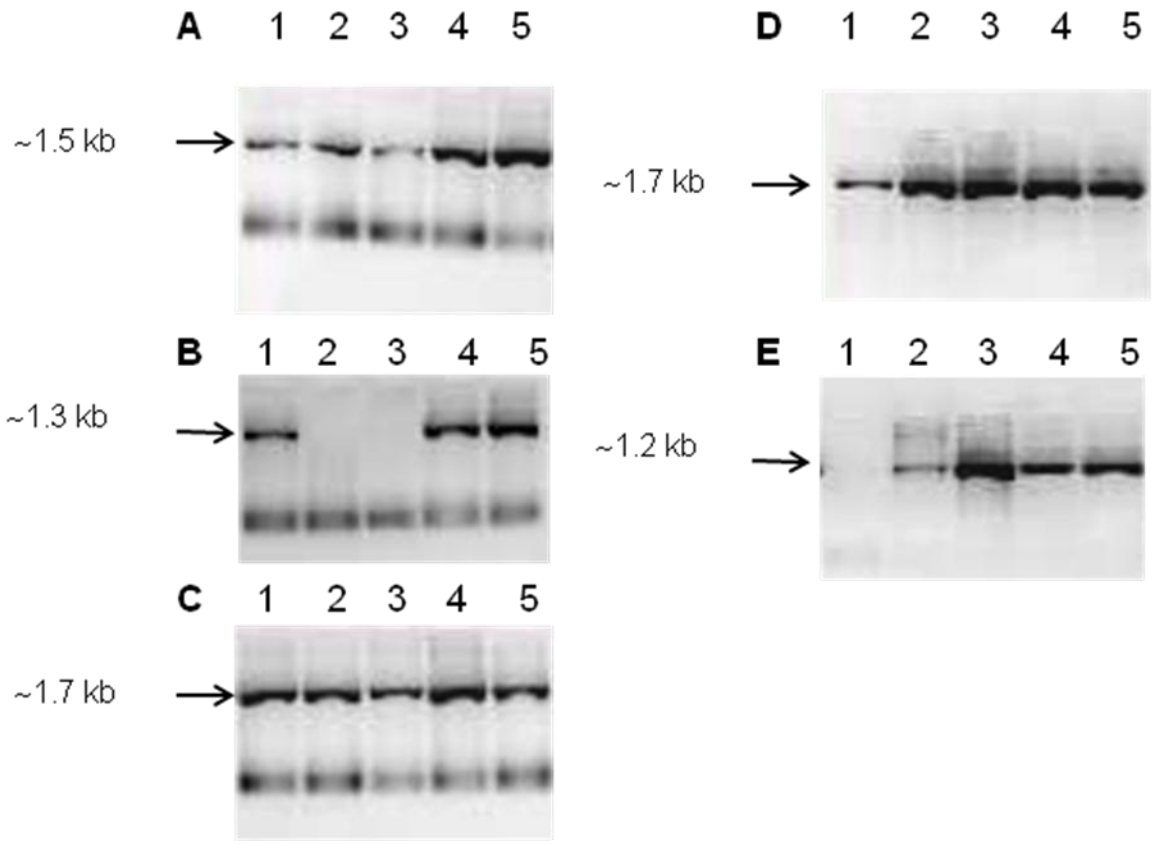


Figure 4.15 Amplification of *BRCA2* exons 10 and 11. The products were separated on a 2% agarose gel and visualized by ethidium bromide staining. **A.** Amplification products of ~1.5 kb for *BRCA2* fragment A. **B.** Amplification products of ~1.3 kb for *BRCA2* fragment B. **C.** Amplification products of ~1.7 kb for *BRCA2* fragment C. **D.** Amplification products of ~1.7 kb for *BRCA2* fragment D. **E.** Amplification products of ~1.2 kb for *BRCA2* fragment E. The fragments represent the amplified products for CAM 2124 (lane 1), CAM 2251 (lane 2), CAM 2123 (lane 3), CAM 2180 (lane 4) and CAM 2204 (lane 5) in each figure.

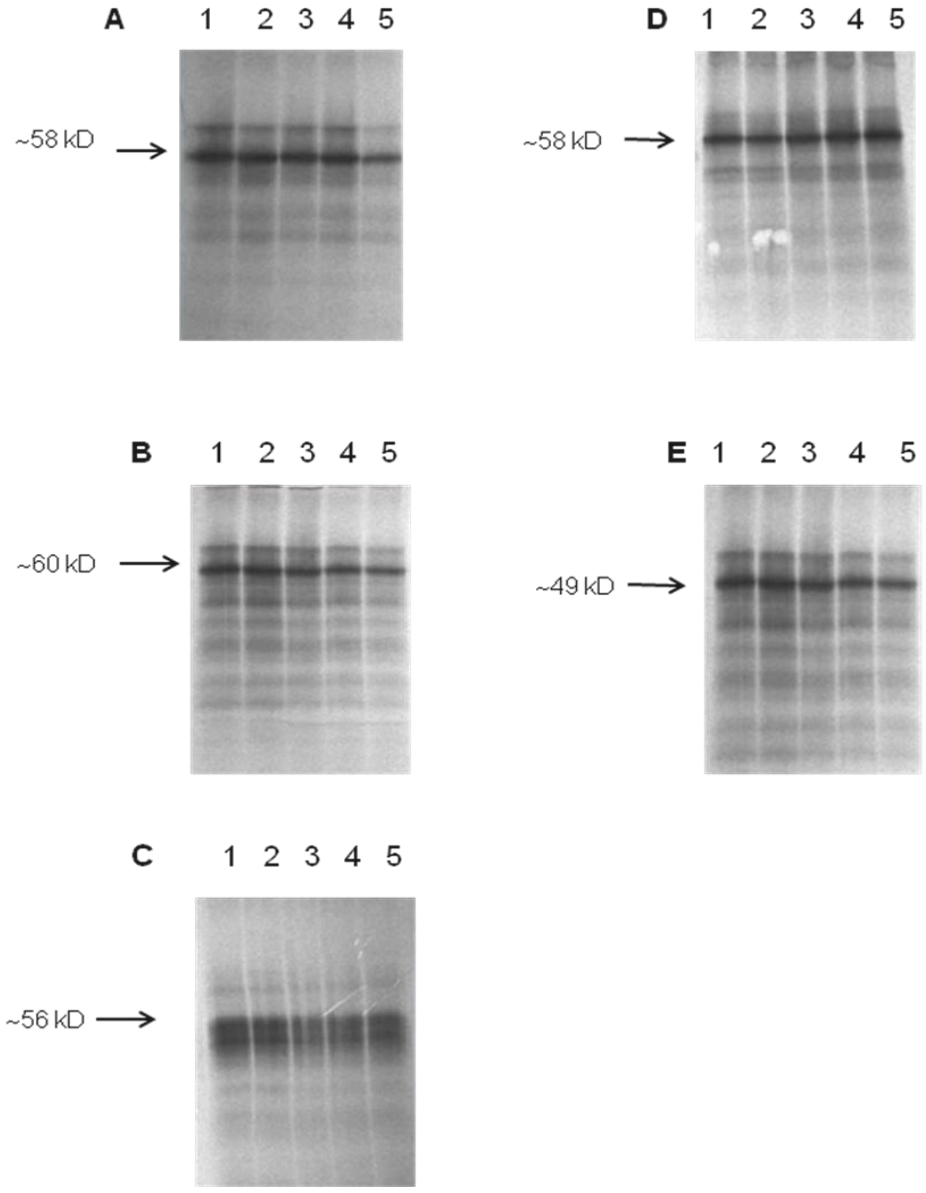


Figure 4.16 Polypeptides generated for *BRCA2* exon 10 and 11. The translated fragments were separated on a 12% poly-acrylamide gel and exposed overnight to an X-ray. **A.** Polypeptides of ~58 kD representing fragment A of *BRCA2* exon 11. **B.** Polypeptides of ~60 kD representing fragment B of *BRCA2* exon 11. **C.** Polypeptides of ~56 kD representing fragment C of *BRCA2* exon 11. **D.** Polypeptides of ~58 kD representing fragment D of *BRCA2* exon 11. **E.** Polypeptides of ~49 kD representing *BRCA2* exon 10. The fragments represent the amplified DNA for CAM 2124 (lane 1), CAM 2251 (lane 2), CAM 2123 (lane 3), CAM 2180 (lane 4) and CAM 2204 (lane 5) in each figure.

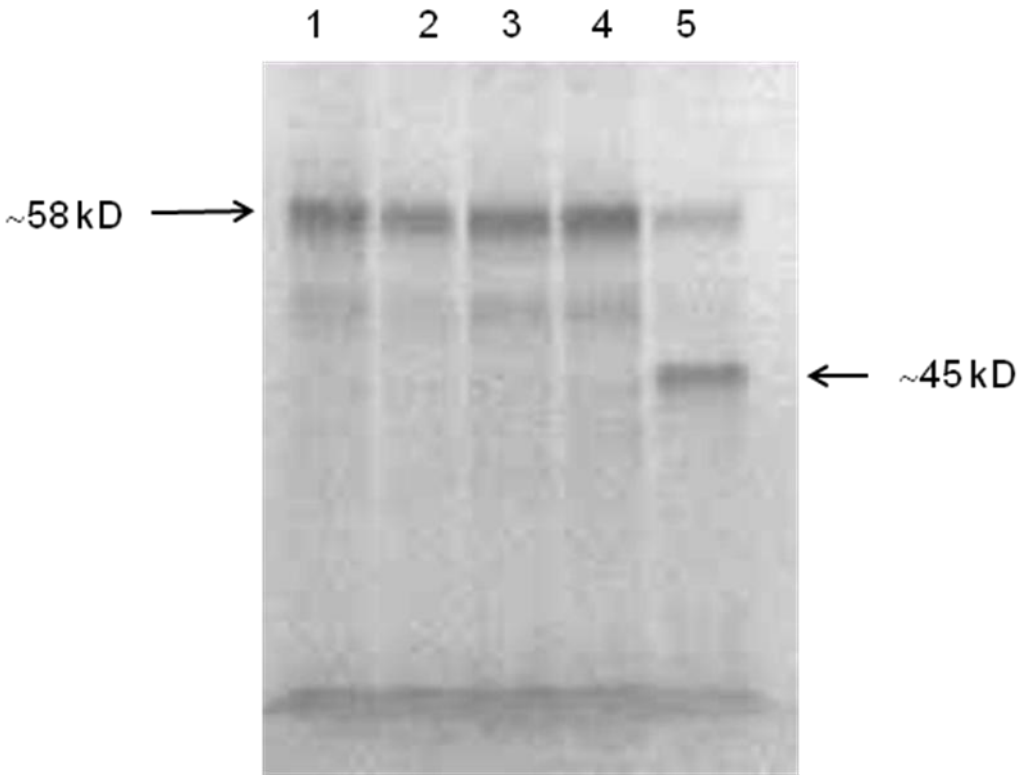


Figure 4.17 Polypeptides generated for fragment D of *BRCA2* exon 11. Translated polypeptides were separated on a 12% poly-acrylamide gel and exposed overnight to an X-ray. A prematurely truncated polypeptide of ~45 kD is visible for CAM 2279 in lane 5 as indicated by the arrow. The fragments represent the amplified products for CAM 2209 (lane 1), CAM 2251 (lane 2), CAM 2252 (lane 3), CAM 2253 (lane 4) and CAM 2279 (lane 5).

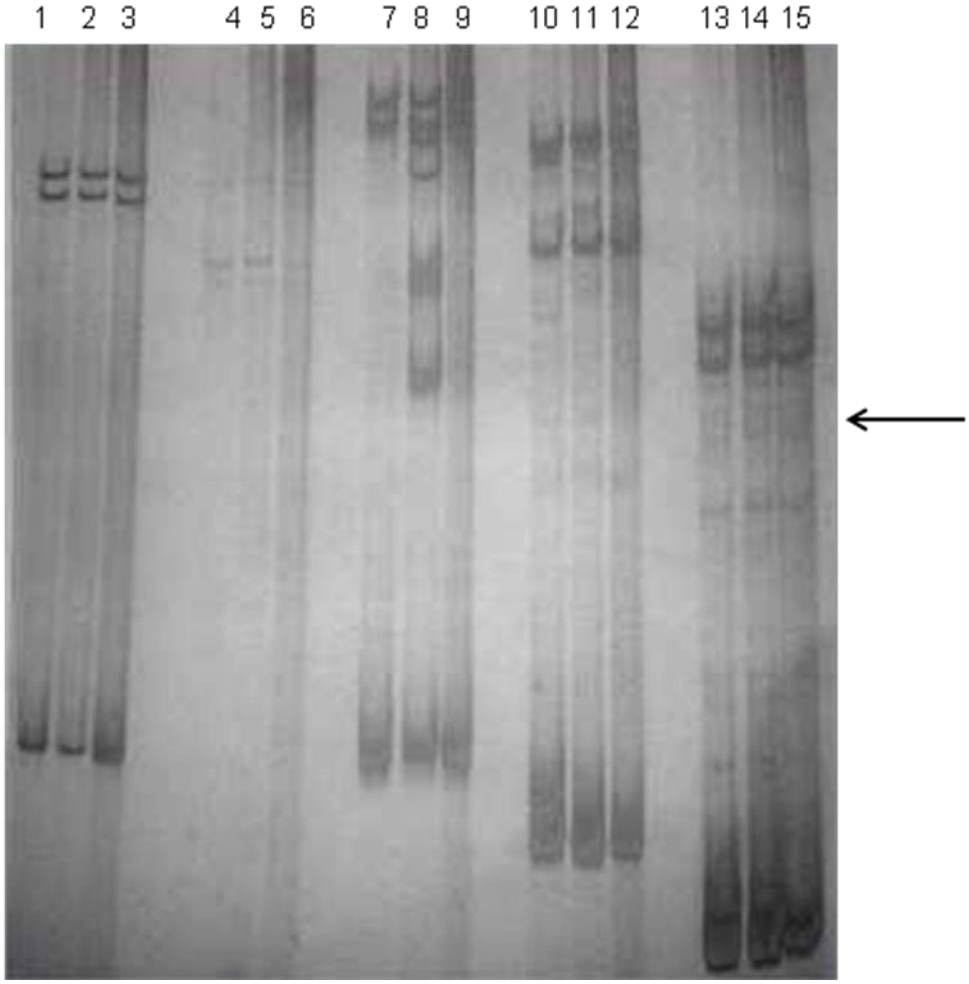


Figure 4.18 SSCP analysis using the smaller primer sets spanning fragment D of *BRCA2* exon 11. Results for SSCP fragment S are presented in lanes 1 to 3, fragment T in lanes 4 to 6, fragment U in lanes 7 to 9, fragment W in lanes 10 to 12 and fragment X in lanes 13 to 15. The different banding pattern visible for CAM 2279 for fragment U is indicated by the arrow. The fragments representing the amplified products for CAM 2279 are presented in lanes 2, 5, 8, 11 and 14, with the control samples of CAM 2300 (lanes 1, 4, 7, 10 and 13) and CAM 2254 (lanes 3, 6, 9, 12 and 15) in the others.

