The molecular pathologies of *BRCA1* in ovarian cancer patients from the West of Scotland

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Decleration

I certify that this thesis does not contain material previously published or written by any other person except where referred to in the text and that the results in this thesis have not been submitted for any other degree or diploma.

Ahmet Dursun

This thesis is dedicated to my family who have always been there for me when I needed them.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

A	Adenine
Arg	Arginine
APS	Ammonium persulphate
Asn	Asparagine
BIC	Breast Cancer Information Core
bp	Base pair
C	Cytosine
°C	Degree Celsius
CCM	Chemical Cleavage Mismatch Analysis
cDNA	Complementary Deoxyribonucleic acid
cm	Centimetre
ddH_2O	Double distilled water
ddNTP	Dideoxyribonucleotide triphosphate
DGGE	Denaturing gradient gel electrophoresis
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	2'-deoxyribonucleoside triphosphate
EDTA	Ethylenediamine tetraacetic acid
G	Guanine
g	Gram
Gln	Glutamine
Glu	Glutamic acid
HA	Heteroduplex Analysis
HBOC	Hereditary Breast-Ovarian Cancer
Ile	Isoleucine

L	Ladder
l	Litre
LOH	Loss of heterozygosity
Lys	Lysine
kb	Kilo base
KD	Kilo Dalton
M	Molar
MDE	Mutation Detection Enhancement
μg	Microgram
μl	Microlitre
μM	Micromolar
mg	Milligram
ml	Millilitre
mM	Millimolar
mm	Millimetre
mRNA	Messenger RNA
ng	Nanogram
PCR	Polymerase Chain Reaction
Phe	Phenylalanine
pmol	Picomole
PTT	Protein Truncation Test
RNA	Ribonucleic acid
r.p.m.	Revolutions per minute
Ser	Serine
SDS	Sodium dodecyle sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SSCP	Single Strand Conformational Polymorphism
SSOC	Site-Specific Ovarian Cancer

T	Thymine
TBE	Tris-borate ethylenediaminetetra acetic acid
TE	Tris ethylenediaminetetra acetic acid
TEMED	N,N,N,N-tetramethylenthylenediamine
THMA	Temperature Modulated Heteroduplex Analysis
Thr	Threonine
Tris	Tris (hydroxymethyl) aminomethane
U	Uracil
UV	Ultraviolet
V	Volt

SUMMARY

Ovarian cancer is one of the most common female malignancies and is the forth leading cause of cancer death in the western world. It is estimated that 3-13% of all ovarian cancers are familial and the tumour suppressor gene, *BRCA1*, is responsible for almost 90% of all these familial cases.

BRCA1 is a tumour suppressor gene which spans 100 kb on chromosome 17q21 and contains 24 exons. The 7.8 kb mRNA encodes a protein of 1863 amino acids. The structure of BRCA1 is not strikingly homologous to other known molecules apart from the presence of a ring zinc finger domain and a 10 amino acid granin motif which are thought to be significant due to fact that they both are conserved in humans and other species studied.

The overall aims of this study were to screen and characterise mutations in the *BRCA1* gene using genomic DNA obtained from ovarian cancer patients from the West of Scotland. As a result of the information obtained from this study, genetic counselling would be possible for those who have inherited the mutations and for those who have a high risk of developing the same condition. In this study, 48 ovarian cancer patients with a family history of breast and/or ovarian cancer were screened for germline mutations in the *BRCA1* gene.

The strategy for germline mutation analysis involved the initial amplification of the *BRCA1* gene by polymerase chain reaction (PCR) using genomic DNA as a template. After initial

amplification, the product was electrophoresed in an 1% agarose gel in order to check for any abnormal alteration in size, quality and quantity of product. If no alteration was identified, further screening of the gene was carried out using the single strand conformational analysis (SSCP) and the protein truncation test (PTT). Any differences in the band pattern, when compared with negative controls (in both techniques) were fully characterised by automated DNA sequencing analysis.

Using the strategy described above, 5 different alterations were detected on analysis of *BRCA1* coding sequences. Of these, two were novel missense mutations, two were polymorphisms and one was an unclassified variant.

The first novel missense mutation was g.3771 A>T, changing serine to cysteine at amino acid position 1218. This mutation is in the granin site at the amino acid positions 1214-1223 and the leucine zipper at amino acid positions 1209-1230. It is well known that cysteine has a very important role in stabilising the three-dimensional protein structure. Two cysteine residues in different parts of the polypeptide chain but adjacent in the three-dimensional structure of a protein can be oxidised to form a disulfide bridge. Disulfide bridges usually occur among secreted proteins and make them less susceptible to degradation.

The second novel missense mutation was g.1380 G>A, changing glutamic acid to lysine at the amino acid position 421. This mutation's effect on the BRCA1 protein is unknown. However, this amino acid is conserved in the murine *BRCA1*

sequence. Two different polymorphisms were also detected in *BRCA1* coding sequence.

Patient no 28 is thought to be homozygous for polymorphism g.3667 A>G was also checked for possible large deletion within the exon 11.

The first polymorphism, g.3667 A>G, was found in four out of forty-eight patients and the second polymorphism, g.4427 T>C, was found in six out of forty-eight patients. These two polymorphisms are the most frequently found in ovarian cancer cases and the normal population. Although, the polymorphisms are generally thought to be a variation in the gene sequence, histopathological studies suggest that the aggressiveness of tumour progress, histological type and survival rate may be related to the host variation.

In order to establish the normal population frequency, one hundred samples from normal population was also screened. Six out of one hundred for polymorphism g.3667 A>G and 10 out of 100 for polymorphism g.4427 T>C were found positive for these polymorphisms.

An insertion of 12 bp of DNA was also found in intron 20 (5'-GTATTCCACTCC-3') at IVS20+60. Three studies suggested that this variant is not present in any of the subject in the control population analysed. It was also reported that this variant did not modify the correct splicing of exon 19-21 by analysing the cDNA obtained from the patient's peripheral blood. It is possible that a relatively large insertion in a region of non-repetitive DNA might affect the kinetics of splicing and result in a lower level of normal mRNA from the mutant allele.

CHAPTER I

INTRODUCTION

1.1 CANCER

Cancer is a cellular disease, characterised by the progressive loss of the organised properties of differentiated cells: growth control, karyotypic stability, particular morphological and biochemical traits, and the definite location of cells in the organism.

Percival Pott, the distinguished British surgeon first identified a environmental cause of cancer 200 years ago, namely cancer of the scrotum amongst chimney sweeps, due to their exposure to soot. The other major ideas on causes, which originated around the beginning of this century, include abnormalities of differentiation, viruses, effects of the immune system, chromosome abnormalities and most importantly somatic mutations.

These ideas can all now be combined into the one main notion that cancer arises from a series of changes in the expression of the genes, mostly genetic alterations that lead progressively from the normal to the malignant cell. It is now established that a tumour arises as a clonal growth, originating from genetic change in a single cell (Bodmer W, 1994).

In recent years, great attention has been focused on two classes of gene alterations that can cause a cancer. One class -the *oncogenes*- encode products that positively regulate cell growth and division. Alterations in oncogenes, which lead to an increased amount of product, can cause uncontrolled cell proliferation and tumour formation. The other class -the *tumour suppressor genes*- encode products that negatively regulate cell

growth and division. If both copies of a tumour suppressor gene are altered, its product is inactive or only partially active, then the negative control may be lost and uncontrolled cell growth may result.

1.2. MECHANISMS RESPONSIBLE FOR TUMORIGENESIS

1.2.1. Oncogenes

The discovery of the first cancer gene was based on work begun long before DNA was known to be the genetic material. It was identified by Peyton Rous in 1909 (Korf R.Bruce, 1996). It was initiated from a series of experiments that started with a chicken which had a lump on its leg. The lump was a soft-tissue sarcoma. Injection of some tumour tissue developed the same soft-tissue sarcoma in other chickens. The active agent was identified as a virus, in fact a retrovirus, and was named the *Rous Sarcoma Virus*. A decade later, one particular gene, named *src*, was found to be responsible for the transforming properties. When *src* was lost or mutated, the virus was no longer oncogenic. By the 1980s, approximately 20 different retroviruses, each with distinctive oncogenes, were known to be associated with cancer (table 1.1).

The breakthrough in understanding non-retroviral malignancies came from recognition that viral oncogenes are homologous with normal eukaryotic genes. These normal cellular genes are referred to as **proto-oncogenes**. The over-expression of a proto-

oncogene due to regulation by the viral genome is responsible for its transforming properties.

On the other hand, **oncogenes** are normal cellular genes that, when their patterns of expression are changed, can cause neoplastic changes in the cell. There are many routes to oncogene activation. One of them is a virus as mentioned above. The most direct route to the oncogene activation is gene amplification, where a block of DNA, including an oncogene and some neighbouring genes, is replicated tens or hundreds of times in the cell, which proliferates faster than other cells in the tissue. One other route to oncogene activation is through chromosome re-arrangements. In most cases, activation of a proto-oncogene is not associated with a visible change in chromosome structure. In bladder cancer, the *ras* oncogene, which is a retrovirus oncogene, is found to be mutated at a single base substitution leading to an altered amino acid.

The normal roles of proto-oncogenes are also important in understanding the characteristics of these genes and their responsibilities for the growth of neoplastic cells. Proto-oncogenes can be divided into four groups.

The **first** group of proto-oncogenes encode growth factors. These growth factors are able to stimulate the proliferation of certain types of cells. Overexpression, or expression of an aberrant protein, leads to enhanced cell proliferation.

The **second** group involves membrane receptors via growth factors. A mutation that keeps a receptor active causes independent cell growth and absence of proliferation control.

The **third** group are membrane associated proteins that are activated by signals transmitted from membrane receptors and transmit a signal by modification of other proteins. This group's mutations lead to overexpression of the activated form of the protein or insensitivity to other proteins that regulate their activity.

The **final** group controls transcription in the nucleus. Direct activation of these proto-oncogenes can cause an abnormal cell proliferation or differentiation in the absence of proper signals transmitted across the cell membrane and through the cytoplasm.

The largest family of oncogenes are the tyrosine kinases, which catalyse the transfer of a phosphate group from ATP to tyrosine residues on specific cellular proteins. Tyrosine kinases can either be cell surface growth factor receptors or intracellular kinases. The receptor tyrosine kinases have conserved overall primary structure which consists of an extracellular ligand binding, transmembrane, juxtamembrane and an intracellular catalytic domain. Specific growth factors have been identified for individual receptors such as epidermal growth factor (EGF) and platelet derived growth factor (PDGF). The growth factors bind to their specific receptors, which results in dimerisation and a consequent conformational change leading to auto-phosphorylation of the receptor on tyrosine residues. Phosphorylation of substrate follows and the signal is transmitted through defined pathways to the nucleus to stimulate transcription (Katsa *et al.*, 1997). The amplitude of

the signal determines the phenotypic response to a signal, namely differentiation or proliferation (Marshall, 1995).

Finally, oncogenes show a dominant effect, which means that overexpression of one copy, contributes to at least some transformed properties to the cell.