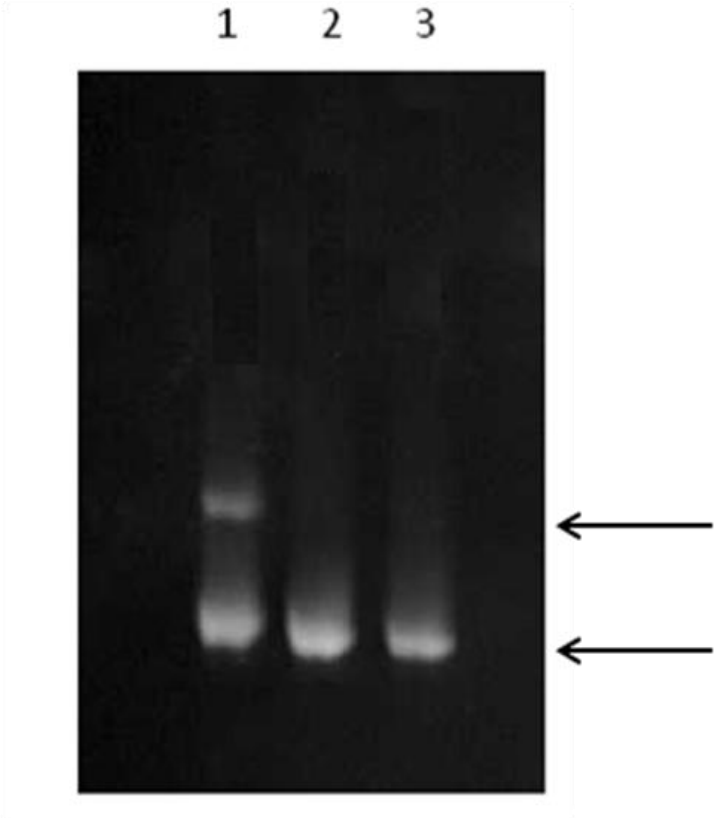


Figure 4.19 Heteroduplex analysis for fragment U of *BRCA2* exon 11. Lane 1 represents affected patient CAM 2279, while lanes 2 and 3 represent control patients CAM 2300 and CAM 2254 respectively. The heteroduplex fragments are indicated by the arrows.

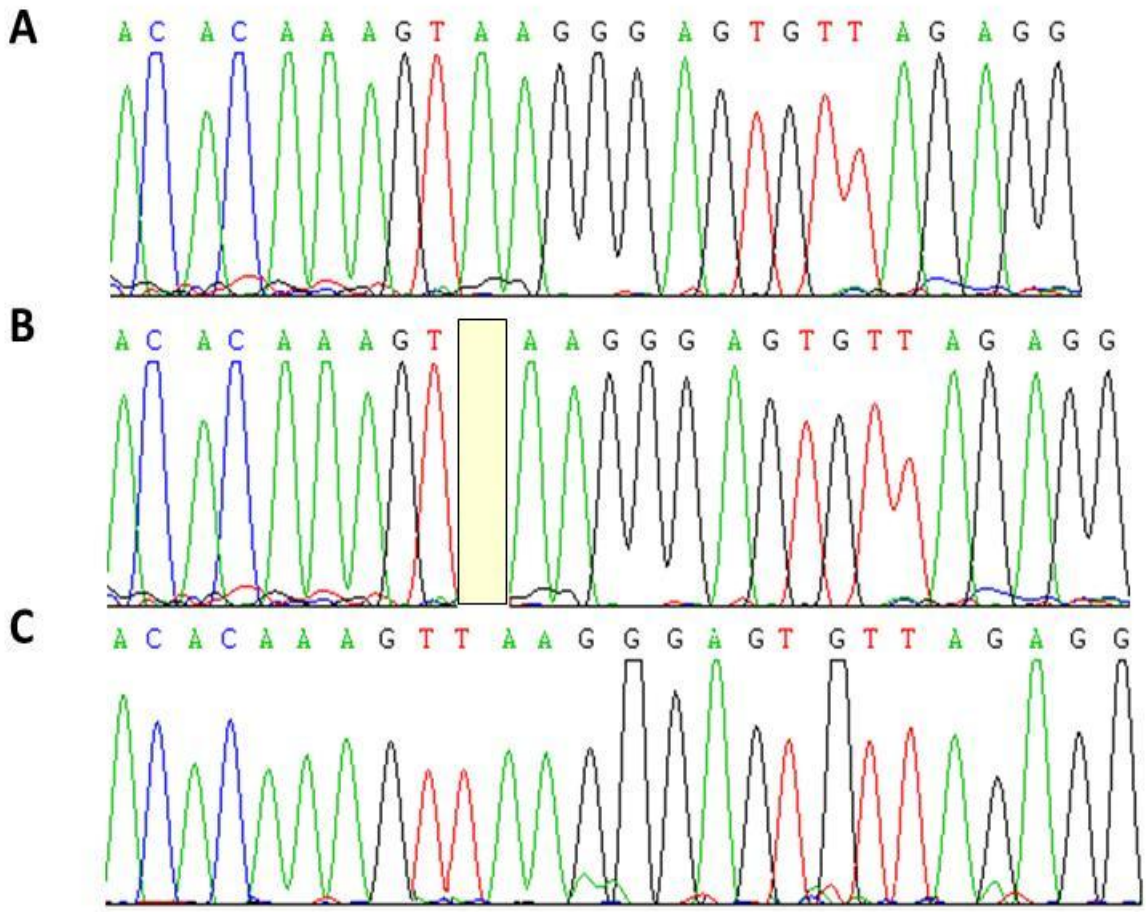


Figure 4.20 Electropherograms illustrating DNA sequencing results for *BRCA2* exon 11. **A.** Electropherogram representing the first excised heteroduplex band for CAM 2279, indicating homozygosity for a single base pair deletion (delT). **B.** Electropherogram of CAM 2279 indicating the position of the deleted T (highlighted by the yellow box). **C.** Electropherogram of the second excised band representing CAM 2279, indicating homozygosity for the normal wild type sequence of *BRCA2*.

p.Lys2075ArgfsX2078. This deletion causes the changing of the Lysine amino acid to Arginine at codon 2075, resulting in a STOP codon at 2078 and a subsequent truncated polypeptide. This mutation has never been reported before.

4.3.1.1 *BRCA2* coding mutations

Two DNA changes were observed within the coding regions of *BRCA2* (Table 4.2), namely *BRCA2* c.7017G>C,p.Lys2339Asn (rs45574331) and *BRCA2* c.8830A>T,p.Ile2944Phe (rs4987047) (Figs. 4.21 and 4.22). Both missense mutations resulted in an amino acid change, are relatively common and have been reported before to the BIC (Table 4.2). The missense mutation rs45574331 was detected in exon 14 for a single patient, namely CAM 1973 (Fig. 4.21). The second single base change rs4987047 was detected in *BRCA2* exon 22 for CAM 2074 (Fig. 4.22). Both these mutations were previously detected for the Native American and African American population groups.

4.3.1.2 Intronic variants within *BRCA2*

In total thirteen intronic variants were identified (Table 4.2). Eight have been previously reported on the BIC and five are regarded as novel variants. Of the eight reported variants detected two were in the 5' untranslated (5′-UTR) region. The variant 203G>A (c.-26G>A) was detected in *BRCA2* exon 2 for patient CAM 1975 (Fig. 4.23). This variant has been previously reported within the Caucasian ethnicity and also on the LOVD. The second 5′-UTR variant 218C>T (c.-11C>T) was detected in *BRCA2* exon 2 for patient CAM 1979 (Fig. 4.24A). This mutation has been previously reported within the African ethnicity and on LOVD database. Both these 5′-UTR variants were detected once in two unrelated patients.

The first intronic variant IVS3-22C/T (c.317-22C>T) was detected in *BRCA2* intron 3 for patient CAM 1973 (Fig. 4.24B). This variant has been reported once within the African ethnicity and also once on the LOVD. Two variants were detected in *BRCA2* intron 6 namely IVS6-4C>G (c.517-4C>G) and IVS6-19C>T (c.517-19C>T) for patients CAM 1989, 1971 and 1973 respectively. The IVS6-4C>G (c.517-4C>G) variant was detected in three unrelated patients (Fig. 4.25).

Table 4.2. Total number of variants detected within *BRCA2* for the 35 patients included for this study. Indicated are the exon or intron number, the nucleotide involved, the base change involved, the amino acid change if any, the new HGVS name, the number of entries onto the BIC, the mutation type, the ethnicity group in which the change has been detected and the rs number if available.

Exon/Intron	Nucleotide number	Codon	Base change	AA change	Designation	HGVS cDNA	HGVS protein	Entries on BIC	Mutation type	Ethnicity	rs number
14A	7245	2339	G>C	Lys to Asn	K2339N	c.7017G>C	p.Lys2339Asn	66	M	African American	rs45574331
22	9058	2944	A>T	Ile to Phe	I2944F	c.8830A>T	p.Ile2944Phe	115	M	Native American/ African American	rs4987047
2	203	-	G>A	Non coding	203G>A	c.-26G>A	-	205	5′UTR	Caucasian	-
2	218	-	C>T	Non coding	218C>T	c.-11C>T	-	48	5′UTR	African	-
I-3	545-22	-	C>T	Non coding	IVS3-22C/T	c.317-22C>T	-	1	IVS	African	rs81002794
I-6	745-4	-	C>G	Non coding	IVS6-4C/G	c.517-4C>G	-	2	IVS	African	rs81002804
I-6	745-19	-	C>T	Non coding	IVS6-19C>T	c.517-19C>T	-	15	IVS	Western Europe Latin American Caribbean	rs11571623

8	909+56	-	C>T	Non coding	IVS8+56C>T	c.681+56C>T	-	3	IVS	Not specified	-
12	7070-175	-	T>G	Non coding	IVS12-175T>G	c.6842-175T>G	-	Novel	IVS	-	-
14A	7663+224	-	A>G	Non coding	IVS14A+224A>G	c.7435+224A>G	-	Novel	IVS	-	-
15	7664-9	-	C>T	Non coding	IVS15-9C>T	c.7436-9C>T	-	Novel	IVS	-	-
22	8983-5	-	C>T	Non coding	IVS21-5C>T	c.8755-5C>T	-	Novel	IVS	-	-
22	8983-66	-	T>C	Non coding	IVS21-66T>C	c.8755-66T>C	-	318	IVS	White South African	rs4942486
26	9876+54	-	G>A	Non coding	IVS26+54G/A	c.9648+54G>A	-	1	IVS	African	rs11571823
27B	9877-62	-	C>T	Non coding	IVS27-62C>T	c.9649-63C>T	-	Novel	IVS	-	-

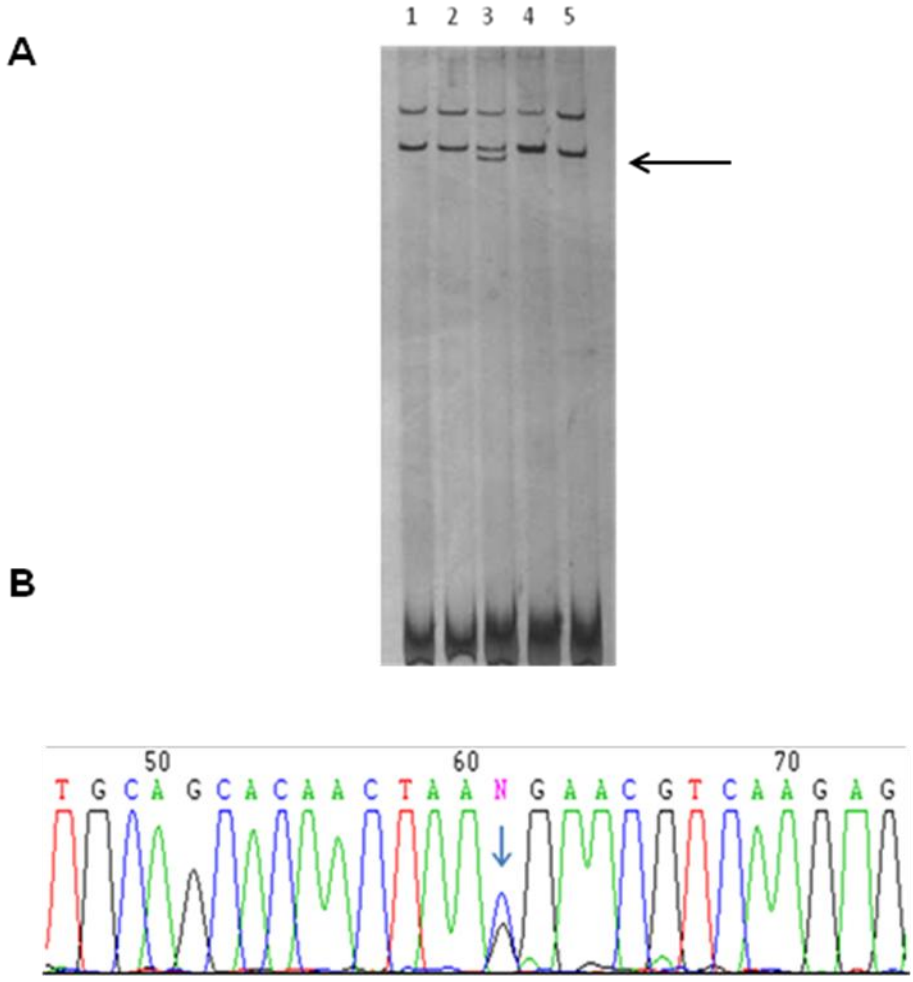


Figure 4.21 Mutation analysis of *BRCA2* exon 14 for CAM 1973. **A.** Illustrated is SSCP and HA analysis results. Presented in lanes 1 to 5 are results for CAM 1971 (lane 1), CAM 1972 (lane 2), CAM 1973 (lane 3), CAM 1974 (lane 4), CAM 1975 (lane 5). The SSCP band shift for CAM 1973 is indicated by the arrow. **B.** DNA sequencing results for *BRCA2* exon 14 indicating the single base pair change, namely a G>C [*BRCA2* c.7017G>C,p.Lys2339Asn (rs45574331)].

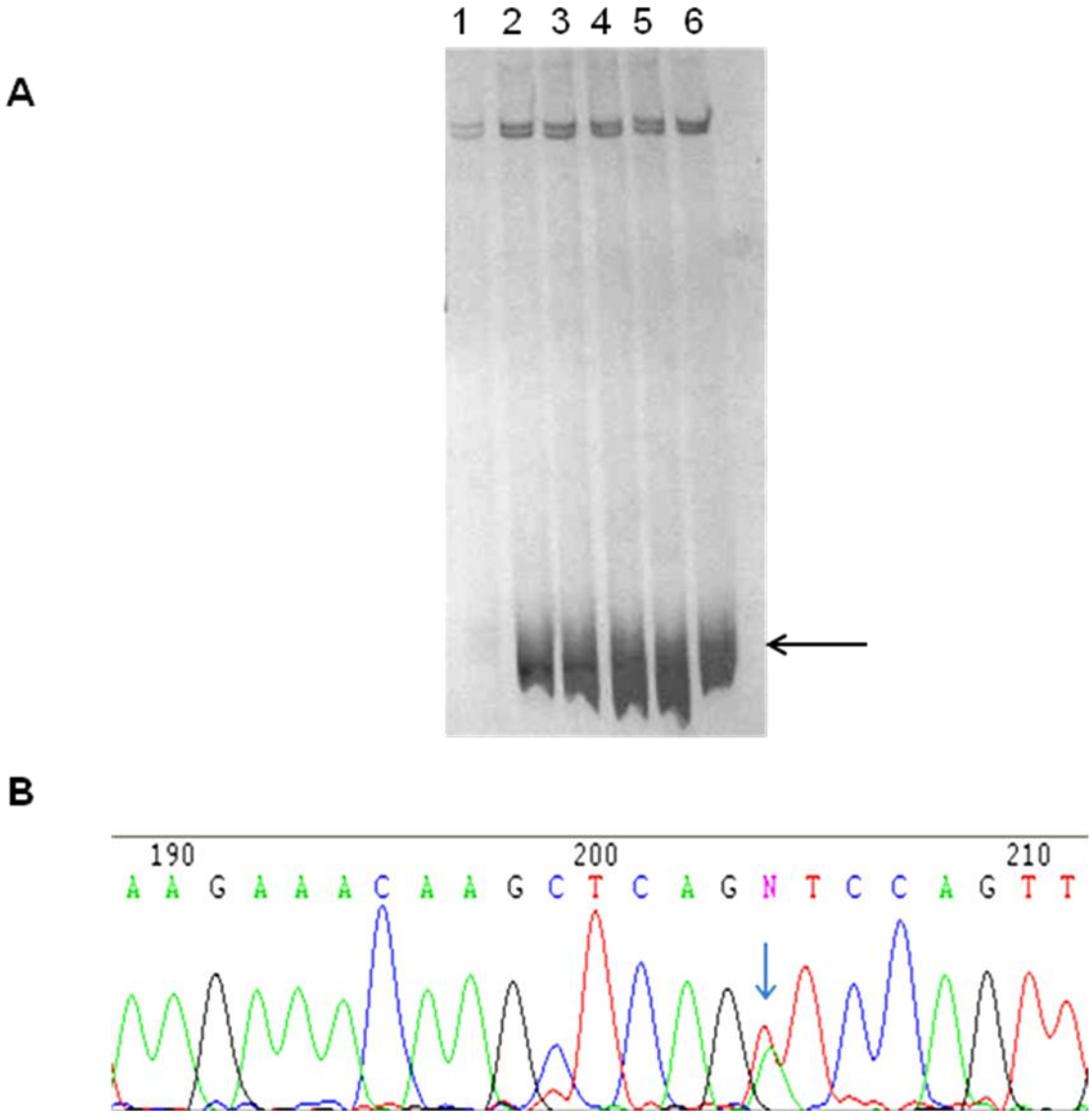


Figure 4.22 Mutation analysis of *BRCA2* exon 22 for CAM 2074. **A.** Illustrated are SSCP and HA analysis results. Presented in lanes 1 to 6 are results for CAM 2074 (lane 1), CAM 2038 (lane 2), CAM 2073 (lane 3), CAM 2075 (lane 4), CAM 2084 (lane 5) and CAM 2122 (lane 6). The separation revealed a HA band shift for CAM 2074 as indicated by the arrow. **B.** DNA sequencing results for *BRCA2* exon 22 indicating the single base pair change, namely a A>T [(*BRCA2* c.8830A>T,p.Ile2944Phe (rs4987047)].

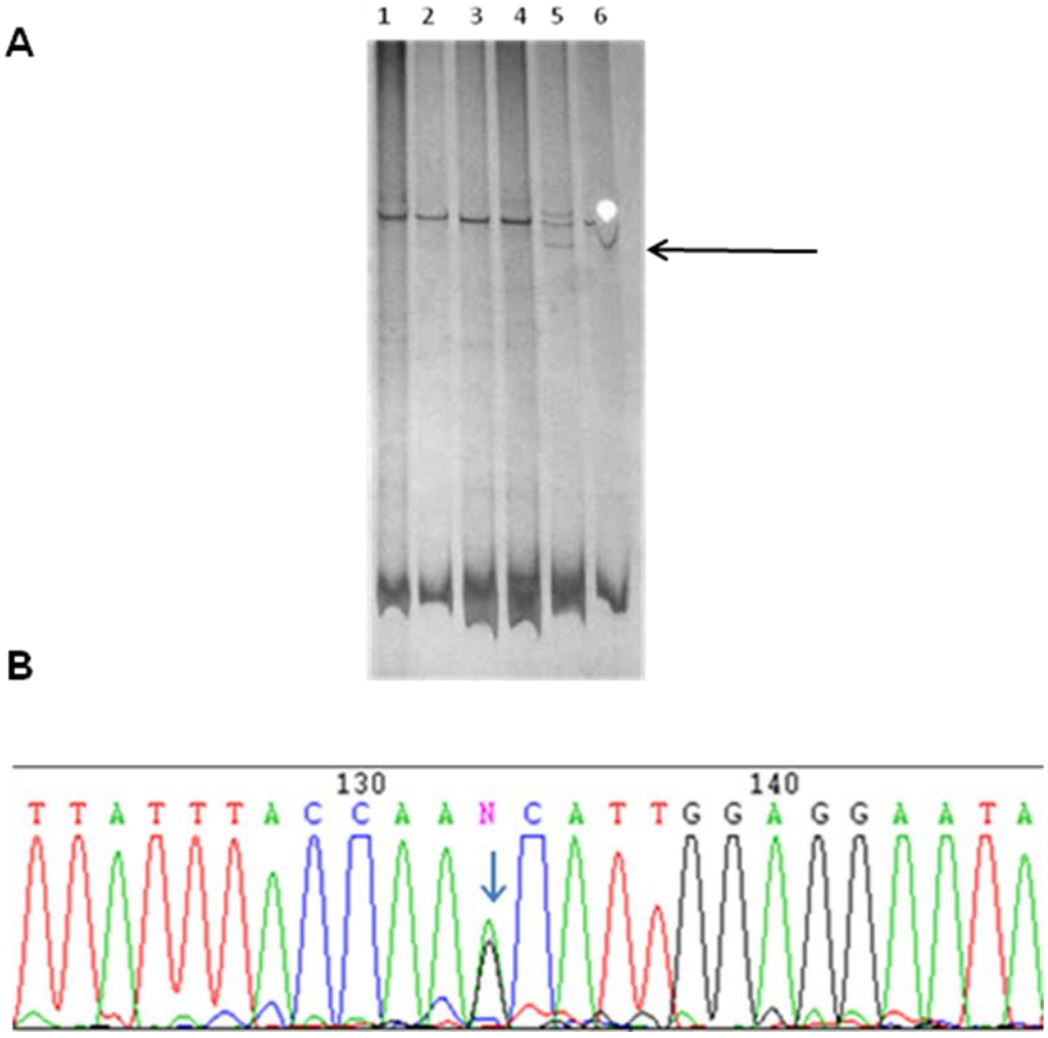
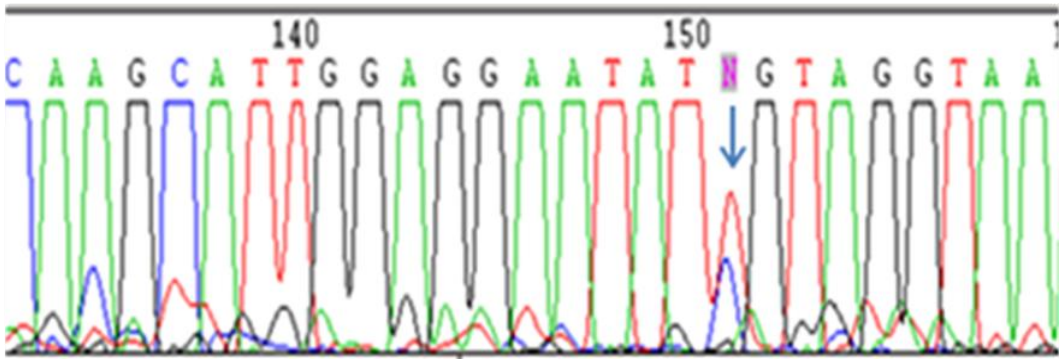


Figure 4.23 Mutation analysis for *BRCA2* exon 2 for patient CAM 1975. **A.** SSCP and HA analysis results. Presented in lanes 1 to 6 are results for CAM 1971 (lane 1), CAM 1972 (lane 2), CAM 1973 (lane 3), CAM 1974 (lane 4), CAM 1975 (lane 5) and CAM 1976 (lane 6). The SSCP band shift for CAM 1975 is indicated by the arrow. **B.** DNA sequencing results for *BRCA2* exon 2 for CAM 1975 indicating the single base pair change, namely a G>A (*BRCA2* c.-26G>A).

A



B

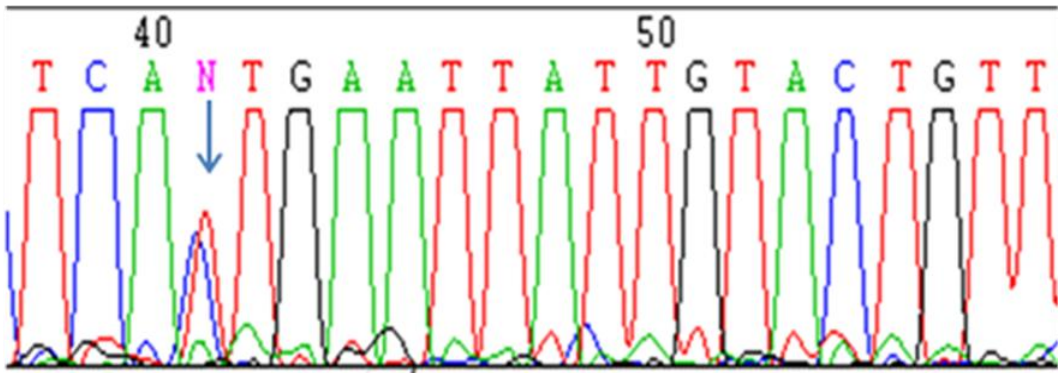


Figure 4.24 Sequence analysis for *BRCA2* exon 2 and *BRCA2* intron 3. **A.** DNA sequencing results for *BRCA2* exon 2 for CAM 1979 indicating the single base pair change, namely a C>T.(*BRCA2* c.-11C>T). **B.** DNA sequencing results for *BRCA2* intron 3 for CAM 1973 indicating the single base pair change, namely C>T (*BRCA2* c.317-22C>T).

The intronic variant IVS6-19C>T (c.517-19C>T) was detected in one patient (Fig. 4.26). Both variants have been previously reported on the BIC within the African and Western Europe, Latin American and Caribbean ethnicities respectively. In *BRCA2* exon 8 variant IVS8+56C>T (c.681+56C>T) was detected for CAM 2028 and CAM 2033 (Fig. 4.27). This variant has been reported on the BIC although ethnicity is not specified. The variant has no functional significance. Novel variant IVS12-175T>G (c.6842-175T>G) was detected in *BRCA2* exon 12 for patient CAM 2033 (Fig. 4.28). This variant has not been reported on the BIC and has no functional significance. Two other novel variants were detected in exons 14A and 15 respectively. The variant IVS14A+224A>G (c.7435+224A>G) in *BRCA2* exon 14A was detected in patient CAM 1980 (Fig. 4.29). The variant IVS15-9C>T (c.7436-9C>T) was detected in *BRCA2* exon 15 for patient CAM 2028.