1.2.2. Tumour Suppressor Genes

Discovery of oncogenes and proto-oncogenes helped to make clear that cancer could also result from inactivation of another group of genes which encode proteins that normally negatively regulate cellular growth. They were named **tumour suppressor genes** and their inactivation would result in unregulated growth (table 1.2).

The crucial point in the cell cycle is the restriction point, which occurs between the G1 and S phases of mitosis. If a cell passes this point it will go on to divide, otherwise it remains in G1. In a normal cell, the tumour suppressor genes are working and keeping a cell in G1 phase and it therefore does not divide. If however the tumour suppressor gene is switched off, the cell becomes uncontrollable and moves out of G1, produces a duplicate set of chromosomes in S phase, and eventually undergoes mitosis to produce daughter cells.

NAME OF THE ONCOGENE	FUNCTION OF THE ONCOGENE	CANCERS WHICH IS CAUSED BY THE ONCOGENE
Erb-B	Codes for a receptor for epidermal growth factor	Involved in glioblastoma, a brain cancer, and breast cancer
erb-B2	Codes for a tumour antigen, which is serologically related to EGFR, also called *HER-2/neu*	Involved in breast, ovarian, and salivary gland cancers
Ki-ras	Codes for a protein that relays a stimulatory signal	Involved in lung, ovarian, colon, and pancreatic cancers
N-ras	Activates growth promoting genes	Involved in leukemias
c-Myc, N-myc, L-myc	Genes for transcription factors that activate growth promoting genes	Involved in leukemias, breast, stomach, and lung cancers (*c-Myc, L-myc*); neuroblastoma (*N-myc*)
Bcl-1	Codes for cyclin D1, a component of the cell cycle clock	Involved in breast, head and neck cancers

Table 1.1: Oncogenes and their role in tumorigenesis.

It was suggested by Knudson (1971) that there is a totally different class of genetic changes in cancers that are essentially recessive in their action. He has suggested that if cancer is due to a series of genetic changes at the somatic level, then sometimes one of those changes may be inherited in the germline and so be present in every cell in the body. He also pointed out that another mutation in the same gene, which occurs somatically, would be knocking out its full function. In other words, while the familial inherited susceptibility behaves as a dominant, the effect at the somatic level, which contributes to tumour progression, is recessive. Knudson's hypothesis has come to be called the *two-hit hypothesis*. Most hereditary cancer syndromes are due to inheritance of a mutant copy of a tumour suppressor gene. Therefore it has been suggested that the *BRCA1* gene is a tumour suppressor gene because the normal copy of *BRCA1* invariably is mutated in breast and ovarian cancers that arise in women who have inherited a mutant copy of *BRCA1* gene.

It has also been shown that BRCA1 expression increases during the cell cycle, suggesting that it may have a role in regulation of the transcription of genes involved in cellular proliferation (Gudas *et al.*, 1995b). Knudson's hypothesis is also applicable to the *BRCA1* gene, because as a tumour suppressor gene, *BRCA1* is recessive at a cellular level and inactivation of both alleles is required for tumorigenesis (Holt *et al.*, 1996).

NAME OF THE TUMOUR SUPPRESSOR GENE	ROLE OF THE TUMOUR SUPPRESSOR GENE	CANCERS CAUSED BY THE TUMOUR SUPPRESSOR GENE
DPC-4	Participates in a cytoplasmic pathway that inhibits cell division	Involved in pancreatic cancer
NF-1	Encodes a GTPase-activating protein (GAP), which activates the RAS GTPase.	Involved in neurofibromas and pheochromocytomas of the nervous system and myeloid leukemia
NF-2	Codes for a nuclear protein.	Involved in meningioma, schwannoma, and ependymoma
RB	Codes for the pRB protein, a nuclearprotein that is a major brake in the cell cycle	Involved in retinoblastoma as well as bone, bladder, small cell lung, and breast cancers
P53	Codes for the cytoplasmic p53 protein that regulates cell division and can induce apoptosis	Involved in a wide range of tumours including Li-Fraumeni syndrome
WT1	Unknown	Involved in Wilm's tumour of the kidneys
BRCA1	Unknown	Involved in breast and ovarian cancer
BRCA2	Unknown	Involved in breast cancer

Table 1.2: Tumour Suppressor Genes and their role in tumorigenesis.

1.3. OVARIAN CANCER

1.3.1. Epidemiology

Ovarian cancer is one of the most common malignancies among women and the fourth leading cause of cancer death in America (Wong P.A. *et al.*, 1995; Parker *et al.*, 1996). Ovarian cancer is predominantly a disease of perimenopausal and postmenopausal women: the mean age at diagnosis is 60 years (DePasquale *et al.*, 1998).

There has been increased interest in familial clustering of ovarian cancer. Although the majority of cases are sporadic in occurrence, it is now estimated that 3 to 13 percent of ovarian malignancies are familial (Lynch *et al.*, 1987; Claus *et al.*, 1996; Narod *et al.*, 1994). In the 1970s, Lynch et al. (1981) and Fraumeni et al. (1978)(Fraumeni *et al.*, 1978) identified several families in which multiple cases of epithelial ovarian cancer had occurred. The clinical characteristics of familial ovarian cancers were noted to be similar in those of women without a family history, however, familial ovarian cancers typically shared advanced-stage papillary serous adenocarcinoma and survival was poor (Piver *et al.*, 1993). Investigators have established at least three distinct phenotypes: site-specific ovarian cancer (SSOC), hereditary breast-ovarian cancer (HBOC), and hereditary nonpolyposis colon cancer/ovarian cancer syndrome (Lynch syndrome II). The most important parameters used in making a diagnosis of hereditary breast or ovarian cancer syndromes are the total number of family members affected with cancer and their early age of onset (Easton *et al.*, 1993).

According to The Breast and Cancer Linkage Consortium, 3 cases of ovarian cancer in first-degree relatives are considered to be hereditary. A family with a total number of 5 cases of breast or ovarian cancers, including 2 cancers of each type, qualifies as the breast-ovarian cancer syndrome (Easton *et al.*, 1993).

1.3.2. Histology

The predominant histological type of hereditary ovarian cancer is serous papillary cystadenocarcinoma (Bewtra *et al.*, 1992). The other major histological type is mucinous carcinoma, however, it has been found less frequently in hereditary ovarian cancers (Greggi *et al.*, 1990; Narod *et al.*, 1994). A third and very rare form is pseudomucinous carcinoma. It has been reported that *BRCA1*-linked ovarian cancers have a higher risk of producing a serous papillary cystadenocarcinoma (Bewtra *et al.*, 1992).

1.3.3. Co-factors

A number of risk factors have been associated with the development of ovarian cancer, including a family history, nulliparity or low parity. Some other factors that increase the risk are, exogenous chemicals like asbestos or talc (Longo & Young, 1979), and dietary factors such as high consumption of animal fats (Rose *et al.*, 1986). There are also a number of factors thought to decrease the risk, including high parity, breast-feeding and oral contraceptive use (DePasquale *et al.*, 1998; Narod *et al.*, 1995).

1.3.4. Age-of-Onset

Early age-of-onset is not as predictable of hereditary ovarian cancer as it is for hereditary breast cancer. However, Amos et al. (1992) found that the age-of-onset of hereditary ovarian cancer patients (those who have two or more relatives affected with ovarian cancer) was younger than expected. The median age of diagnosis of hereditary ovarian cancer was 47 years, which was 14 years earlier than the US median for all ovarian cancers.

On the other hand, although the penetrance of breast and ovarian cancer in carriers of the BRCA1 gene are high, it is likely that estimates based on selected large multicase families are inflated by selection bias. In addition, the proportion of breast or ovarian cancers related to the genes is similarly lower in population-based samples than in referred selected families, and, even for subjects with cancer onset at young ages or with a family history, it is quite small (Elwood, 1999).

1.3.5. Population Genetics

A family history of ovarian cancer is a consistent risk factor for ovarian cancer. Based on the records of the Office of Population Censuses and Surveys (UK), it was found that the life-time risk of death from ovarian cancer was 1 in 40 when one relative was affected, which is 3 times greater than the general population risk. If a woman had an affected mother and sister, or two affected sister, the risk became 30-40% (Ponder *et al.*, 1991).

It is established that familial ovarian cancer often strikes during the fourth and fifth decades of life and the estimated lifetime

risk of ovarian cancer is 60% in BRCA 1 carriers (Easton *et al.*, 1995; Easton *et al.*, 1993; Easton *et al.*, 1993).

1.3.6. Molecular Basis of Ovarian Cancer

Mary-Claire King's group first demonstrated in 1990 that a fraction of familial breast cancer was linked to a location on chromosome 17q (Hall J. *et al.*, 1990). The following year, Narod et al. (Narod *et al.*, 1991) reported that families with both breast and ovarian cancer also showed linkage to 17q. The responsible gene was mapped to (Bowcock *et al.*, 1993; Kelsell *et al.*, 1993; Neuhausen *et al.*, 1994) and cloned and named *BRCA1* in 1994(Miki *et al.*, 1994a). In addition to the discovery of *BRCA1*, evidence has been found for the existence of a second gene, *BRCA2*, which is located on chromosome 13q and which also confers increased risk of breast and ovarian cancer within high-risk breast and ovarian cancer families (Wooster *et al.*, 1994). According to studies in families selected on the basis of strong family history and early age of onset, germline mutations in *BRCA1* are responsible for approximately 50% of hereditary breast cancers and 90% of hereditary ovarian cancers (Ford & Easton, 1995; Easton *et al.*, 1993).

Inheritance of familial cancer syndromes usually follows an autosomal dominant mendelian pattern, as a tumour suppressor, *BRCA1* is recessive at the cellular level because inactivation of both copies is required for tumourigenesis (Berchuck *et al.*, 1998a). It is now well established that the age-specific and lifetime risk of developing ovarian cancer is predicted to be high for carriers of the *BRCA1* gene (Berchuck *et al.*, 1998a).

1.4. *BRCA1* GENE

1.4.1. Molecular Genetics of the *BRCA1* Gene

BRCA1 spans 100 kb on chromosome 17q21 (Bowcock *et al.*, 1993; Kelsell *et al.*, 1993; Neuhausen *et al.*, 1994) and contains 24 exons. The 7.8 kb mRNA encodes a protein of 1863 amino acids (Miki *et al.*, 1994a). The largest *BRCA1* exon is exon 11, which has a 3.4kb long cDNA. The structure of BRCA1 is not strikingly homologous to other known molecules apart from the presence of a **ring zinc finger domain** at the amino terminus of the protein (Saurin *et al.*, 1996) and a granin consensus sequence (BRCA1 amino acids 1214-1223) (Jensen *et al.*, 1996) (figure 1.1). The ring finger domain is being found in an increasing number of proteins and might be involved in mediating protein-protein interactions. The functional importance of this motif is supported by the observation that it is highly conserved in other species (Bennett *et al.*, 1995). Jensen *et al.* (1996) suggested that BRCA1 exhibits properties of the 10 amino acid granin that is also conserved across species.