Two intron 21 variants were detected. The intronic variants IVS21-5C>T (c.8755-5C>T) was detected in patient CAM 2028 and IVS21-66T>C (c.8755-66T>C) in patients CAM 2073, 2075 and 2084 respectively (Fig. 4.30). The former has never been reported on the BIC, while the latter has been reported and was identified in four unrelated patients. The c.8755-66T>C variant is reported on the BIC within the white South African population.

The variant IVS26+54G>A (c.9648+54G>A) in *BRCA2* exon 26 was detected in CAM 1971 and 1973 (Fig. 4.31). This variant has been reported on the BIC for African patients. The last variant detected IVS27B-62C>T (c.9649-63C>T) was in *BRCA2* exon 27B for patient CAM 2035 (Fig. 4.32). This variant has not been reported on the BIC and has no effect on the protein.

4.3.2 MLPA

To detect large deletions or insertions, the *BRCA2* gene was screened using MLPA. Although all of the 35 samples were sent, results were only obtained for 14. All 14 patients tested negative for the presence of major insertions or deletions within *BRCA2*. The patients included CAM 1971, CAM 1973, CAM 1974, CAM 1978, CAM 2027, CAM 2033, CAM 2034, CAM 2074, CAM 2075, CAM 2084, CAM 2122, CAM 2252 CAM 2253 and CAM 2279. One patient (CAM 2251) did not amplify for *BRCA2*.

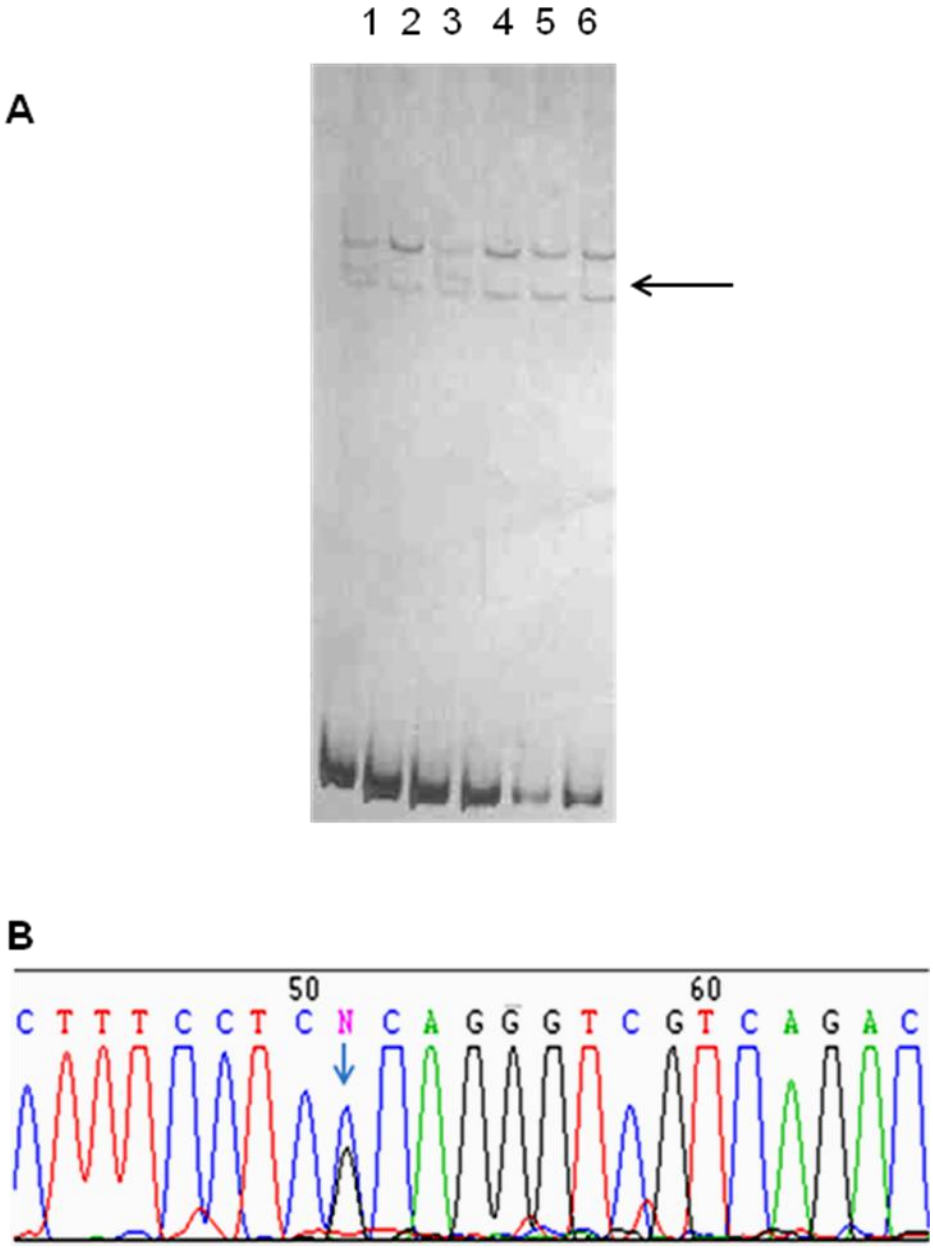


Figure 4.25 Mutation analysis for *BRCA2* intron 6 for patient CAM 1971 and CAM 1973. **A.** SSCP and HA analysis results. Presented in lanes 1 to 6 are results for CAM 1971 (lane 1), CAM 1972 (lane 2), CAM 1973 (lane 3), CAM 1974 (lane 4), CAM 1975 (lane 5) and CAM 1976 (lane 6). The SSCP band shift for CAM 1971 and CAM 1973 are indicated by the arrow. **B.** DNA sequencing results for *BRCA2* intron 6 for CAM 1971 and CAM 1973 indicating the single base pair change, namely C>G.(*BRCA2* c.517-4C>G).

A

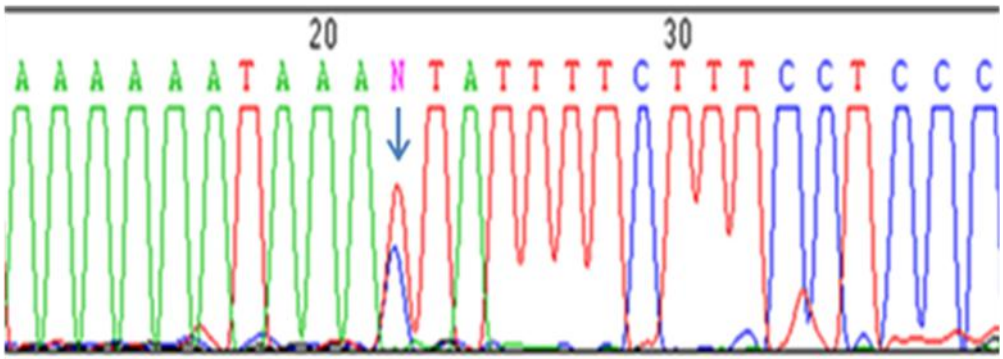


Figure 4.26 Mutation analysis for *BRCA2* intron 6 for patient CAM 1989. DNA sequencing results for *BRCA2* intron 6 for CAM 1989 indicating the single base pair change namely, (C>T) (*BRCA2* c.517-19C>T) indicated with the arrow.

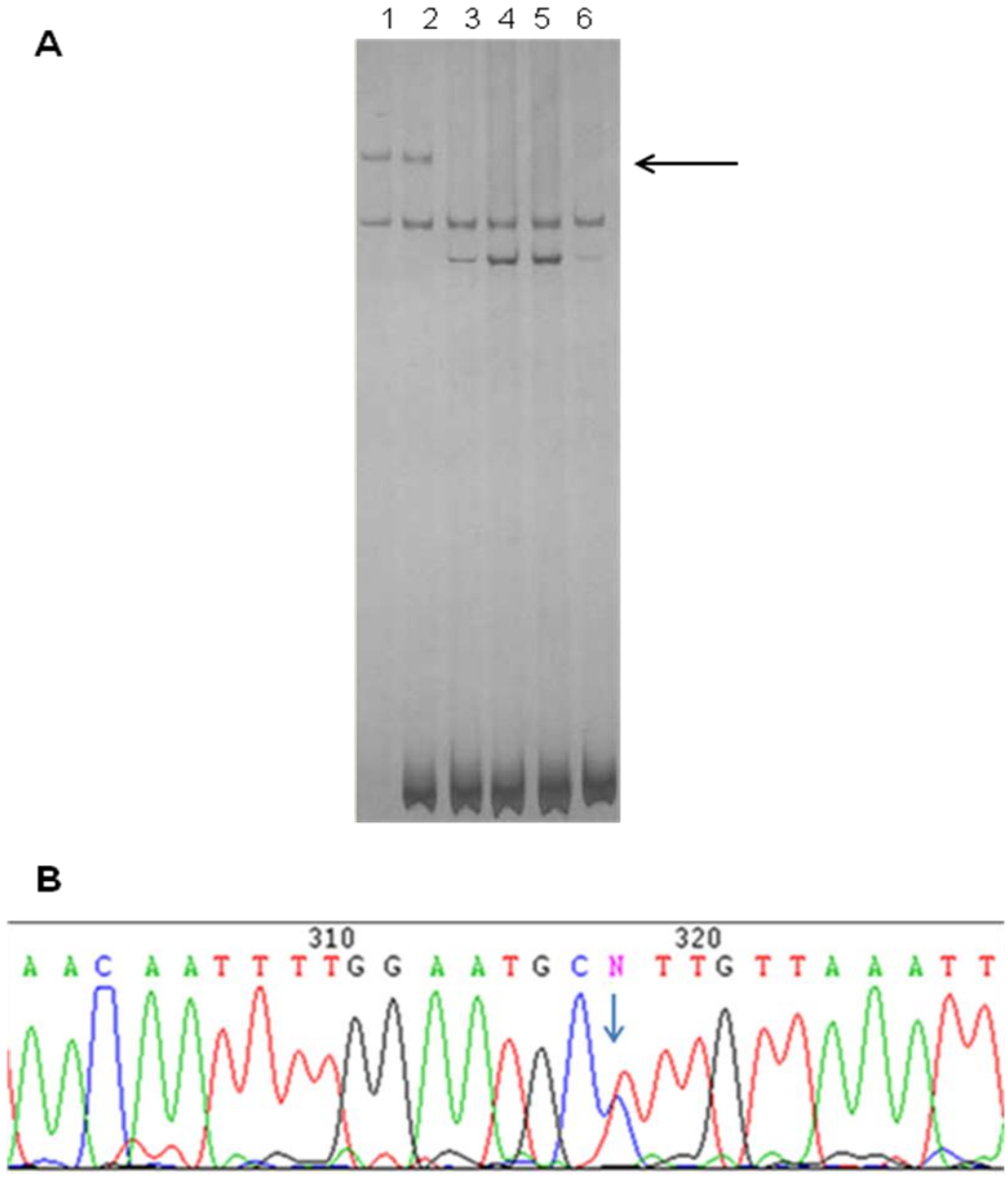


Figure 4.27 Mutation analysis for *BRCA2* exon 8 for patient CAM 2028 and CAM 2033. **A.** SSCP and HA analysis results. Presented in lanes 1 to 6 are results for CAM 2028 (lane 1), CAM 2033 (lane 2), CAM 2034 (lane 3), CAM 2035 (lane 4), CAM 2036 (lane 5) and CAM 2037 (lane 6). The SSCP band shift for CAM 2028 and CAM 2033 is indicated by the arrow. **B.** DNA sequencing results for *BRCA2* exon 8 for CAM 2028 indicating the single base pair change C>T (*BRCA2* c.681+56C>T).

]