BRCA1 has also two "BRCT" repeats at the carboxyl terminus (Koonin *et al.*, 1996; Callebaut & Mornon, 1997). The BRCT repeat is a poorly conserved domain found in a range of proteins many of which are involved in either DNA repair or metabolism (Koonin *et al.*, 1996; Callebaut & Mornon, 1997).

1.4.2. Location of BRCA 1 Protein

There is a controversy about the sub-cellular location of BRCA1 protein. In normal cells, there is an agreement that BRCA1 is a nuclear protein (Chen *et al.*, 1995; Aprelikova *et al.*, 1996; Shao *et al.*, 1996; Wilson *et al.*, 1997; Scully *et al.*, 1996; Chen Y. *et al.*, 1996). However, there is a discussion about the location of protein in tumour tissue. It has been reported (Chen *et al.*, 1996) that BRCA1 is present in the cytoplasm of cancer cells whereas Scully et al. (1996) have suggested that the protein still remains in the nucleus of these cells. Splice variants of *BRCA1* that lack either exon 11 or exons 9 and 10 have been described. It has been suggested that some splice variants may lose a nuclear localisation domain, which might explain the conflict between results obtained by various groups (Chen *et al.*, 1996; Wilson *et al.*, 1997).

On the other hand, it was suggested (Jensen *et al.*, 1996; Steeg, 1996) that BRCA 1 is a secreted protein which has a 10 amino acid granin motif (granin is a member of secreted proteins), and its secretion is triggered by activation of cyclic AMP and induced by estradiol. It has also been reported that BRCA1 is present in the Golgi complex.

1.4.3 Molecular Organisation and Regulation of the BRCA 1 Gene

Many tumours arising in patients with germline mutations display loss of heterozygosity (LOH), which uniformly involves loss of the wild-type *BRCA1* allele. It has been suggested that *BRCA1* is a tumour suppressor gene. Furthermore, it has been

shown in cultured cells that expression of BRCA1 increases as cells move out of G1 into the S phase of the cell cycle (Vaughn *et al.*, 1996). In addition, BRCA1 antisense strategies using cultured cells resulted either in acceleration of proliferation (Thompson *et al.*, 1995) or cell transformation (Rao *et al.*, 1996), supporting the tumour-suppressive function of BRCA1.

BRCA1 has been shown to have a specific interaction with Rad51 protein (figure 1.1) mediated through a *BRCA1* exon 11 domain (Scully *et al.*, 1997b). Rad51, in mammals, is involved with the homologous pairing of DNA during the process of recombination and the recombinational repair, supporting the proposition that *BRCA1* is involved in DNA repair processes (Sung & Robberson, 1995). The interaction between these two proteins was first found from the observation of similar staining patterns during S and M phases of cell cycle (Scully *et al.*, 1996; Scully *et al.*, 1997b). There are reports that BRCA1 undergoes dramatic changes in phosphorylation and sub-nuclear localisation in response to DNA damage. The same changes in localisation apply to Rad51 suggesting that they may have a physiological interaction that is triggered by DNA damage (Scully *et al.*, 1997a). Although the interaction seems to be specific, its functional basis is still not clear. It has been reported that the evidence for BRCA1 function in DNA repair suggests that it may not be a direct tumour suppressor gene but may be involved in the maintenance of genome integrity (Bertwistle & Ashworth, 1998).

The loss of genome integrity, however, would lead to DNA damage and activation of normal checkpoints on the cell

division involving, in particular, the p53 pathway (Kinzler & Vogelstein, 1976; Brugarolas & Jacks, 1997). P53 was identified in 1990 by Dr David Malkin and his colleagues (Malkin *et al.*, 1990) and that it was mutated in 5 out of 5 families studied with the Li-Fraumeni syndrome. It is a tumour suppressor gene and mutated in a very wide variety of human cancers. It encodes a 53KD protein. It affects cell cycle in the G1 phase of the cell cycle. It was hypothesised that loss of the wild-type *BRCA1* allele and p53 pathway are both required for tumourigenesis. There are two ways that this could occur (Kinzler & Vogelstein, 1976; Brugarolas & Jacks, 1997).

First, the loss of the wild type *BRCA1* gene in a carrier of a *BRCA1* mutant might lead to a defect in DNA repair. DNA damage would result in the induction of p53, which mediates growth arrest or apoptosis. Loss of the p53 pathway would reduce the growth-arrest allowing cell division. Growth with a failure of the DNA repair pathway might lead to the activation of an oncogene and neoplasia.

Second, cells carrying a heterozygous mutation in *BRCA1* might lose the p53 pathway. Loss of the wild-type *BRCA1* allele would then be driven by a main defect caused by loss of p53 function. Thus, failure of a DNA repair mechanism might lead to the activation of an oncogene and neoplasia (Bertwistle & Ashworth, 1998) (Figure 1.2).

Crook et al. has reported that in their study, p53 mutations have been found in one familial breast and three familial ovarian cancer patients. It has been also suggested that loss of BRCA1 function activates a genome guardian checkpoint which mediates the block to

cell cycle progression, suggesting that p53 may be such a checkpoint and that BRCA1 tumorigenesis may require not only the inheritance of a mutant germline BRCA1 allele, but also an acquired p53 mutation.

Two separate promoters exist for *BRCA1*, generating transcripts that differ in the first exon, which is a part of the untranslated region (Xu *et al.*, 1995). Both promoters contain oestrogen response elements, suggesting that BRCA1 may be involved in hormone signalling pathways (Xu *et al.*, 1997b). Although evidence suggests that this may be an indirect and nonspecific effect, which is related to changes in the cell cycle rather than to interactions between BRCA1 and a control pathway (Spillman & Bowcock, 1996;; Marks *et al.*, 1997; Gudas *et al.*, 1995).
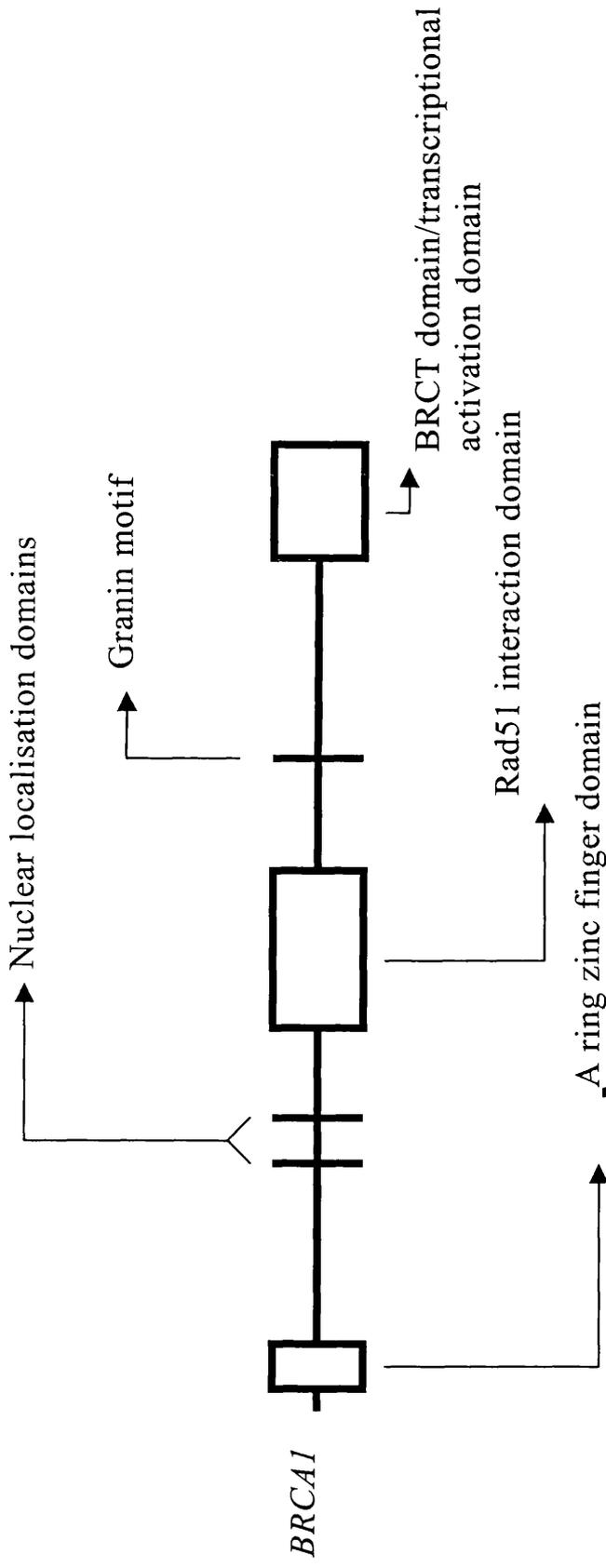
Figure 1.1: The functional domains in the *BRCA1* gene. The 1863 amino acid BRCA1 protein contains an amino-terminal RING finger and two BRCT domains. A rad51 interaction domain and nuclear localisation sequence have also been defined in *BRCA1*. In addition, a granin motif is present (amino acid position 1214-1223)(Bertwistle & Ashworth, 1998).

1.4.4. Molecular Pathologies in *BRCA1* Gene:

BRCA1 is not mutated in sporadic Breast tumours and only rarely in sporadic ovarian cancers (Takahashi *et al.*, 1996; Futreal *et al.*, 1994; Merajver *et al.*, 1995; Hosking *et al.*, 1995; Foster *et al.*, 1996). Mutations in the *BRCA1* gene reported so far, disrupt the normal function of the protein. In early reports, approximately 85% of the alterations in *BRCA1* were either nonsense mutations in which a single nucleotide substitution produced a stop codon or frameshift mutations in which one or more nucleotides were deleted producing a stop codon somewhere downstream (Shattuck-Eidens *et al.*, 1995; Couch & Weber, 1996). In either case the predicted result is production of a smaller than usual BRCA1 protein. In these reports, missense mutations that only change a single amino acid formed about 10% of *BRCA1* mutations.

The recent reports show that the nonsense, frame-shift mutations and splice variants in the *BRCA1* gene that produce short products are not as frequent as previously thought. (Breast cancer information Core http://www.nhgri.nih.gov/). Almost 850 different mutations have been reported out of nearly 4000 entries and more than 500 (57%) of them have not been previously reported.

More than half of those mutations (57%) are small insertions, deletions, nonsense mutations, splice variants and regulatory mutations that are predicted to affect the size and the molecular functions of the BRCA1 protein. Nearly, one third of those mutations (29%) are missense mutations that change functions of BRCA1 protein. Where missense mutations involve highly

conserved functional domains in BRCA1 it is likely they are deleterious. More than 10% of all entries in the database are identified as polymorphisms. The most frequently identified mutation to date is "185delAG" which is predicted to produce a short 39 amino acid peptide.

On the other hand, the frequency of these mutations, including nonsense mutations, small deletions and insertions, missense mutations occurring at crucial aminoacid positions within well conserved domains and mutations affecting splice sites is lower than expected from data, based on linkage analysis (Szabo & King MC., 1997). Some of these mutations can be attributed to the major genomic rearrangements of the BRCA1 gene, which escape most of the current PCR-based mutation detection methods (Petrij-Bosch *et al.*, 1997; Swensen *et al.*, 1997; Puget *et al.*, 1997).

It has been noted that various *BRCA1* mutations may tend to result in the development of different cancers. It has been suggested that mutation in the carboxyl terminus of the gene may result in a higher frequency of breast cancer, relative to ovarian cancer (Shattuck-Eidens *et al.*, 1995; Gayther *et al.*, 1995b). On the other hand, mutations in the proximal amino end of the gene may result in a higher likelihood of developing ovarian cancer. Studies on phenotype/genotype correlation suggests that carboxyl terminal *BRCA1* mutants effectively inhibit growth of ovarian, but not breast, cancer cells (Holt *et al.*, 1996).
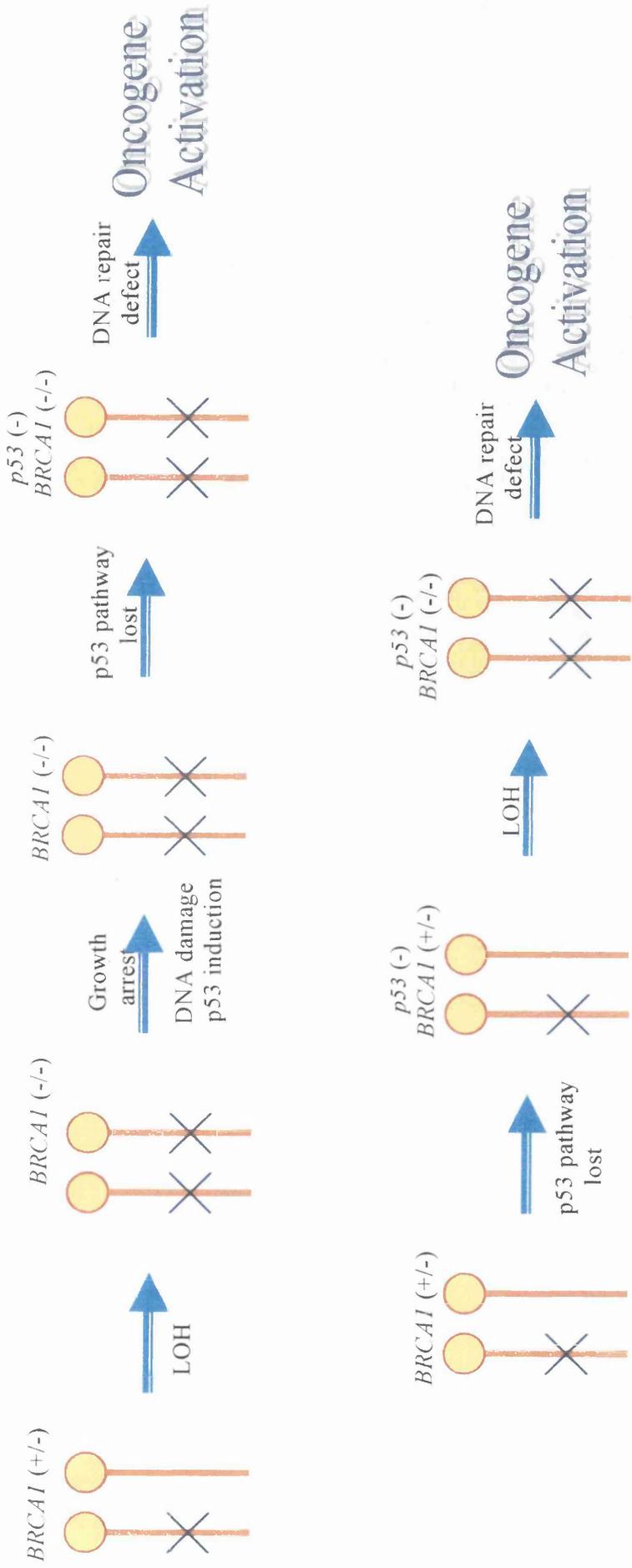
Figure 1.2: Models for possible genetic changes in carriers of BRCA 1 mutations (Bertwistle & Ashworth, 1998).

1.5. MUTATIONS RESPONSIBLE FOR GENETIC ALTERATIONS

Mutation is the result of any detectable change that affects DNA's chemical or physical constitution, its replication, its phenotypic function, or the sequence of one or more DNA base pairs. If a mutant cell gives rise only to somatic cells, it cannot be passed to the next generation. This type of mutation is called a **somatic mutation**. However, mutations in the germline can be transmitted to the next generation as the mutation is in both the somatic and the germ line cells. These kind of mutations are called **germline mutations**.

1.5.1. Types of Mutations:

A change in the organisation of a chromosome is called a **chromosomal mutation or aberration**. A mutation in a gene sequence is called **gene mutation**. These mutations can occur spontaneously or induced by the application of a **mutagen**. Mutations that result from treatment with mutagens are called **induced mutations**, naturally occurring mutations are **spontaneous mutations**. Gene mutations can involve any number of alterations of the DNA sequence of the gene, including base-pair substitutions, and insertions or deletions of one or more base pairs.

I.5.1.1. A base-pair substitution mutation:

It is a chance in a gene that one base pair is replaced by another base pair. There are two different ways that a base-pair substitution can occur: **a transition mutation** and **a transversion mutation**.

A **transition mutation** is a specific type of base-pair substitution mutation involving a change from one purine base pair to the other purine base pair. The four types of transition mutations are: A to G, G to A, T to C, and G to T (Figure 1.3).

A **transversion mutation** is another specific type of base-pair substitution mutation involving, a change from a purine base pair to a pyrimidine base pair. The four types of transversion mutations are: A to T, G to C, A to C, and G to T (Figure 1.3). Mutations can also be defined according to their effects on amino acid sequences in proteins.

Missense mutations: This is a base-pair substitution mutation in which a base-pair change in the DNA causes a change in an mRNA codon so that a different amino acid is inserted into the polypeptide in place of the one specified by the wild type codon, resulting in an altered protein (figure 1.4).

Nonsense mutations: This is a base-pair change in DNA that results in a change of an mRNA codon from one that specifies an amino acid to a chain-terminating codon (UAG, UAA, or UGA) (figure 1.4).

Neutral mutations: This base-pair substitution in a gene changes a codon in the mRNA such that the resulting amino acid produces no detectable change in the function of the protein translated from the message (Figure 1.5).

Silent Mutations: This is also a kind of missense mutation in that a base pair change in a gene alters a codon in the mRNA such that the same amino acid is inserted in the protein (Figure 1.5). The **wobble hypothesis,** proposed by Francis Crick (Crick, 1979), has shown that the base at the 5' end of the anticodon is not as constrained as the other two bases. This feature allows for less exact base pairing so that the base at the 5' end of the anticodon can potentially pair with one of three different bases at the 3' end of the codon. The protein, in this case, has wild-type function.

1.5.1.2. Point mutations:

Point mutations occur when only one nucleotide is deleted or inserted. It also occurs when a nucleotide substitutes to another nucleotides in the same position resulting in a missense mutation.

1.5.1.3. Frameshift mutations:

A frameshift mutation results from the addition or deletion of one or more base pairs in a gene (when only one base pair is involved, the frameshift mutation is also called a point mutation).

1.5.1.3.1. Deletions:

A deletion is a loss of one or more base pairs in a gene. It shifts the mRNA's reading frame by one or more bases so that incorrect amino acids are added to the polypeptide chain after the mutation sites. Often, deletions generate new codons

resulting in a stop codon, which may shorten the protein, or they result in readthrough of the normal stop codon, resulting in longer than normal proteins. In either case, deletions usually result in a non-functional or semi-functional protein (Figure 1.6).

1.5.1.3.2 Insertions:

Insertion is a gain of one or more base pairs in a gene that result in change to the reading frame of a gene from the site of mutation onward. Like deletions, there are two possible outcomes from this change. Mutated mRNA can create either a short product, because the mutation would produce an unexpected stop codon, or a long product, because insertion would change the reading frame and when the translation reaches the original stop codon, it would no longer be recognised as such, and the translation process would continue beyond the end (figure 1.7).

a) Transition mutation (TA to CG in this example)

5' ... TCT·CAA·TAA·TTT·ACG ... 3'
3' ... AGA·GTT·ATT·AAA·TGC ... 5'

→

5' ... TCTCAACAATTTACG ... 3'
3' ... AGAGTTGTTAAATGC ... 5'

b) Transversion mutation (GC to TA in this example)

5' ... TCT·CAA·AGA·TTT·ACG ... 3'
3' ... AGA·GTT·TCT·AAA·TGC ... 5'

→

5' ... TCT·CAA·ATA·TTT·ACG ... 3'
3' ... AGA·GTT·TAT·AAA·TGC ... 5'

Figure 1.3: Transition (a) and Transversion (b) mutations.

a) Missense mutation (change from one amino acid to another)

5' ··· TCT·CAA·AAA·TTT·ACG ··· 3'
3' ··· AGA·GTT·TTT·AAA·TGC ··· 5'
··· Ser · Gln · Lys · Phe · Thr ···

→

5' ··· TCT·CAA·GAA·TTT·ACG ··· 3'
3' ··· AGA·GTT·CTT·AAA·TGC ··· 5'
··· Ser · Gln · Glu · Phe · Thr ···

b) Nonsense mutation (change from an amino acid to stop codon)

5' ··· TCT·CAA·AAA·TTT·ACG ··· 3'
3' ··· AGA·GTT·TTT·AAA·TGC ··· 5'
··· Ser · Gln · Lys · Phe · Thr ···

→

5' ··· TCT·CAA·TAA·TTT·ACG ··· 3'
3' ··· AGA·GTT·ATT·AAA·TGC ··· 5'
··· Ser · Gln · STOP ---

Figure 1.4: Missense (a) Nonsense (b) mutation.

c) Neutral mutation (change from one amino acid to another amino acid with similar chemical properties)

5' ··· TCT·CAA·AAA·TTT·ACG ··· 3'
3' ··· AGA·GTT·TTT·AAA·TGC ··· 5'
··· Ser · Gln · Lys · Phe · Thr ···

→

5' ··· TCT·CAA·AGA·TTT·ACG ··· 3'
3' ··· AGA·GTT·TCT·AAA·TGC ··· 5'
··· Ser · Gln · Arg · Phe · Thr ···
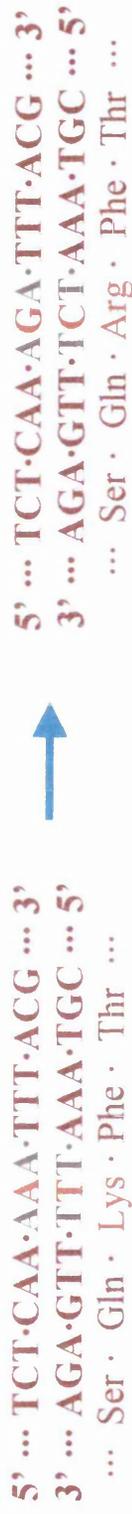
d) Silent mutation (change in codon that the same amino acid is specified)

5' ··· TCT·CAA·AAA·TTT·ACG ··· 3'
3' ··· AGA·GTT·TTT·AAA·TGC ··· 5'
··· Ser · Gln · Lys · Phe · Thr ···

→

5' ··· TCT·CAA·AAG·TTT·ACG ··· 3'
3' ··· AGA·GTT·TTC·AAA·TGC ··· 5'
··· Ser · Gln · Lys · Phe · Thr ···

Figure 1.5: Neutral (a) Silent (b) mutation.