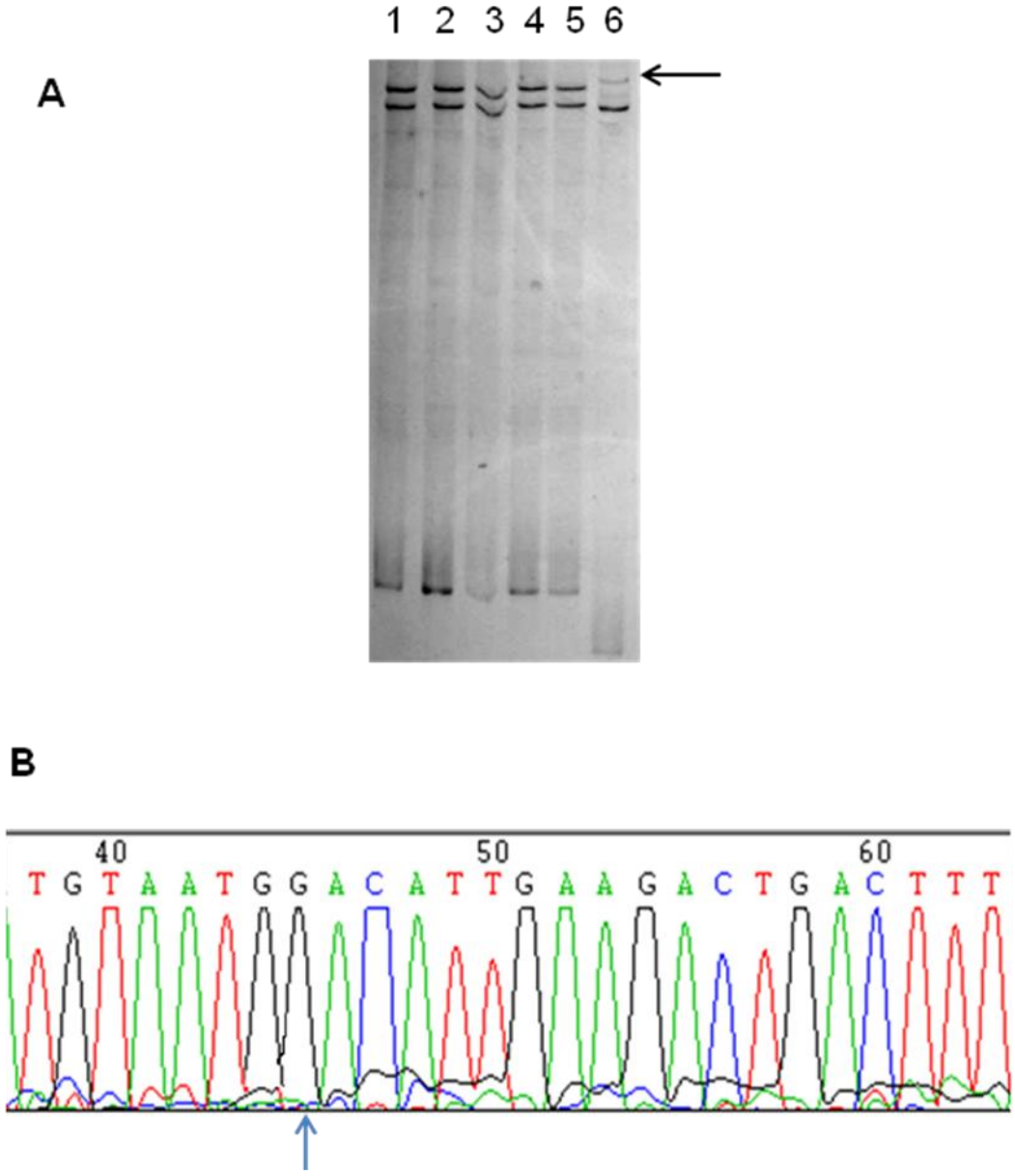


Figure 4.28 Mutation analysis for *BRCA2* exon 12 for patient CAM 2033. **A.** SSCP and HA analysis results. Presented in lanes 1 to 6 are results for CAM 2028 (lane 1), CAM 2037 (lane 2), CAM 2034 (lane 3), CAM 2035 (lane 4), CAM 2036 (lane 5) and CAM 2033 (lane 6). The SSCP band shift for CAM 2033 is indicated by the arrow. **B.** DNA sequencing results for *BRCA2* exon 12 for CAM 2033 indicating a homozygous change for a single base pair, namely a T>G as indicated by the arrow (*BRCA2* c.6842-175T>G).

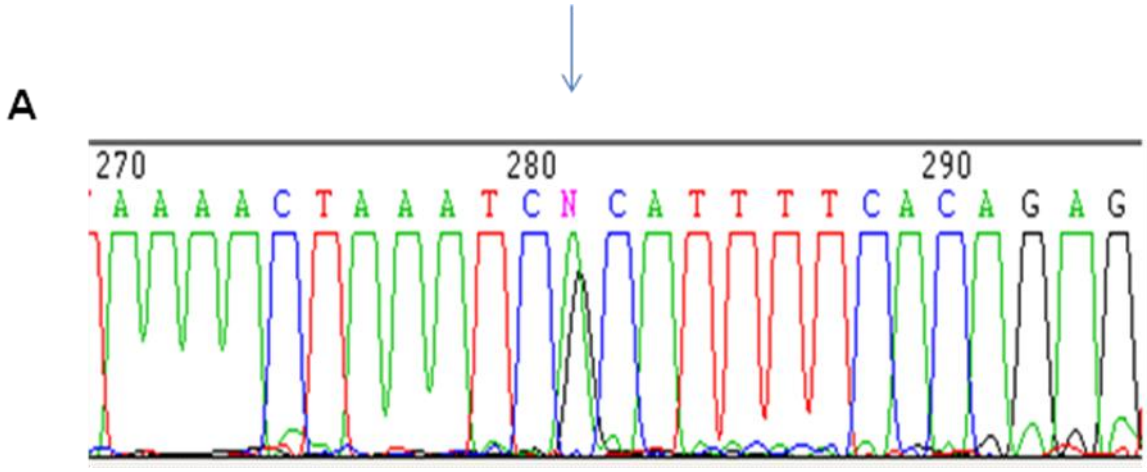


Figure 4.29 Mutation analysis for *BRCA2* exon 14A for patient CAM 1980. DNA sequencing results for *BRCA2* exon 14A for CAM 1980 indicating a single base pair change A>G (*BRCA2* c.7435+224A>G) indicated by arrow.

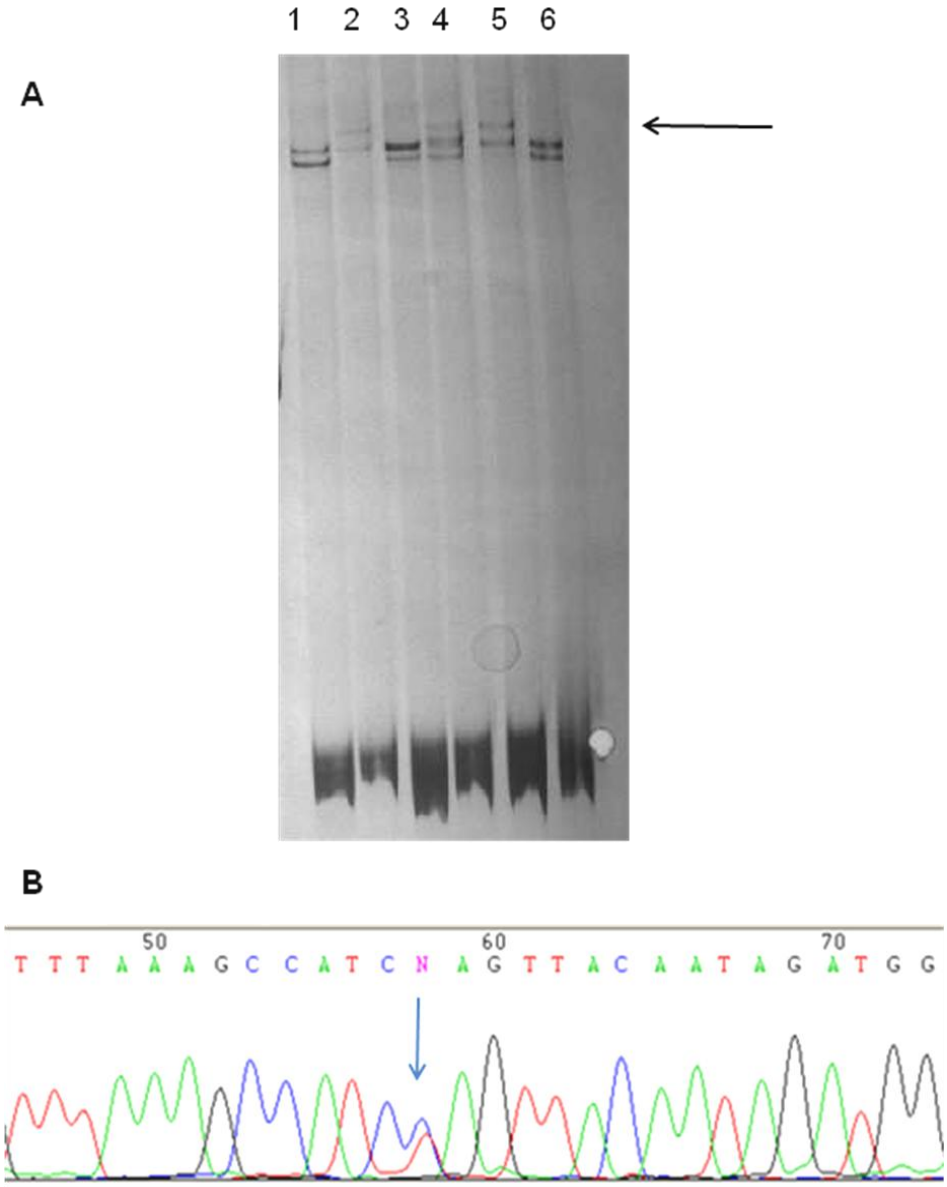


Figure 4.30 Mutation analysis for *BRCA2* intron 21 for patient CAM 2073, 2075 and 2084. **A.** SSCP and HA analysis results. Presented in lanes 1 to 6 are results for CAM 2038 (lane 1), CAM 2073 (lane 2), CAM 2074 (lane 3), CAM 2075 (lane 4), CAM 2084 (lane 5) and CAM 2122 (lane 6). The SSCP band shifts for CAM 2073, 2075 and 2084 are indicated by the arrow. **B.** DNA sequencing results for *BRCA2* intron 21 for CAM 2075 indicating the single base pair change T>C (*BRCA2* c.8755-66T>C).

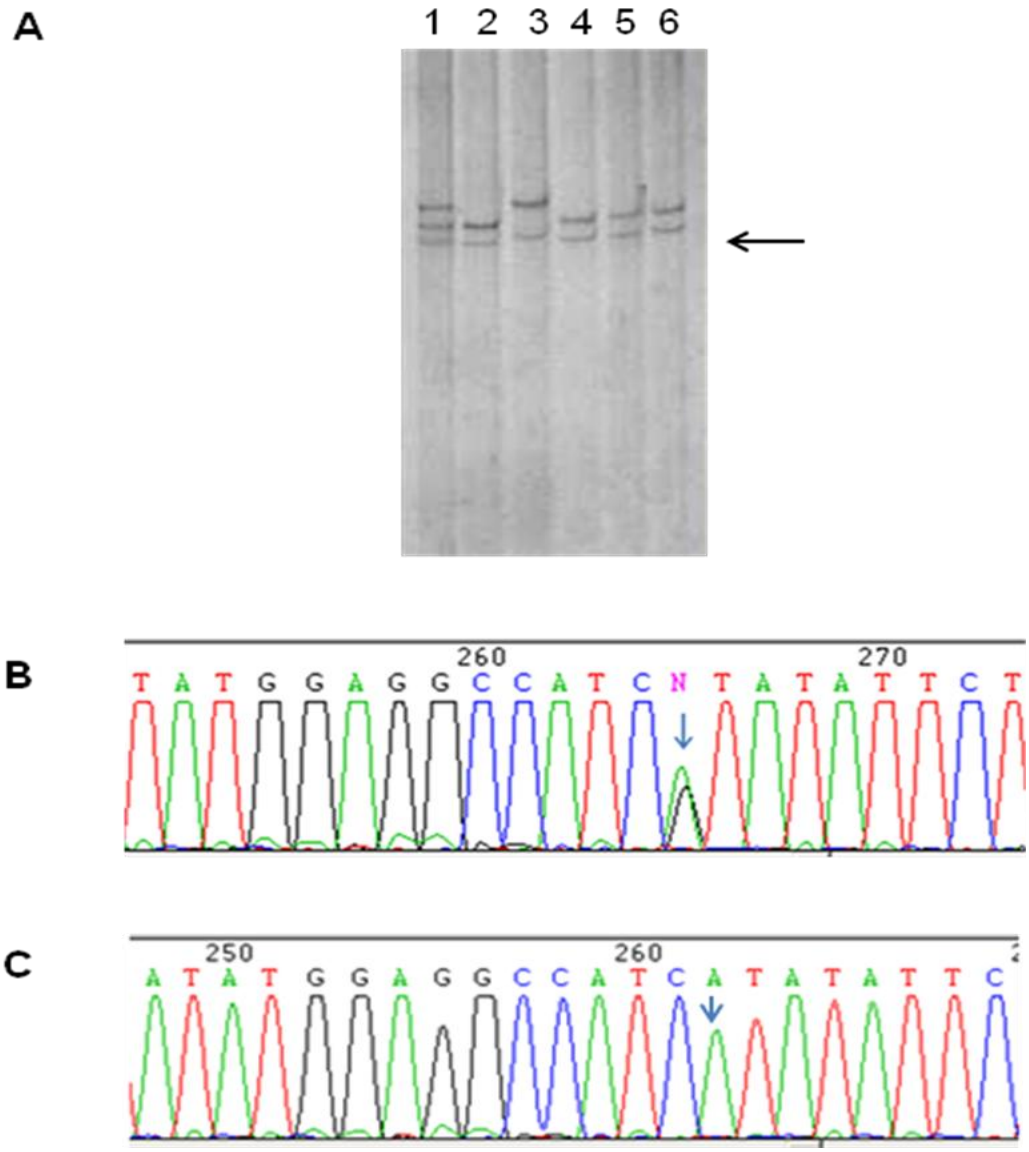


Figure 4.31 Mutation analysis for *BRCA2* exon 26 for patient CAM 1971 and CAM 1973. **A.** SSCP and HA analysis results. Presented in lanes 1 to 6 are results for CAM 1971 (lane 1), CAM 1972 (lane 2), CAM 1973 (lane 3), CAM 1974 (lane 4), CAM 1975 (lane 5) and CAM 1976 (lane 6). The SSCP band shift for CAM 1971 and CAM 1973 are indicated by the arrow. **B.** DNA sequencing results for *BRCA2* exon 26 for CAM 1971 indicating heterozygosity for a single base pair change G>A (*BRCA2* c.9648+54G>A). **C.** DNA sequencing results for *BRCA2* exon 26 for CAM 1973 indicating homozygosity for the single base pair change G>A (*BRCA2* c.9648+54G>A).

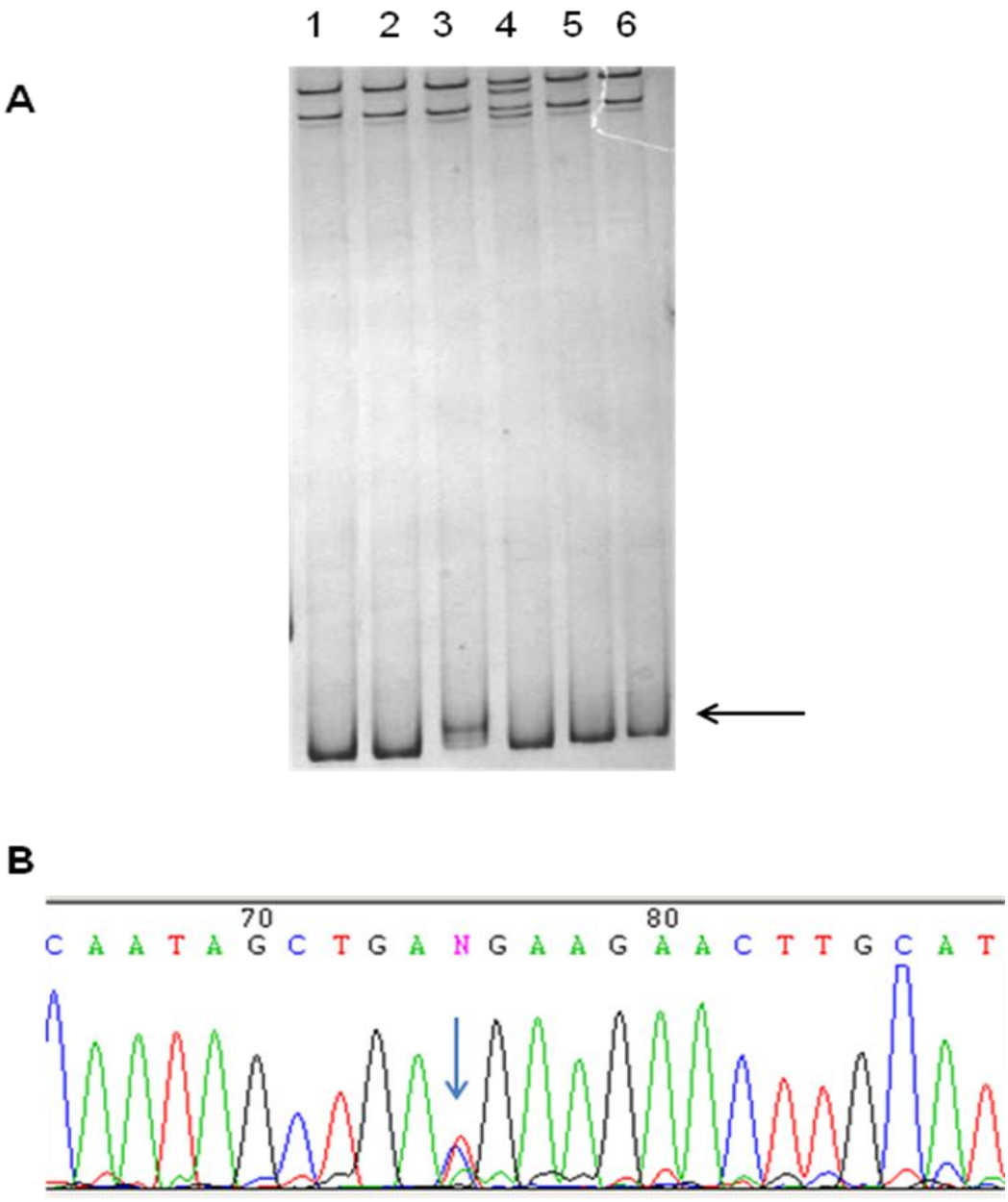


Figure 4.32 Mutation analysis for *BRCA2* exon 27B for patient CAM 2035. **A.** SSCP and HA analysis results. Presented in lanes 1 to 6 are SSCP and HA results for CAM 2033 (lane 1), CAM 2034 (lane 2), CAM 2035 (lane 3), CAM 2036 (lane 4 - heterozygous for a benign single base change in the 3'UTR of exon 27 at position c.10362A>C), CAM 2037 (lane 5) and CAM 2038 (lane 6). The HA band shift for CAM 2035 is indicated by the arrow. **B.** DNA sequencing results for *BRCA2* exon 27B for CAM 2035 indicating the single base pair change, namely C>T (*BRCA2* c.9649-62C>T).

Chapter 5

Discussion

The Black ethnic groups of the vast African continent have not been widely studied where BC is concerned. Internationally, the focus of familial BC was mainly on the industrialized Western world. A similar scenario occurred within South Africa where the Afrikaner population with its European heritage was primarily targeted for BC screening (Reeves *et al*., 2004). Comparisons between the Afrikaner and the rest of the World's Western population groups are relatively uncomplicated as they share similar lifestyles and socio-cultural environments. They also have the highest age-standardized BC incidence rates worldwide (Mosher *et al*., 2003; Parkin *et al*., 2005).

In contrast, much less is known about BC in the developing world, particularly within population groups indigenous to sub-Saharan Africa. Africans differ from their Western counterparts in terms of ethnicity, lifestyle and environmental exposure (Strassmann and Dunbar, 1999; Ramachandran *et al*., 2005). Although BC is also the first or second most common female malignancy in Africa, the age-adjusted incidence rate in indigenous African women is much lower than that in Western women (Parkin *et al*., 2003; Parkin *et al.,* 2005).

This is also the case for the Sotho/Tswana population of SA. Thousands of female patients are diagnosed yearly at the tertiary National and Universitas Hospitals within the Free State, with what seem to be an early age at onset. In an attempt to determine the genetic component of their disease, we embarked on a search for the presence of disease-causing mutations within the familial breast cancer genes *BRCA1* and *BRCA2*. This study is the first to focus on this specific ethnic group residing in the Free State province of SA.

5.1 Selection criteria

The selection criteria for inclusion in this study were Sotho/Tswana women with an early age of BC diagnosis, the presence of a positive BC family history and the presence of triple-negative IHC breast tumours. The ethnicity of the patients was confirmed through self-identification and with Sotho/Tswana being their preferred first language.

The probability of finding a disease-causing *BRCA* mutation within a patient is normally correlated with a combination of the above mentioned selection criteria. Since only 40% of Sotho/Tswana patients reported a positive breast cancer family history and only five patients had triple negative tumours, sporadic cases with an early onset of the disease were also included (Table 3.1). This resulted in a bias towards an early onset age within the selection criteria.

The average age at onset for this study was 45.3 years of age. This corresponds with reports from various other African countries where the median age of onset (48 years) is more than ten years younger than the European/American median of 61 (Laraqui *et al.*, 2013). Laraqui *et al*. (2013) observed an increase in the age specific rates from 35 onwards, which reached a peak in the 40 to 54 year age group. The rates thereafter decreased in the older age groups. Wainstein (2012) compared the age of BC onset between the South African Black and Caucasian populations and concluded that the Black population showed a similar shift towards a younger median age compared to Caucasians. Wainstein (2012) speculated that the use of a young age of BC diagnosis may not be a good criterion to use in a population where the age distribution is so significantly varied. This implied that the majority of patients included in our study might have been sporadic cases as more patients with sporadic BC might have been included in this study than anticipated. Gershoni-Baruch *et al*. (1999) made use of a retrospective cohort of Jewish women and showed that 82% of bilateral BC patients with early onset of disease carried a *BRCA* mutation. In contrast, only 7.9% of the patients not associated with an early age of onset were found to be mutation carriers. They concluded that when bilateral BC and early age of onset are taken together as selection criteria, the association with the presence of *BRCA1/BRCA2* mutations becomes very strong. This perception was strengthened by their observation that young women with unilateral BC matched

for family history were less likely to harbor a mutation. As only one patient with bilateral BC was included in the current study, it can be concluded that the cohort included more sporadic patients than females with heritable BC.

Various factors could account for a patient's perception of a negative family BC history. These include a small family size, the predominance of males, incomplete penetrance (Gayther *et al.*, 1997), non-disclosure of cancer information to family members and the influence of traditional healing which could result in patients possibly passing away undiagnosed. The majority of the patients in our study (60%) could not recall or did not have any family history suggestive of BC and/or other cancer types associated with mutations within the familial *BRCA* genes.

The discussions with the familial cases showed that African women with BC often hid their diagnosis from their close relatives and also failed to report potential or unverifiable cancers. This tendency was also observed by Wainstein (2012) during a genetic counseling based study of Black women. The stigma surrounding BC is prevalent in many African countries and communities. Many women are afraid to seek medical assistance and very few disclose their diagnosis to immediate family members. A patient from Ghana has for example kept her double mastectomy secret for 20 years due to fear of stigmatization. Only her immediate family members knew about it (Carlucci and Abayie, 2011). Cases like these indicate that it is common within the Black African women to keep their diagnosis and surgery a secret out of fear from being shunned by their husbands or families due to so-called disfigurement.

Population groups in African countries such as SA, Ghana (Carlucci and Abayie, 2011), Uganda and Sudan (Gardner-Purdue, 2013) are religious. According to the 2001 national census, Christians accounted for 79.7% of SA's population, of which the African Indigenous Churches made up the largest group (http://www.statssa.gov.za/publications/SAStatistics/SAStatistics2012.pdf). Many pastors tell their flocks that cancer is a spiritual illness, and that the answer is prayer, not surgery. As a result, the majority of African women do not seek medical attention until the tumours have spread and it is too late for life-saving treatment. This contributes to patients presenting with late-stage BC that has spread to other organs which makes treatment ineffective. This late stage presentation has being documented in various African countries including Nigeria

(Gukas *et al.*, 2005; Adesunkanmi *et al.*, 2006), Ghana (Clegg-Lamptey and Hodasi, 2007) and Uganda (Gakwaya *et al*., 2008). This contributes to the poor prognostic factors for BC within sub-Saharan African women. It could also explain the disproportionate mortality rate in African women when compared to BC incidence and mortality in Caucasian women. This could also be the case for the Sotho/Tswana population with the majority of patients already being at Stages III or IV when they were referred to Universitas or the National Hospitals in Bloemfontein for treatment.

Complicating matters is the strong belief in traditional healing. This is especially applicable to older and more rural generations among the Sotho/Tswana population. The WHO estimated in 2003 that up to 80% of the population in Africa makes use of traditional medicine. In SA, there were an estimated 190 000 traditional health practitioners in 2007 that treated an array of health-related problems and culture-bound syndromes or ailments (Gqaleni *et al.*, 2007; Peltzer, 2009). According to Nxumalo *et al.* (2011), the users of traditional medicine are likely to be poor, unemployed, living in rural areas and aged between 25 and 49 years with no medical aid. Traditional healers have the potential to serve as crucial components of a comprehensive health care strategy. However, this strong believe in traditional healers by older family members, might have caused many Black Africans to die unaware of their cancer diagnosis.

Historically, BC received scant attention in sub-Saharan countries where the main focus was on communicable diseases like malaria and HIV/AIDS. It is only now that non-communicable diseases such as BC are becoming the leading cause of death in many of these African countries, that the situation regarding BC awareness and possible prevention is changing (Carlucci and Abayie, 2011).

5.2 Pathogenic *BRCA* mutations

Mutations in *BRCA1* and *BRCA2* are responsible for the majority of familial BC cases (Thompson and Easton, 2004; Levy-Lahad and Friedman, 2007). According to literature, 10% of ovarian and 3–5% of BC cases are associated with mutations in either one or both of these genes (Clark *et al.,* 2012). Although the two familial BC genes do not show substantial sequence similarity, their encoded proteins both associate with Rad51. Furthermore, each gene has an unusual

gene structure (large exon 11). These genes contribute to DNA repair and transcriptional regulation in response to DNA damage and cell cycle control (Yoshida and Miki, 2004). An inherited mutation in either of these genes combined with loss of heterozygosity predisposes cells to chromosomal instability. This greatly increases the probability of malignant transformation and cancer development.

Our study involving the Sotho/Tswana women revealed a single disease-causing mutation in exon 11 of both *BRCA1* and *BRCA2*, namely *BRCA1* c.2069_2072delAAAG,p.Lys653SerfsX699 for patient CAM 1973 and *BRCA2* c.6455_6455delT,p.Lys2075ArgfsX2078 for patient CAM 2279. Only the latter patient reported a family history of BC. The other patient represented a sporadic case as she could not recall any family member affected with BC or any other related cancer type. Both these mutations are novel.

5.2.1 *BRCA1* c.2069_2072delAAAG

BRCA1, a multi-domain protein, is most frequently mutated in one of three domains or regions, namely the N-terminal RING domain (exons 2 – 7), the BRCT domain (exons 16 – 24) and exons 11 – 13 (Clark *et al.*, 2012). The RING domain is responsible for the E3 ubiquitin ligase activity of *BRCA1* and mediates interactions between *BRCA1* and other proteins (Meza *et al.*, 1999; Brzovic *et al*., 2001). The BRCT domain on the other hand binds to phosphoproteins with specific sequences recognized by both *BRCA1* and ATM/ATR kinases (Williams *et al.*, 2001; Williams *et al.,* 2004). Structural studies of these domains revealed the molecular basis by which cancer causing mutations impact the function of *BRCA1*.

The effect of mutations in the coding regions of exons 11 to 13 also plays a major role in the tumour suppressor function of the associated protein. Exons 11 – 13 cover more than 65% of the entire coding sequence of the gene and encode two nuclear localization sequences (NLS) and binding sites for several proteins including the Retinoblastoma protein (RB), cMyc, Rad50 and Rad51 (Deng and Brodie, 2000; Clark *et al.*, 2012).

The region also contains portions of a coiled-coil domain which mediates interactions with PALB2 (amino acids 1364 – 1437), as well as a portion of a serine containing domain (SCD, amino acids 1280 – 1524) that is phosphorylated

by ATM (Clark *et al.*, 2012). All these binding partners are involved in a wide range of cellular pathways.

The *BRCA1* c.2069_2072delAAAG,p.Lys653SerfsX699 mutation detected for patient CAM 1973 falls within exon 11. The binding of the Retinoblastoma protein, a well-known tumor suppressor that controls growth by regulating progression through the cell cycle, will not be affected by the mutation as binding to *BRCA1* occurs specifically at amino acids 304 – 394 (Classon and Harlow, 2002).

This mutation is situated within the binding region of one of the two proteins involved in DNA repair, namely Rad50 (Clark *et al.*, 2012). Rad50 functions in a complex together with MreII (Meiotic recombination 11 - MIM 600814) and Nbs1 (Nijmegen Breakage Syndrome 1 - MIM 602667) to participate in both non-homologous end joining (NHEJ) as well as homologous recombination (HR). The detected mutation could prevent Rad50 binding, leading to impaired NHEJ or HR that could result in the proliferation of abnormal cells.