FRAMESHIFT MUTATIONS

a) Deletion (deletion of one base pair leads to a change in reading frame)

5' ··· TCT·CAA·AAA·TTT·ACG ··· 3'
3' ··· AGA·GTT·TTT·AAA·TGC ···5'
··· Ser · Gln · Lys · Phe · Thr ···

→

5' ··· TCT·CAA·AAT·TTA·CGA ··· 3'
3' ···AGA·GTT·TTA·AAT·GCT ···5'
··· Ser · Gln · Asn · Leu · Arg ···

b) Deletion (leads to a change in reading frame, creates a stop codon)

5' ··· TCT·CAA·AAA·TTT·AAG ··· 3'
3' ··· AGA·GTT·TTT·AAA·TTC ···5'
··· Ser · Gln · Lys · Phe · Thr ···

→

5' ··· TCT·AAA·ATT·TAA·G ···3'
3' ··· AGA·TTT·TAA·ATT·C ···5'
··Ser · Lys · Ile · Stop ···

Figure 1.6: Frameshift Mutations caused by deletions.

FRAMESHIFT MUTATIONS

c) Insertion (insertion of one base pair leads to a change in reading frame)

5' ⋯ TCT·CAA·AAA·TTT·ACG ⋯ 3'
3' ⋯ AGA·GTT·TTT·AAA·TGC ⋯ 5'
⋯ Ser · Gln · Lys · Phe · Thr ⋯

→

5' ⋯ TCT·CAA·GAA·ATT·TAC·G ⋯ 3'
3' ⋯ AGA·GTT·CTT·TAA·ATG·C ⋯ 5'
⋯ Ser · Gln · Glu · Ile · Tyr · ⋯

d) Insertion (leads to a change in reading frame, creates a larger protein than expected)

5' ⋯ TCT·CAA·AAA·TAA·ACG ⋯ 3'
3' ⋯ AGA·GTT·TTT·ATT·TGC ⋯ 5'
⋯ Ser · Gln · Lys · Stop ⋯⋯⋯⋯

→

5' ⋯ TCT·GCA·AAA·ATA·AAC·G ⋯ 3'
3' ⋯ AGA·CGT·TTT·TAT·TTG·C ⋯ 5'
⋯ Ser · Ala · Lys · Ile · Asn · ⋯

Figure 1.7: Frameshift Mutations caused by insertions.

1.6 APPLICATIONS FOR MUTATION DETECTION AND SCREENING

There is no one technique, which is ideally suited for all mutation detection. All applications have advantages and disadvantages. Some are straightforward to perform and rapid but less sensitive than other techniques, whereas others have high sensitivity but are time consuming, labour intensive and less cost-effective. The choice of application can also be dependent on other factors, including the material to be analysed, whether it is genomic DNA or cDNA. Some available methods are detailed below.

- Single Strand Conformation Polymorphism Analysis (SSCP/SSCA)
- Protein Truncation Test (PTT)
- Heteroduplex Analysis (HA)
- Chemical Cleavage Mismatch Analysis (CCM)
- Denaturing Gradient Gel Electrophoresis (DDGE)
- Nucleic Acid Sequencing

1.6.1. Single Strand Conformation Polymorphism Analysis (SSCP/SSCA):

Single strand conformation polymorphism analysis was originally described by Orita et al. (Orita et al., 1989a; Orita et al., 1989b). With this method, regions of DNA thought to contain mutations are amplified using the polymerase chain reaction (PCR), and rendered single-stranded by heating. The

separated strands are then fractionated on a non-denaturing polyacrylamide gel or the mutation detection enhancement (MDE) gels under conditions that may resolve two molecules differing by a single base (figure 1.8). Because conformational changes and the consequent alterations in electrophoretic mobility cannot be predicted, separation using several different conditions may be required to detect all mutations. SSCP analysis can be best applied if the products are 150-350 bp in length. It has been suggested that if the product is under 200 bp, the detection rate will be maximised.

SSCP analysis is today being widely used, because it is technically easy and can be used in the screening of large numbers of samples. It has been accepted that in 80%-100% of cases a single base change is sufficient to cause altered electrophoretic mobility, if the product is 150-350 bp in length.

1.6.2. Protein Truncation Test (PTT):

The protein truncation test (PTT) is a mutation detection method that specifically detects mutations which lead to the premature termination of mRNA translation and subsequent protein truncation. It detects mutations which lead to the shortened protein by producing an early stop codon: **nonsense mutation** where a single nucleotide substitution which produces a stop codon (TGA, TAA or TAG) and mRNA translation stops at the point of mutation, **frameshift mutation** where one or more nucleotides are either inserted or deleted and if the number of bases is not divisible by 3, a change in the reading frame will occur which will alter the remainder of the

translation of the mRNA, and frequently a stop codon is introduced prematurely.

PTT is carried out by amplification of target DNA by PCR. This can be as long as 3kb, without introns. The upstream primer has a T7 promoter at its 5' end and is added to a coupled *in vitro* transcription-translation system. For detection, a labelled amino acid is included during amplification, this is usually methionine, leucine or cysteine. The label can either be a **radionucleotide** such as [^{35}S] which is visualised by autoradiography, or **biotin** which is detected by a colorimetric Western blot employing a streptavidin-biotin-alkaline phosphatase complex. The polypeptides produced are separated by size using an SDS-PAGE gel. If the only product visualised is full length, no truncating mutation is present. Truncating mutations result in shorter products, the size of which gives the approximate position of the mutation (Figure 1.9).

The most valuable advantage of PTT is that it allows the analysis of large stretches of coding sequence, as long as 3kb. It only detects mutations that create a stop codon, therefore polymorphisms are not identified. It can only detect a mutation in a coding sequence thus it is suitable for analysis of large exons. For genes with small exons, RNA extraction and cDNA preparation will be needed. It has been reported that PTT has a 100% detection rate.

DIAGRAM OF SSCP REACTION
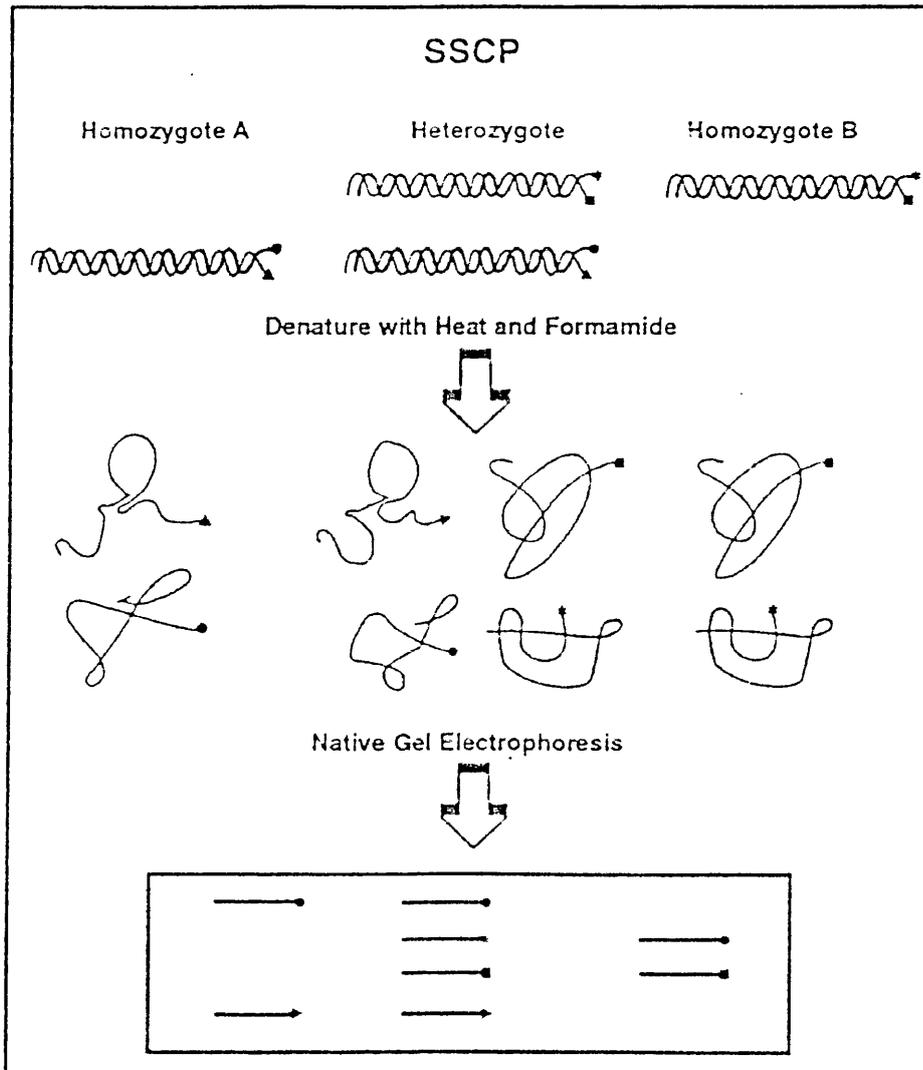
Figure 1.8: Diagram of SSCP reaction: The SSCP analysis is dependent on conformational differences in single stranded DNA. Regions of DNA thought to contain mutations are amplified using the polymerase chain reaction (PCR), and rendered single-stranded by heating. The separated strands are then fractionated on a non-denaturing polyacrylamide gel or mutation detection enhancement (MDE) gel.

Figure 1.9: Protein Truncation Test (PTT). (MRCPath Self-help course, 1998-1999 Course Birmingham. (http://209.185.131.251/cgi-bin/).

Forward primer.

5'   3'
TAATACGACTCACTATAGGGAGACCACC**ATG**GAGAACAACTGTCTACAAACT

T7 promoter   translation   gene-pecific
                   initiation   sequence
                   sequence

Reverse primer

a) The forward primer carries at its 5' end a T7 promoter, followed by a eukaryotic translation initiation sequence, which includes an ATG start codon.
Next is a gene-specific sequence designed so that the sequence amplified reads in-frame from the ATG.

Size markers

Full length transcript

Truncated transcripts

b) If the product is full length, no truncating mutation is present. Truncating mutations result in shorter products, the size of which gives the approximate position of the mutation.

1.6.3. Heteroduplex Analysis (HA):

This technique can be used to identify point mutations or single base pair polymorphisms in heterozygous individuals. It takes advantage of the fact that heteroduplex molecules containing single base pair mismatches can be separated under particular conditions of gel electrophoresis from nearly identical molecules containing no mismatches (White *et al.*, 1992). RNA or DNA from a potentially heterozygous individual is amplified by PCR and the products are run on non-denaturing mutation detection enhancement (MDE) or polyacrylamide gels. Hybrid molecules containing a mismatch, migrate more slowly than their corresponding homoduplexes. It has been shown that MDE gels have significantly greater ability than polyacrylamide gel to resolve and point mutations.