5.2.2 *BRCA2* c.6455_6455delT

In contrast to the multi-domain *BRCA1* protein, *BRCA2* is evolving at a faster rate than most other cancer susceptibility or tumour suppressor genes (Bennett *et al.*, 1995). This gene has no obvious homology to any known gene and the protein contains no well-defined functional domains (Bignell *et al.*, 1997; Ibrahim *et al.*, 2010). Exon 11 of the *BRCA2* gene codes for the large central region of the protein that houses eight relatively conserved 35 amino acid BRC repeats, six of which are known to bind Rad51 (Davis and Pellegrini, 2007). The spacing between the individual motifs varies from 60 to 300 amino acids. Another Rad51 binding motif is present in the extreme C-terminus of *BRCA2* encoded by exon 27. These binding motifs are responsible via their interaction with Rad51 for DNA repair by homologous recombination (Davis and Pellegrini, 2007).

Very little is known about the individual function of each of these different repeats, but together through interaction, they recruit Rad51 to sites of DNA damage (Wong *et al.*, 1997; Chen *et al.*, 1998; Chen *et al.*, 1999; Carreira and Kowalczykowski, 2011). Two groups of BRC repeats with unique functional characteristics exist (Rajendra and Venkitaraman, 2010). Group 1 includes BRC1

to 4, which are responsible for the binding of free Rad51 with high affinity. These repeats are responsible for the reduction of ATPase activity of Rad51 and enhance DNA strand exchange by Rad51. Although Group 2 (BRC5 to BRC8) binds to free Rad51 with low affinity, it binds with high affinity to the Rad51-ssDNA filament (Carreira and Kowalczykowski, 2011). Although these two groups work through different mechanisms, all eight are responsible (together with the C-terminal of *BRCA2*) to ensure the efficient formation of a Rad51 filament on ssDNA by promoting its nucleation and growth (Carreira and Kowalczykowski, 2011). The *BRCA2* c.6455_6455delT,p.Lys2075ArgfsX2078 mutation detected within CAM 2279 is located within BRC repeat 8 (amino acids 2036 – 2110) located within exon 11 (Bork *et al.*, 1996). It is therefore hypothesized that the presence of a deleterious mutation within this specific *BRCA2* domain could affect the formation of the ssDNA filament by Rad51. The mutation will create a premature truncation of the protein, which will in the end influence the DNA repair capacity of the Rad51 complex.

5.2.3 *BRCA* missense mutations

Four missense mutations were detected for the 35 BC patients (Tables 4.1 and 4.2). The two mutations present within *BRCA1* [*BRCA1* c.114G>A,p.Lys38= (rs1800062); *BRCA1* c.4308T>C,p.Ser1436= (rs1060915)] were synonymous and described as polymorphisms. Both these mutations were validated by various methods that include multiple, independent submissions to the refSNP cluster, by frequency or genotype data that include minor alleles observed in at least two chromosomes, genotyping by the HapMap project and sequencing in the 1000 Genome project. Rs1060915 was much more common than rs1800062 that was detected only 17 times. This also reflects in their global minor allele frequency (MAF), which for rs1060915 is currently 0.304/662 compared to 0.017/37 for rs1800062 (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref).

The two missense mutations detected in *BRCA2* [*BRCA2* c.7017G>C,p.Lys2339Asn (rs45574331); *BRCA2* c.8830A>T,p.Ile2944Phe (rs4987047)] occurred at a much lower MAF, namely 0.006/13 and 0.008/17 respectively (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref). Both these mutations have been validated as benign polymorphisms by a number of methods

including the HapMap and 1000 Genome screen. The lower MAF might be due to the fact that the 1000 genomes screened for mutations mostly represented Caucasian/European populations. Although both these mutations were observed in only two patients (CAM 1973 and CAM 2074 respectively), they have regularly been reported for the native African American populations (reported in ≥66 and ≥115 individuals respectively, Table 4.2).

5.2.4 *BRCA* intronic variants

The intronic variants detected within the 35 BC patients ranged from a 5 bp deletion (*BRCA1* c.-19-89delTTTAC) in intron 1 to single base pair changes (for example *BRCA2* c.517-19C>T) (Tables 4.1 and 4.2). The majority of intronic variants represented single base pair changes, as no insertions or deletions were observed within the intronic areas of *BRCA2* (Table 4.2).

Fifty percent of the intronic variants detected within *BRCA1* were novel, compared to 38.46% for *BRCA2*. This observation was expected as Africa has been indicated to be the most genetically diverse region of the world, with more than 2000 distinct ethnic groups and languages (Tishkoff and Williams, 2002).

Only two *BRCA1* intronic variants were recurrent, namely *BRCA1* c.548-64_548-64delT and *BRCA1* c.5152+73G>A (Table 4.1). Although each of these variants have been reported only once before, it was each detected within three unrelated Sotho/Tswana patients. The screening of *BRCA2* revealed more recurrent variants (38.46%) which included *BRCA2* c.517-19C>T (detected for three patients), *BRCA2* c.517-4C>G (g.32900632C>G) (three patients), *BRCA2* c.681+56C>T (two patients) and *BRCA2* c.8755-66T>C (three patients) (Tables 4.1 and 4.2).

The single nucleotide change detected within intron 6 (rs81002804) was the most interesting (Table 4.2). As this variant is located 4 bp from the start of exon 7, it was hypothesized that this variant might be pathogenic. It was detected in three patients in a heterozygous state and has also been reported for five other African BC patients (South African and Kenyan) referred to the NHLS Molecular Genetics Laboratory for diagnostic screening. For SA, this variant is restricted to the Black population, as it was not detected in any of the other population groups studied thus far within this laboratory.

This variant is definitely rare and was first detected in an African patient in 1998 by an American laboratory (BIC). They detected this variant during an attempt to produce a comprehensive summary of *BRCA2* sequence variants in 95 representatives of 40 diverse global populations which served as normal controls for 71 Austrian BC families. In their study (Wagner 1999), recorded a total of 71 different simple sequence variants within the controls, of which *BRCA2* c.517-4C>G was one.

The analysis of control individuals from a wide variety of populations for the purpose of identifying polymorphisms had particular significance at the time. The control populations consisted of unaffected individuals of the same ethnic background as the Austrian families. They argued that a sequence variation with an extremely rare occurrence among the African population, though more frequent in for example the Caucasian population, will not turn up in an African control population. They (Wagner 1999) stated that 63 (80%) of these variants were specific for a certain continent and 51 (65%) were detected only in specific populations. They concluded that all the variants detected with a frequency of >1% in both the global and specific populations may be classified reliably as a polymorphism. As *BRCA2* c.517-4C>G was detected within a control individual of African origin, they postulated that this variant is most likely benign.

It is postulated that this variant could have clinical significance as it was detected only in one individual in a heterozygous state (Wagner *et al.*, 1999). According to the NCBI data, this variant has a very low MAF of 0.000/1, which makes it extremely rare. It has been detected once within the Bushman genome during the 1000 genome screen (www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs81002804).

It has been proven that both exonic and intronic sequence changes can alter pre-mRNA splicing by directly disturbing the natural splice sites (Di Giacomo *et al.*, 2013). The consensus sequences of the 5' and 3' splice sites which define the intron-exon boundaries, comprise not only of intronic nucleotides (including the highly conserved AG and GT dinucleotides), but also of the last three and first nucleotides of the next exon, respectively (Cartegni *et al.*, 2002; Chasin, 2007). As this intronic variant is located only four bp from the start of exon 7, it could play a role in creating an alternative splice site.

This variant has also been detected in another SA study where the molecular aetiology of inherited BC in the Black population of Gauteng SA was studied (Chen *et al.*, 2013). Although the specific tribal group was not stipulated, it was detected within three Black BC patients. The authors also reported this variant to be benign. Although many variant-induced splicing alterations can be predicted with bioinformatics tools that evaluate the strength of splice sites, it is recommended that experimental evidence in a molecular diagnostics setting must be obtained to determine the possibility of this variant disturbing the natural splice site of exon 7 (Sanz *et al*., 2010; Houdayer *et al*., 2012; Colombo *et al*., 2013). These additional studies will help to complete and clarify the possible involvement of this variant at the onset of the disease in these patients.

5.2.5 Founder or recurrent mutations in the Black population of SA

The current study did not reveal the presence of founder mutations for the Sotho/Tswana population. The two disease-causing mutations were both limited to a single BC patient. These mutations did not correspond with the pathogenic mutations detected by Chen *et al.* (2013) for the Gauteng region. They recorded three disease-causing mutations in three of the 33 Black BC patients tested, namely *BRCA1* c.431dupA,p.Asn144LysfsX15 (novel mutation), *BRCA2* c.582G>A,p.Trp194X (rs80358810) and *BRCA2* c.7712A>G,p.Glu2571Gly (rs55689095). Only two founder mutations have thus far been documented for the continent of Africa, namely *BRCA1* c.422T>G,p.Y101X in exon 7 (Fackenthal *et al.*, 2005) and *BRCA2* c.5771_5774del,p.Ile1924ArgfsX38 in exon 11 (van der Merwe *et al.*, 2012). The *BRCA1* c.422T>G,p.Y101X mutation was identified within the indigenous Yoruban tribe in Nigeria. Although this population is large and ancient, it exhibits extensive genetic diversity which is in contrast with typical founder populations (Zhang *et al.*, 2008). Founder mutations are typically an indicator of low genetic diversity such as the Afrikaner and are often associated with bottlenecks in population size (Zhang *et al.*, 2008).

The *BRCA2* c.5771_5774del,p.Ile1924ArgfsX38 mutation was described for the Xhosa and Mixed Ancestry populations from the Western Cape Province of SA (van der Merwe *et al.*, 2012). This mutation has not been detected for any other populations or regions within SA.

Both these mutations have previously been described for a few BC patients of European ancestry. The *BRCA1* c.422T>G,p.Y101X mutation was found within a patient of European ancestry, which included Irish, English, German and Yugoslavian backgrounds. As this Nigerian patient shared a common haplotype with the Yoruban BC patients, they concluded that the mutation arose due to a single mutational event many years before (Zhang *et al.*, 2008). Although the haplotype analysis of the patients carrying the *BRCA2* c.5771_5774del,p.Ile1924ArgfsX38 mutation for each country shared a common haplotype, the haplotype for the patients representing each of the countries differed. This indicated that this mutation arose independently on two different occasions and that the mutation event was not shared by the two populations.

Recurrent mutations might be absent from African populations due to the ethnic mosaic nature of these populations, which is reflected in the diverse mutational spectrum (Zhang *et al.*, 2008; Zhang *et al.*, 2012). Studies using mitochondrial and nuclear DNA markers consistently indicate that Africa is the most genetically diverse region of the world (Tishkoff and Williams, 2002; Olopade *et al.*, 2003). The ancient African migration events are complex, owing to historical fluctuations in geology, ecology and climate, including periods of glaciations and warming that could have affected population expansion and contraction (Lahr and Foley, 1998). Another contributing factor can be the polygamous nature of many African tribes including the Xhosa and Zulu tribes of SA (Hayase and Liaw, 1997).

5.2.6 Large genomic insertions or deletions in the Black population of SA

The majority of pathogenic mutations detected thus far for *BRCA1/2* consists of single base changes or small deletions/insertions that result in premature protein truncation, disruption of messenger RNA processing or amino acid substitutions in critical domains that have significant impact on protein function.

A small percentage of mutations are represented by large genomic rearrangements of DNA segments that totally disrupt the function of the protein. These mutations involve mostly deletions and duplications of one or more of the coding regions. Most of these mutations are caused by recombination between the Alu repeats present within these genes, specifically *BRCA1* (Smith *et al.*,

1996). Within *BRCA1*, a duplicated region at the 5' end of the gene that contains the *BRCA1* pseudogene represents an additional source of illegitimate recombination (Puget *et al.*, 2002).

The proportion of genomic rearrangements compared to the overall deleterious mutations reported for breast/ovarian cancer families seems to be population-dependent, varying from 8% in a series of American families (Puget *et al.*, 1999) to 36% in Dutch patients (Petrij-Bosch *et al.*, 1997). In one mega study by Myriad, the difference in prevalence of these mutations for the various ancestries was confirmed (Judkins *et al.*, 2012). The percentage for patients of Latin American/Caribbean descent was as high as 21.4%, while the percentage was 16.7% for patients of Near East/Middle East descent. For the former group, the single deletion mutation of exons 9 – 12 within *BRCA1* accounted for 37% of all large genomic rearrangements reported (Judkins *et al.*, 2012).

The data from Myriad (Judkins *et al.*, 2012) indicated that a number of large genomic rearrangements involving the same exonic regions in *BRCA1* and *BRCA2* are strongly associated with a single ancestry, possibly due to founder effects. This was typical for the African population as only 3 different mutations (*BRCA1* del exons 1 – 19 detected for 6 patients; *BRCA1* del exon 8 detected for 5 patients and *BRCA1* dup exons 18 – 19 detected for 15 patients) were responsible for 60.5% of all large genomic rearrangements detected for the entire African continent. Of this percentage, the *BRCA1* duplication of exon 18 – 19 alone constituted 33% of the total percentage detected (Judkins *et al.*, 2012).

The percentage of large genomic rearrangements within the Black population of SA seems to be very low as none were detected during this study. These results are corroborated by Chen *et al.* (2013) who also did not find any large genomic rearrangements within 33 Black South African patients.

5.3 Value of contribution

This study is the first to search for the presence of disease-causing *BRCA* mutations within the Sotho/Tswana population residing in the catchment area of the tertiary Universitas and the National state hospitals. Although this pilot study only included 35 patients affected with breast cancer, it contributed to the knowledge of the mutational spectrum of the *BRCA* genes within this population

group. The cohort of this study should be enlarged in future in order to make the results representative of this specific Black ethnic group. Once the mutational spectrum is known, the appropriate and population specific mutations can be incorporated onto the diagnostic platform specific for the Sotho/Tswana BC patients.