PCR products of more than 500 bp in length are not suitable for single base pair mutation detection.

1.6.4. Chemical Cleavage Mismatch Analysis (CCM):

This was first described by Cotton et al. (1988). Mutation screening by the chemical cleavage method is based on the fact that mismatched cytosine (C) and thymidine (T) are much more reactive with the compounds hydroxylamine and osmium tetroxide, respectively, than Watson and Crick-paired cytosine and thymidine bases. In this protocol, an excess of unlabeled target DNA is hybridised with labelled, wild type DNA probe and heteroduplexes are formed. One part is treated with hydroxylamine, which reacts with mismatched C bases, the other part is treated with osmium tetroxide, which reacts with

mismatched T bases. The reactions are mixed with piperidine which cleaves the strands at the sites where hydroxylamine and osmium tetroxide react. Cleaved fragments are then electrophoresed and sized on polyacrylamide gels, identifying the point of cleavage. Mismatched G and A bases will not be directly detected, but they are transposed to mismatched C and T bases respectively, by use of a probe of the opposite sense for detection.

Although it was first described as a screen for point mutations, deletions and insertions can also be detected by this method. It has been claimed to have an almost 100% success rate for single base pair mutation detection.

1.6.5. Denaturing Gradient Gel Electrophoresis (DDGE):

Separation of DNA fragments by denaturing gradient gel electrophoresis (DDGE) was first described by Fischer and Lerman (Fischer & Lerman, 1978). The separation principle of denaturing gradient gel electrophoresis is based on the melting behaviour of the DNA double helix of a given fragment. This melting behaviour is sequence dependent and generally occurs in discrete domains rather than at single bases. Strand separation at a domain in a DNA fragment is detected as a reduction in the mobility of the fragment when it moves through an acrylamide gel containing a chemical denaturant gradient. Two double-stranded DNA fragments of the same size, but differing in sequence, melt at different points in a denaturing gradient and can be distinguished by differential migration.

PCR products should be in the 100-800 bp range, however, more than two or three melting domains in a fragment would be the limitation for a successful result. It has been reported that between 90-100% of all possible mutations in the area screened can be reliably detected using this method.

1.6.6. Nucleic Acid Sequencing:

Although nucleic acid sequencing became commonplace in studies of molecular systems in the late-1970s, only stretches of DNA 15-20 base pairs in length had been sequenced. Breakthroughs in nucleic acid sequencing were published almost simultaneously by Maxam and Gilbert (Maxam & Gilbert, 1977) and Sanger et al. (Sanger *et al.*, 1977).

Maxam and **Gilbert**, or chemical DNA sequencing relies on the use of base-specific modification and cleavage reactions. In this method, a DNA fragment is electrophoretically separated into its two complementary strands, and each of them end labelled with [^{32}P]. Sequencing was accomplished by dividing the target DNA into four sub samples and then treating the sub samples with a series of base-specific chemical reagents that partially cleave the DNA. The radioactively labelled fragments from the four sub samples are then electrophoretically separated by size on a denaturing polyacrylamide gel and visualised by exposing the dried gel to an X-ray film to produce an autoradiograph.

Sanger sequencing, or controlled interruption of enzymatic DNA replication, uses dideoxynucleotide analogs in primer-directed DNA extension to produce discrete DNA fragments.

The doubled-stranded DNA is first denatured to produce a single-stranded DNA.

Next, using a primer, the sample is divided into four sub samples, to each of which is added the four deoxynucleotides and DNA polymerase. In addition, one of four dideoxynucleotide (ddNTP) is added to each of the tubes. The primer has a free 3'-OH group on its deoxyribose, to which additional nucleotides can be attached. The DNA polymerase using the target DNA as a template extends the DNA sequence. At some point, the polymerisation is terminated because the ddNTP lacks a 3'-OH group. The radioactive fragments are then separated by denaturing polyacrylamide gel electrophoresis and visualised by autoradiography (figure 1.10).

Later on, another method, called **cycle sequencing**, was introduced. Cycle sequencing is based on the dideoxynucleotide chain-termination method of Sanger et al., but utilizes a linear polymerase reaction to amplify labelled DNA that is complementary to the target DNA (Murray, 1989; Craxton, 1991). In this method, first, a segment of the candidate gene is PCR amplified from the genomic DNA of an affected individual. The PCR product is then subjected to multiple round of further amplification in a thermal cycler using a heat-stable DNA polymerase in the presence of different dideoxynucleotides and a radiolabeled primer. The resulting [^{32}P]-labeled sequence reaction products are fractionated on a denaturing polyacrylamide gel and visualised by autoradiography.
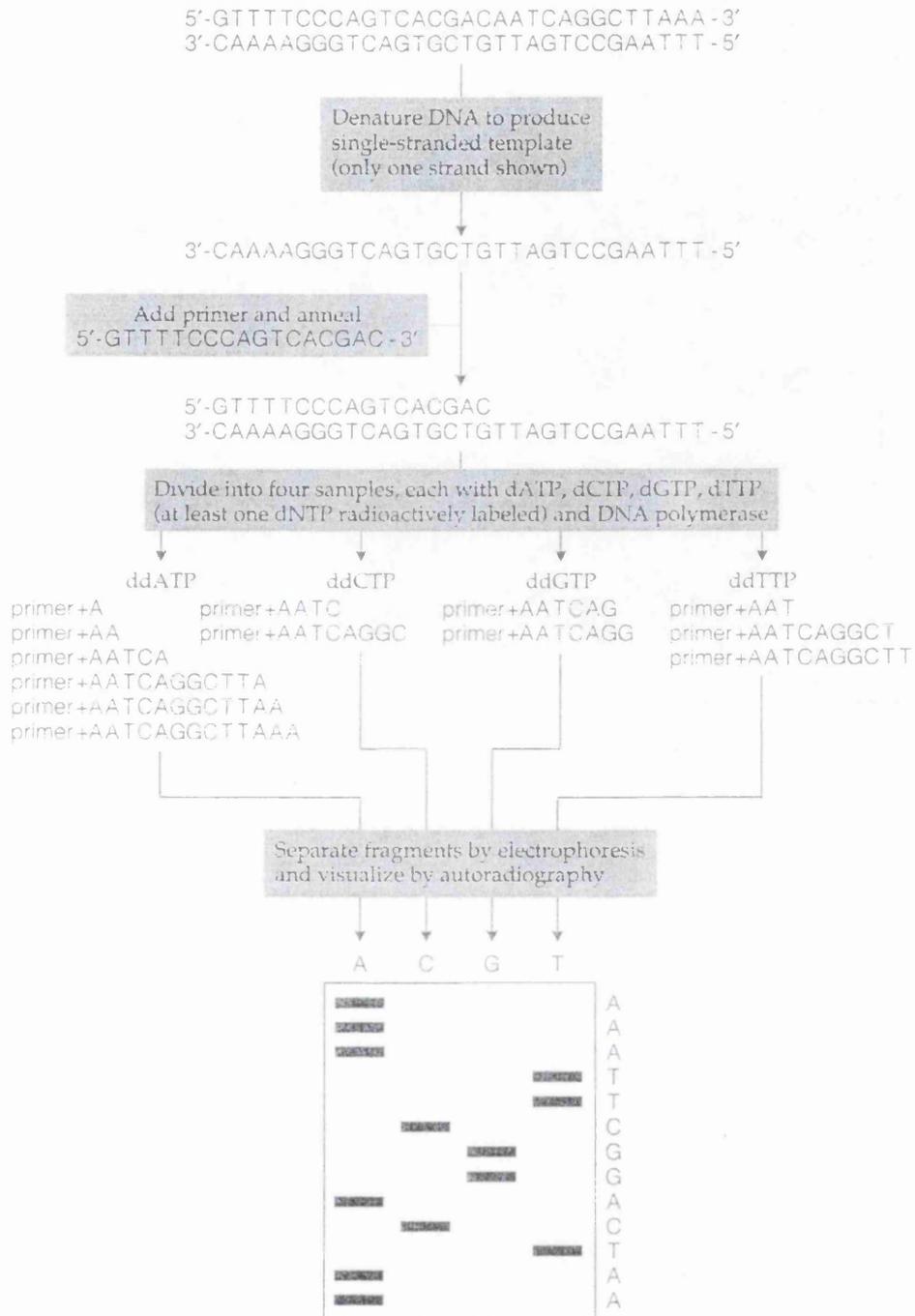
Figure 1.10: Sanger (enzymatic) sequencing. The DNA replication, uses dideoxynucleotide analogs in primer-directed DNA extension to produce discrete DNA fragments.(David *et al.*, 1996)

Cycle sequencing is an efficient method to screen for mutations compared with those methods, mentioned above. Fragments up to 300 bp in length can be searched for mutations. However, recent development in sequencing have given an opportunity to search for mutations more quickly and more cost-effectively.

This new technique is known as **automated sequencing**. There are a number of types of automated sequencing protocols, but the most common is Sanger sequencing with fluorescently labeled DNA fragments. The method involves the synthesis of a DNA strand by a DNA polymerase, using a single stranded DNA template. The synthesis is initiated at the site where a primer anneals to the template. Elongation is facilitated from the 3' end of the annealed primer by a DNA polymerase in the presence of dNTPs and is terminated by the incorporation of a ddNTP, which will not support continued DNA elongation. Since each ddNTPs are tagged with a different fluorescent label, (G=black, T=red, C=Blue and A=Green) the fluorescent sequencing instrument recognises the distinct label colours to give complete sequencing information. These fragments, which are invisible with the naked eye, are detected during the electrophoresis with the use of a tunable laser. The laser is stationary with respect to the electrophoresis apparatus, and fragments are recorded as they pass a single point. The system does not need autoradiography or manual recording of results. Instead, the sequence is recorded and analysed directly in a computer and can be printed out for further analysis.

The maintenance of this system is expensive, however, it can be very cost effective in the long run. It is also much quicker and

more reliable in comparison to manual sequencing that may take a week to have a result.

1.7. AIMS OF THIS STUDY

The aim of this project was to identify and characterise mutations in *BRCA1* in patients from the West of Scotland with ovarian cancer. Patients, who had one or more first or second degree relatives with breast or ovarian cancers, would be selected. Well-established mutation screening techniques such as single strand conformational polymorphism analysis (SSCP) and the protein truncation test (PTT) would be employed. Suspected mutations will be confirmed by automated DNA sequencing.

CHAPTER II

MATERIALS AND METHODS

2.1. PATIENTS

Forty-eight ovarian and breast-ovarian cancer patients with between one and six affected first or second degree relatives have been selected from patients attending the cancer clinic of the department of Medical genetics (table 2.1a,b). The range of onset for these patients is between 22 and 78 years of age and the median age of onset is 49.8 years. All those patients were screened for BRCA 1 gene using DNA as a template by SSCP and exon 11 was also screened by PTT.

2.2. EXTRACTION OF DNA FROM PERIPHERAL BLOOD CELLS

DNA from peripheral lymphocytes was isolated according to the method described in the PureGene Kit (REF). 10ml of whole blood was added to 30ml ice-cold red cell lysis solution (155mM Ammonium Chloride, 10mM Potassium Hydrogen Carbonate, 1mM EDTA). The mixture was incubated at room temperature for 15-20 minutes and inverted occasionally to mix. It was then centrifuged at 2000 r.p.m. for 5-10 minutes at room temperature. The supernatant was discarded and 10 ml of cell lysis solution (25mM EDTA, 2% SDS) were added to the pellet to fully lyse the cells. When the solution was homogenous, (and now stable at room temperature for at least 18 months) 3.5ml protein precipitation solution (10M Ammonium Acetate) was added and the mixture vortexed vigorously. It was then centrifuged at 3000 r.p.m. for 20 minutes at room temperature to pellet the proteins. The supernatant was transferred to a clean

tube containing 10ml isopropanol. It was mixed thoroughly by inversion to form a DNA precipitate. DNA was spooled out by using a sealed Pasteur pipette and washed in 70% ethanol. It was then air dried and dissolved in an appropriate volume (100-800μl) of TE buffer (10mM Tris/HCl pH 8.0, 1mM EDTA) and stored at -20°C until required.

2.3. DESIGN OF OLIGONUCLEOTIDE PRIMERS

The oligonucleotides used in this study for SSCP analysis have been published by Friedman et al. (Friedman et al., 1994) and those for PTT have been published by Hogervorst et al. (Hogervorst et al., 1995). Oligonucleotide primers were generally designed in the range of 18-24 bases except for those that were used for PTT analysis (56-58 bases). In order to screen the entire BRCA1 gene, there were 46 sets of primers (table 2.2-2.3-2.4) for SSCP analysis and to screen exon 11, there were 3 sets of PTT primers (table 2.5). Two oligonucleotide primers were produced for each polymerase chain reaction (PCR). The forward primer (F) was complementary to the sense strand and the reverse primer (R) was complementary to the antisense strand. For PTT, the forward primer contained a T7 primer with a promoter sequence and a sequence for the initiation of translation (Hogervorst et al., 1995).

All the oligonucleotides were synthesised on an APPLIED BIOSYSTEM (ABI) 391 DNA SYNTHESISER. Ammonium hydroxide was used to elute the primers from the solid support

and deprotection was carried out at 55°C for 12-16 hours. In order to purify the deprotected oligonucleotides, a 250μl primer aliquot was taken out and left in the fume hood to allow rapid evaporation of ammonia then left at -20°C until required. The bulk of the primers were stored in their ammonia solutions at -20°C until required.

Patient Sample No:	Ovarian Cancer (Age)	Breast Cancer (Age)	Other Cancers	Family Members (Num/Mean Age)
1	41	--	--	1/50
2	39	41	--	2/43
3	60	45	--	3/51
4	43	56	--	3/64
5	42	42	--	4/42
6	66	66	--	2/62
7	60	--	--	1/35
8	35	40	--	3/40
9	67	--	--	1/65
10	65	--	--	1/67
11	47	--	--	1/56
12	60	--	--	1/70
13	32	--	--	1/60
14	66	48	--	2/59
15	74	--	--	2/64
16	54	66	--	1/54
17	22	--	--	1/32
18	39	32	--	6/46
19	56	--	--	4/43
20	62	--	--	4/43
21	42	39	--	4/43
22	44	45	--	2/37

Table 2.1a: Characteristic of ovarian cancer patients (patients no, 1-22) who have been tested for *BRCA1* mutations in this study.

Patient Sample No:	Ovarian Cancer (Age)	Breast Cancer (Age)	Other Cancers	Family Members (Num/Mean Age)
23	45	33	--	2/40
24	54	58	--	1/54
25	45	--	--	1/30
26	56	43	--	1/40
27	69	--	--	1/40
28	48	46	--	5/47
28	78	61	--	4/53
30	63	59	63	2/75
31	44	--	--	3/39
32	63	--	--	2/67
33	66	--	--	2/51
34	40	--	--	1/47
35	60	--	59	1/63
36	43	--	--	2/55
37	24	--	--	1/60
38	49	--	--	3/74
39	52	--	--	4/81
40	25	--	--	1/70
41	50	--	--	2/52
42	49	--	--	2/46
43	28	--	--	3/56
44	26	--	--	2/35
45	38	55	--	2/45
46	59	--	--	5/42
47	48	--	--	3/42
48	56	48	--	1/53

Table 2.1b: Characteristic of ovarian cancer patients (patients no, 23-48) who have been tested for *BRCA1* mutations in this study.

Primer Pair	Size (bp)	DNA for amplification	Forward Primer	Reverse Primer
Exon 1	315	Genomic	5'-TAG CCC TTG GTT TCC GTG-3'	5'-TCA CAA CGC CTT ACG CCT C-3'
Exon 2	250	Genomic	5'-GAA GTT GTC ATT TTA TAA ACC TTT-3'	5'-TGT CTT TTC TTC CCT AGT ATG T-3'
Exon 3	300	Genomic	5'-TCC TGA CAC AGC AGA CAT TTA-3'	5'-TTG GTA TTT CGT TCT CAC TTA-3'
Exon 4	200	Genomic	5'-GTC AAA GAG ATA GAA TGT GAG C-3'	5'- CCC GTC TCT ACA GAA ACC AC-3'
Exon 5	200	Genomic	5'-CTC TTA AGG GCA GTT GTG AG-3'	5'- TTC CTA CTG TGG TTG CTT CC-3'
Exon 6	200	Genomic	5'-CTT ATT TTA GTG TCC TTA AAA GG-3'	5'- TTT CAT GGA CAG CAC TTG AGT G-3'
Exon 7	250	Genomic	5'-CAC AAC AAA GAG CAT ACA TAG GG-3'	5'-TCG GGT TCA CTC TGT AGA AG-3'
Exon 8	220	Genomic	5'-TGT TAG CTG ACT GAT GAT GGT-3'	5'-ATC CAG CAA TTA TTA TTA AAT AC-3'
Exon 9	200	Genomic	5'-CCA CAG TAG ATG CTC AGT AAA TA-3'	5'-TAG GAA AAT ACC AGC TTC ATA GA-3'
Exon 10	220	Genomic	5'-TGG TCA GCT TTC TGT AAT CG-3'	5'-GTA TCT AAC CAC TCT CTT CTT CAG-3'

Table 2.2: The 10 primer pairs were used for SSCP analysis of exons 1-10 (Friedman *et al.*, 1994).

Primer Pair	Size (bp)	DNA or cDNA for amplification	Forward Primer	Reverse Primer
Exon 11Ai	301	Genomic	5'-GGA ATT AAA TGA AAG AGT ATG AGC-3'	5'-CTT CCA GCC CAT CTG TTA TGT TG-3'
Exon 11A	309	Both	5'-AAC ACC ACT GAG AAG CGT GCA G-3'	5'-CTC ACA CAG GGG ATC AGC ATT C-3'
Exon 11B	300	Both	5'-CAA CAT AAC AGA TGG GCT GGA AG-3'	5'-ACG TCC AAT ACA TCA GCT ACT TTG G-3'
Exon 11B2	209	Both	5'-CCT AGA GAT ACT GAA GAT GTT CCT TGG-3'	5'-GCC AGT AAG TCT ATT TTC TCT GAA GAA CC-3'
Exon 11C	295	Both	5'-GGT TCT GAT GAC TCA CAT GAT GGG-3'	5'-TCT GTG GCT CAG TAA CAA ATG CTC-3'
Exon 11D	254	Both	5'-GAA AAC CTA TCG GAA GAA GGC AAG-3'	5'-TCA TCA CTT GAC CAT TCT GCT CC-3'
Exon 11D2	272	Both	5'-GAG CCA CAG ATA ATA CAA GAG CGT C-3'	5'-GCA GAT TCT TTT TCG AGT GAT TCT ATT GGG-3'
Exon 11E	269	Both	5'-ATC AGG GAA CTA ACC AAA CGG AG-3'	5'-CGC ATG AAT ATG CCT GGT AGA AG-3'
Exon 11E2	410	Both	5'-TCA GGG AAC TAA CCA AAC GGA G-3'	5'-CCA TGA GTT GTA GGT TTC TGC TG-3'
Exon 11F	273	Both	5'-AGG CTG AGG AGG AAG TCT TCT ACC-3'	5'-CAG CTC TGG GAA AGT ATC GCT G-3'
Exon 11 G	319	Both	5'-GCA ACT GGA GCC AAG AAG AGT AAC-3'	5'-CCT GAG TGC CAT AAT CAG TAC CAG G-3'
Exon 11 H	312	Both	5'-CAG CGA TAC TTT CCC AGA GCT G-3'	5'-TCT GTT TTT GCC TTC CCT AGA GTG-3'
Exon 11H2	286	Both	5'-AAG TGT CTA ATA ATG ATG AAG ACC CC-3'	5'-CCC AAT GGA TAC TTA AAG CCT TCT G-3'
Exon 11I	280	Both	5'-GCA CTC TAG GGA AGG CAA AAA CAG-3'	5'-CAT TCC TCT TCT GCA TTT CCT GG-3'
Exon 11I2	333	Both	5'-GAA GGC TTT AAG TAT CCA TTG GG-3'	5'-CTT ATC TTT CTG ACC AAC CAC AGG-3'
Exon 11J	288	Both	5'-GCC AGT CAT TTG CTC CGT TTT C-3'	5'-CGT TGC CTC TGA ACT GAG ATG ATA G-3'
Exon 11K	305	Both	5'-TGC AGG CTT TCC TGT GGT TG-3'	5'-GGC TAA TTG TGC TCA CTG TAC TTG G-3'
Exon 11K2	296	Both	5'-GCA ACG AAA CTG GAC TCA TTA CTC-3'	5'-AAT ACT GGA GCC CAC TTC ATT AGT AC-3'
Exon 11L	270	Both	5'-TCA ATG TCA CCT GAA AGA GAA ATG G-3'	5'-CAG GAT GCT TAC AAT TAC TTC CAG G-3'
Exon 11M	270	Both	5'-TTG AAT GCT ATG CTT AGA TTA GGG G-3'	5'-GAC GCT TTT GCT AAA AAC AGC AG-3'
Exon 11N	253	Both	5'-GTT TGT TCT GAG ACA CCT GAT GAC C-3'	5'-AGT GTT GGA AGC AGG GAA GCT C-3'
Exon 11O	289	Both	5'-GAG TCC TAG CCC TTT CAC CCA TAC-3'	5'-GTG ATG TTC CTG AGA TGC CTT TG-3'
Exon 11P	314	Both	5'-CGT TGC TAC CGA GTG TCT GTC TAA G-3'	5'-AGC CCG TTC CTC TTT CTT CAT C-3'
Exon 11Pi	200	Genomic	5'-AAA GCC AGG GAG TTG GTC TGA G-3'	5'-GTG CTC CCA AAA GCA TAA A-3'

Table 2.3: The 24 primer pairs were used for SSCP analysis (Friedman *et al.*, 1994).