Chapter 6

References

6.1 General references

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Chapter 7

Summary

Screening for mutations within the *BRCA1* and *BRCA2* genes is a daunting task due to the length of the genes and the absence of mutational hotspots. An additional contributing factor is the genetic diversity within the Black ethnic groups of Africa, including the Sotho/Tswanas of the Free State. As no information was available regarding the prevalence of *BRCA* mutations within this group, this pilot study was launched in an attempt to determine the genetic component attributable to *BRCA* mutations in BC development.

The selection criteria were not optimal and possible sporadic or single cases were included which according to literature, is not associated with mutations within the two familial BC genes. This resulted in a low percentage of disease-causing mutations being detected. It is proposed that the selection criteria in the future should emphasize the selection of bilateral BC cases with or without a positive family history. This characteristic seems to be more closely associated with familial BC in the Black patients than an early age at onset. The latter could be masking the familial BC cases, as the median age of onset of the disease in Black ethnic groups is 48.

Two disease-causing mutations were identified, one within each of the genes. Both mutations were detected with PTT as they are located within exon 11. This indicates that this technique, although based on older technology, is still a valuable screening technique as it is cost-effective and less time consuming than screening the larger exons with for example high resolution melting (HRM).

The two mutations are both situated within critical regions of the genes. *BRCA1* c.2069_2072delAAAG,p.Lys653SerfsX699 is located within the binding domain of Rad50 whereas *BRCA2* c.6455_6455delT,p.Lys2075ArgfsX2078 is located in BRC repeat 8. The presence of both these mutations will result in a

truncated protein that would probably not be able to participate in DNA repair in response to DNA damage and cell cycle control (Green and Lin, 2012). This could result in chromosome instability and therefore tumour formation.

Both mutations are novel and have not been detected internationally nor in the Black population residing in Gauteng SA. As all mutations detected thus far for the Black SA population seem to be limited to a single family and with no founder mutations found, full screening of both these genes remains the golden standard.

Functional studies should be performed for the intronic variant *BRCA2* c.517-4C>G (g.32900632C>G, rs81002804) detected in various patients. As this intronic variant is located only four bp from the start of exon 7, it could play a role in creating an alternative splice site. These studies together with the analysis of control individuals from the various Black SA ethnic groups will resolve the question whether this variant has the potential to be disease-causing.

Keywords: familial breast cancer, mutation screening, *BRCA1*, *BRCA2*, Sotho/Tswana population, SSCP, HA, PTT, DNA sequencing.

Chapter 8

Opsomming

Sifting vir mutasies in die oorerflike borskanker gene *BRCA1* en *BRCA2* is ‘n senutergende taak. Dit word bemoeilik deur die grootte van elkeen van hierdie gene sowel as die afwesigheid van spesifieke areas waarin foute algemeen voorkom. Die genetiese variasie teenwoordig in die swart bevolkingsgroepe, insluitend die Sotho/Tswana etniese groep van die Vrystaat, bemoeilik hierdie taak verder. Hierdie studie is ge-inisieer weens die gebrek aan inligting aangaande die teenwoordigheid van *BRCA* mutasies vir hierdie bevolkingsgroep. Die studie het dus gepoog om te bepaal tot watter mate *BRCA* mutasies bydra tot die genetiese komponent van oorerflike borskanker vir hierdie groep.

Die seleksie kriteria tydens hierdie studie was nie optimaal nie. Die vermoede bestaan dat verskeie sporadiese borskanker gevalle ingesluit was, wat daartoe gelei het dat die persentasie positiewe resultate baie laag was. Volgens literatuur, is daar geen verband tussen sporadiese borskanker gevalle en mutasies in die oorerflike borskanker gene nie. Aangesien bilaterale borskanker in swart pasiënte wel ‘n assosiasie toon met die oorerflike tipe, word daar voorgestel dat toekomstige studies hierdie eienskap sal gebruik om sodoende meer potensiëel oorerflike gevalle in te sluit. Indien slegs ‘n vroëe ouderdom van diagnose gebruik word as insluitings kriterium, kan dit die persentasie oorerflike gevalle verskuil aangesien die gemiddelde ouderdom van borskanker diagnose 48 is vir die swart bevolkingsgroepe.

Twee siekte-veroorsakende mutasies is gevind, een in *BRCA1* en een in *BRCA2*. Hierdie mutasies is albei in ekson 11 van die onderskeie gene geleë en is geïdentifiseer deur van die verkorte proteïen toets (PTT) gebruik te maak. Die resultate het die waarde van PTT opnuut geïllustreer ten spyte daarvan dat dit eintlik ou tegnologie is. Die betrokke metode is baie meer koste effektief en tyd

besparend om groot koderende areas te deursoek, wanneer dit byvoorbeeld met HRM vergelyk word.

Die twee siekte-veroorsakende mutasies is in kritiese areas van beide hierdie gene geleë. *BRCA1* c.2069_2072delAAAG,p.Lys653SerfsX699 lê binne die bindingsgebied van Rad50 met BRCA1, terwyl *BRCA2* c.6455_6455delT,p.Lys2075ArgfsX2078 geleë is in BRC herhaling 8. Die teenwoordigheid van beide hierdie mutasies lei heel waarskynlik tot die vorming van verkorte polipeptiede wat nie die selsiklus kan beheer en gevolglike DNA skade kan herstel nie (Green and Lin, 2012). Dit kan weer lei tot onstabiele chromosome wat op hulle beurt aanleiding tot die vorming van gewasse kan gee.

Nie een van die twee mutasies is al voorheen vir beide internasionale of die plaaslike swart bevolking beskryf nie. Die mutasies bekend is dus huidiglik beperk tot enkele families. Aangesien geen stigters- of herhalende mutasies tot dusver gevind is nie, bemoeilik dit diagnostiese toetsing. Die DNA van swart pasiënte moet dus tans volledig deurgesoek word met behulp van DNA volgordebepaling as die goue standard.

Funksionele studies is egter nodig om te bepaal of die *BRCA2* c.517-4C>G (g.32900632C>G, rs81002804) variant teenwoordig in intron 6 siekte-veroorsakend kan wees. Aangesien hierdie variant vier basisse vanaf die begin van ekson 7 lê, kan die verandering moontlik ‘n nuwe herkenningsgebied vir die uitsny van ekson 7 te weeg bring. Hierdie addisionele studies, sowel as die toets van normale kontrole individue vir die teenwoordigheid van die variant, sal meer lig op die moontlikheid werp.

Sleutelwoorde: oorerflike borskanker, mutasiesifting, *BRCA1*, *BRCA2*, Sotho/Tswana bevolking, SSCP, HA, PTT, DNA volgorde bepaling.

Appendix A

UNIVERSITEIT VAN DIE VRYSTAAT
UNIVERSITY OF THE FREE STATE
YUNIVESITHI YA FREISTATA

Direkteur: Fakulteitsadministrasie / Director: Faculty Administration
Fakulteit Gesondheidswetenskappe / Faculty of Health Sciences
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Ms H Strauss 2008-05-23

DR NC VAN DER MERWE
DIVISION OF HUMAN GENETICS
FACULTY OF HEALTH SCIENCES
UFS

Dear Dr van der Merwe

ETOVS NR 65/08
PROJECT TITLE: SCREENING OF YOUNG SOUTH AFRICAN BREAST CANCER PATIENTS FOR THE PRESENCE OF DELETERIOUS BRCA1 AND BRCA2 MUTATIONS.

- You are hereby informed that the Ethics Committee approved the revised version of a more detailed Informed Consent in English and Afrikaans at the meeting on 20 May 2008.
- Committee guidance documents: Declaration of Helsink, ICH, GCP and MRC Guidelines on Bio Medical Research. Clinical Trial Guidelines 2000 Department of Health RSA; Ethics in Health Research: Principles Structure and Processes Department of Health RSA 2004; the Constitution of the Ethics Committee of the Faculty of Health Sciences and the Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines.
- Any amendment, extension or other modifications to the protocol must be submitted to the Ethics Committee for approval.
- The Committee must be informed of any serious adverse event and/or termination of the study.
- A progress report should be submitted within one year of approval of long-term studies and a final report at completion of both short term and long term studies.
- Kindly refer to the ETOVS reference number in correspondence to the Ethics Committee secretariat.

Yours faithfully

for **PROF WH KRUGER**
CHAIR: ETHICS COMMITTEE

339, Bloemfontein 9300 RSA ☎ (051) 405 2812 gndkhs.md@ufs.ac.za
Republiek van Suid Afrika / Republic of South Africa

Appendix B

UNIVERSITEIT VAN DIE VRYSTAAT
UNIVERSITY OF THE FREE STATE
YUNIVESITHI YA FREISTATA

Afdeling Mensgenetika / Division of Human Genetics
Skool vir Geneeskunde / Medical School
Fakulteit Gesondheidswetenskappe / Faculty of Health Sciences

Dr N van Zyl
The Head of Clinical Services
Room 1129
First Floor
Universitas Hospital
BLOEMFONTEIN

02 November 2009

Dear Dr N van Zyl

PERMISSION FOR USING LABORATORY SPACE AND TO OBTAIN BLOOD SAMPLES FROM CLINIC PATIENTS

We are in the process of launching a research project on the screening of young black South African breast cancer patients for the presence of deleterious *BRCA1* and *BRCA2* mutations.

I would therefore like to ask your permission to use the laboratory space and equipment of the Division of Human Genetics (Molecular Laboratory) for our project. I am also seeking your permission to approach patients attending the breast clinic at Universitas Hospital in order to obtain 10ml of EDTA blood should they qualify for the project. We would like to commence with the selection of patients as soon as possible. We are also planning to publish the data and to present it at the local Faculty Forum as well as on other national congresses. The duration of the study will be from January 2010 to December 2011.

Your prompt reply will be appreciated.

Yours sincerely

Dr NC vd Merwe PhD
Principal investigator
E-mail: vanderMerweNC.MD@ufs.ac.za

339 (G11), Bloemfontein 9300, Republiek van Suid-Afrika/Republic of South Africa
(051) 405 3047 (051) 444 4195

Appendix C

UNIVERSITEIT VAN DIE VRYSTAAT
UNIVERSITY OF THE FREE STATE
YUNIVESITHI YA FREISTATA

Afdeling Mensgenetika / Division of Human Genetics
Skool vir Geneeskunde / Medical School
Fakulteit Gesondheidswetenskappe / Faculty of Health Sciences

Prof Du Toit
The Head of Surgery
Universitas Academic Laboraries
Ground Floor, Block C
BLOEMFONTEIN

02 November 2009

Dear Prof RS Du Toit

PERMISSION TO APPROACH AND INCLUDE PATIENTS ATTENDING BREAST CLINIC AT UNIVERSITAS HOSPITAL IN A RESEARCH PROJECT

We are in the process of launching a research project on the screening of young black South African breast cancer patients for the presence of deleterious *BRCA1* and *BRCA2* mutations.

I am seeking your permission to approach and counsel patients attending the department's breast clinic in order to involve them in our project. Ten millimetres of EDTA blood and tumor samples (where possible) will be required from all selected patients. We are planning to publish the data and to present it at the local Faculty Forum as well as on other national congresses. The duration of the study will be from January 2010 to December 2011.

Your prompt reply will be appreciated.

Yours sincerely

Dr NC vd Merwe PhD
Principal investigator
E-mail: vanderMerweNC.MD@ufs.ac.za

339 (G11), Bloemfontein 9300, Republiek van Suid-Afrika Republic of South Africa
(051) 405 3047 (051) 444 4195

Appendix D

UNIVERSITEIT VAN DIE VRYSTAAT
UNIVERSITY OF THE FREE STATE
YUNIVESITHI YA FREISTATA

Afdeling Mensgenetika / Division of Human Genetics
Skool vir Geneeskunde / Medical School
Fakulteit Gesondheidswetenskappe / Faculty of Health Sciences

Prof M Theron
The Head of Human Genetics
Universitas Academic Laboraries
Ground Floor, Block C
BLOEMFONTEIN

02 November 2009

Dear Prof M Theron

PERMISSION FOR USING LABORATORY SPACE AND EQUIPMENT

We are in the process of launching a research project on the screening of young South African breast cancer patients for the presence of deleterious *BRCA1* and *BRCA2* mutations.

I would therefore like to ask your permission to use the space and equipment of the Division of Human Genetics (Molecular Laboratory) for our project. We are also planning to publish the data and to present it at the local Faculty Forum as well as on other national congresses. The duration of the study will be from January 2010 to December 2011.

Your prompt reply will be appreciated.

Yours sincerely

Dr NC vd Merwe PhD
Principal investigator
E-mail: vanderMerweNC.MD@ufs.ac.za

339 (G11), Bloemfontein 9300, Republiek van Suid-Afrika/Republic of South Africa
☎ (051) 405 3047 (051) 444 4195

Appendix E

National Health Laboratory Service
Human Genetics \ Mensgenetika

Tel: +27 51 4053047
Fax: +27 51 4441161
Practice/Praktyk no: 920 296

Block C
Faculty of Health Sciences
University of the Free State
Bloemfontein

P.O. Box 339 (G11)
UFS
Bloemfontein 9300

Prof H Pieters
NHLS Tertiary Laboratory Business Manager
Room 301
First Floor
Chemical Pathology, Block C
Bloemfontein

Dear Prof H Pieters

02 November 2009

PERMISSION FOR USING LABORATORY SPACE

We are in the process of launching a research project on the screening of young South African breast cancer patients for the presence of deleterious *BRCA1* and *BRCA2* mutations.

I would therefore like to ask your permission to use the space and equipment of the Division of Human Genetics (Molecular Laboratory) for our project. We are also planning to publish the data and to present it at the local Faculty Forum as well as on other national congresses. The duration of the study will be from January 2010 to December 2011.

Your prompt reply will be appreciated.

Yours sincerely

Dr NC vd Merwe PhD
Principal investigator
E-mail: vanderMerweNC.MD@ufs.ac.za