Primer Pair	Size (bp)	DNA for amplification	Forward Primer	Reverse Primer
Exon 12	220	Genomic	5'-GTC CTG CCA ATG AGA AGA AA-3'	5'-TGT CAG CAA ACC TAA GAA TGT-3'
Exon 13	280	Genomic	5'-AAT GGA AAG CTT CTC AAA GTA-3'	5'-ATG TTG GAG CTA GGT CCT TAC-3'
Exon 14	250	Genomic	5'-CTA ACC TGA ATT ATC ACT ATC A-3'	5'-GTG TAT AAA TGC CTG TAT GCA-3'
Exon 15	250	Genomic	5'-TGG CTG CCC AGG AAG TAT G-3'	5'-AAC CAG AAT ATC TTT ATG TAG GA-3'
Exon 16	375	Genomic	5'-AAT TCT TAA CAG AGA CCA GAA C-3'	5'-AAA ACT CTT TCC AGA ATG TTG T-3'
Exon 17	350	Genomic	5'-GTG TAG AAC GTG CAG GAT TG-3'	5'-TCG CCT CAT GTG GTT TTA-3'
Exon 18	350	Genomic	5'-GGC TCT TTA GCT TCT TAG GAC-3'	5'-GAG ACC ATT TTC CCA GCA TC-3'
Exon 19	220	Genomic	5'-CTG TCA TTC TTC CTG TGC TC-3'	5'-CAT TGT TAA GGA AAG TGG TGC-3'
Exon 20	220	Genomic	5'-ATA TGA CGT GTC TGC TCC AC-3'	5'-GGG AAT CCA AAT TAC ACA GC-3'
Exon 21	275	Genomic	5'-AAG CTC TTC CTT TTT GAA AGT C-3'	5'-GTA GAG AAA TAG AAT AGC CTC T-3'
Exon 22	275	Genomic	5'-TCC CAT TGA GAG GTC TTG CT-3'	5'-GAG AAG ACT TCT GAG GCT AC-3'
Exon 23	250	Genomic	5'-CAG AGC AAG ACC CTG TCT C-3'	5'-ACT GTG CTA CTC AAG CAC CA-3'
Exon 24	275	Genomic	5'-ATG AAT TGA CAC TAA TCT CTG C-3'	5'-GTA GCC AGG ACA GTA GAA GGA-3'

Table 2.4: The 13 primer pairs were used for SSCP analysis in exons 12-24 (Friedman *et al.*, 1994).

NAME OF THE PRIMER	FORWARD PRIMER WITH INITIATION SEQUENCE	REVERSE PRIMER
EXON 11-A 793-2125	GCTAATACGACTCACTATAGGAACAGACCACCATGG AACACTTAAAAGACTCTGCC	TACTCAACATCCAAAGACGACA
EXON 11-B 1921-3383	GCTAATACGACTCACTATAGGAACAGACCACCATGG TGTTAAGTTTTCGTGGATTTTTC	TTGGGGATTAGATTCGTATCGTAAG
EXON 11-C 3061-4183	GCTAATACGACTCACTATAGGAACAGACCACCATGG GTGGTGAAAAAGGGGTAGTTCAG	AATAAAAAGAAGGTTCGGGCAAGG

Table 2.5: Protein Truncation Test primers. Forward primers have T7 promoter and initiation sequences for translation (Hogervorst *et al.*, 1995).

2.4. THE POLYMERASE CHAIN REACTION (PCR) USING GENOMIC DNA AS A TEMPLATE

2.4.1. Polymerase Chain Reaction for SSCP

Polymerase chain reactions were performed by using a PCR Beads "ready-to-go" kit (Amersham Pharmacia Biotech). In this kit, dried PCR Beads were provided that were stable at room temperature and each bead contained all of the necessary reagents for performing a $25\mu l$ PCR amplification reaction. Beads were pre-dispensed in 0.5ml thin-walled tubes.

Each reaction contained 1.5 units of *taq* DNA polymerase, 10mM Tris-HCl (ph 9.0 at room temperature), 50mM KCl, 1.5mM $MgCl_2$, $200\mu M$ of each dNTP, 30-50pmol of each primer and $0.1\mu g$ genomic DNA. Double distilled water was used to bring the volume to $25\mu l$. The concentration of primers and DNA was optimized to get the best possible PCR result.

The cycles of PCRs were generally carried out as follows; two minutes at 95°C for one cycle (initial denaturation) followed by one minute at 95°C (denaturation), one minute at 58°C (primer annealing) and one minute at 72°C (primer extension) for 30 cycles. The final extension was 7 minutes at 72°C for one cycle (table 2.6).

INITIAL DEANTURATION	DENATURATION	ANEALING	EXTENTION	FINAL EXENSION
95°C, 2 MIN	95°C, 1 MIN	58°C, 1 MIN	72°C, 1 MIN	72°C, 7 MIN
1 CYCLE	30 CYCLES	30 CYCLES	30 CYCLES	1 CYCLE

Table 2.6: PCR conditions for SSCP

PCR products were visualized by 1% agarose gel analysis, ethidium bromide staining and transillumination using UV light.

2.4.2. Polymerase Chain Reaction for PTT

Polymerase chain reactions were performed by using a PCR Beads "ready-to-go" kit (Amersham Pharmacia Biotech). Total reaction volume was designed to be $50\mu l$. Therefore two PCR beads were used in one 0.5ml thin-walled tube.

Each reaction contained 3 units of *taq* DNA polymerase, 20 mM Tris-HCl (ph 9.0 at room temperature), 100mM KCl, 1.5mM $MgCl_2$, $400\mu M$ of each dNTP, 60-100pmol of each primer and $0.2\mu g$ genomic DNA. The ddH_2O was used to bring the volume to $50\mu l$. The concentration of primers or DNA was optimized to produce the best possible PCR result.

The PCR conditions were set as follows; two minutes at 95°C for one cycle (initial denaturing) followed by one minute at 95°C (denaturation), one minute at 57°C (primer annealing) and two minute at 72°C (primer extension) for 35 cycles. The final extension was six minutes at 72°C for one cycle (table 2.7). PCR products were visualized by 1% agarose gel electrophoresis, ethidium bromide staining and transillumination using a UV light.

INITIAL DEANTURATION	DENATURATION	ANEALING	EXTENTION	FINAL EXENSION
95°C, 2 MIN	95°C, 1 MIN	57°C, 1 MIN	72°C, 2 MIN	72°C, 6 MIN
1 CYCLE	35 CYCLES	35 CYCLES	35 CYCLES	1 CYCLE

Table 2.7: PCR conditions for PTT

2.5. IDENTIFICATION OF PCR PRODUCTS

Products were visualized by mixing $5\mu l$ of PCR product with $2\mu l$ of loading mix (0.03% bromophenol blue, 0.03% xylene cyanol, 0.4% Orange G, 10mM Tris-HCl (pH 7.5) 15% Ficoll® 400 (Promega cat no. G1741) and loading on a 1% agarose gel containing ethidium bromide. A 1% agarose gel was prepared by adding 0.3-0.6g agarose powder (GIBCO BRL life technologies cat no. 15510-027) in 30-60ml 1xTAE buffer (depending on the size of the gel). The mixture was boiled and cooled down to 60°C. Ethidium bromide ($4\mu l$ from 10mg/ml. Sigma cat no. E1510) was added to the solution and the gel poured into a gel tray. The samples were loaded on the gel and electrophoresis at a constant 100 volts for 30 minutes. The results were then visualized using a standard UV transilluminator (Fotodyne, UV-transilluminator) and printed using a gel documentation (UVP Imagestor 5000) system.

2.6. PURIFICATION OF PCR PRODUCTS

At the end of the PCR, the amplified DNA was purified by removing the excess primers, dNTPs, and salts using different techniques as follows.

2.6.1. GFX^{TM} PCR DNA and Gel Band Purification Kit

A GFX^{TM} DNA purification kit uses a chaotropic agent that denatures protein, dissolves agarose and promotes the binding of double-stranded DNA to a glass fiber matrix. This kit was used to clean double-stranded DNA of over 100 bp in size.

Capture buffer (buffered acetate and chaotrope), wash buffer (Tris-EDTA buffer and 48ml 80% ethanol), GFX^{TM} columns (microspin columns pre-packed with a glass fiber matrix), and collection tubes (2ml capless microcentrifuge tubes) were supplied with the kit. The maximum volume of PCR product that could be processed with it was 100μl.

For the cleaning procedure, each GFX^{TM} column was placed in a collection tube. 500μl capture buffer was added to the GFX^{TM} column and the mineral oil-free PCR product was mixed into the solution by pipetting the sample up and down 4-6 times. The mixture was then centrifuged in a microcentrifuge at 12000 r.p.m. for 30 seconds. The residue was discarded from the collection tube and the GFX^{TM} column was replaced into it. 500μl of wash buffer was added in to the column and centrifuged in a microcentrifuge at 12000 r.p.m. for 30 seconds. The collection tube was discarded and the GFX^{TM} column transferred to a fresh 1.5ml microcentrifuge tube. The required amount of double-distilled water (25-50μl) was directly applied to the top of the glass fiber matrix in the GFX^{TM} column without touching it. The concentration of the final product could be produced at this stage by adding less or more ddH$_2$O. After incubating the sample at room temperature for one minute, a final centrifuge stage was carried out at 12000 r.p.m. for one minute. Recovered DNA was then collected from the microcentrifuge tube and stored for further usage.

2.6.2. Isopropanol Precipitation of PCR Products

This method was used to remove excess dNTPs, salts and primers from PCR products for PTT analysis. Mineral oil-free PCR product was added to one volume of 4M ammonium acetate and two volumes of isopropanol into a 1.5ml microcentrifuge tube and left on ice for 10 minutes. The mixture was then centrifuged at 12000 r.p.m. for 20 minutes. The supernatant was discarded and the DNA pellet was twice washed in 70% ethanol and briefly centrifuged. After discarding the ethanol, the pellet was air-dried and dissolved in an appropriate amount of Rnase-free (DEPC treated) water for further usage.

2.7. METHODS FOR MUTATION DETECTION

2.7.1 Mutation Detection by Single Strand Conformational Polymorphism Analysis (SSCP)

This technique allows the detection of single base changes in short DNA fragments, preferably under 300 bp, based on mobility differences of single stranded molecules. DNA fragments differing in sequence by as little as 1 bp will have different three-dimensional structure due to the alteration of the intra molecular interactions. DNA fragments with different three-dimensional structures may move at a different rate to each other when electrophoresis through mutation detection gels

such as non-denaturing polyacrylamide or a mutation detection enhancement (MDE™) gels.

SSCP analysis was used to screen all *BRCA1* exons 1 to 24. All the *BRCA1* genes thought to contain mutations were amplified by using polymerase chain reaction as described before. PCR products were then heated to generate single strands and fractionated on MDE™ gels. Silver staining was used for visualizing and detecting the product.

2.7.1.1. Gel Preparation and Pouring

The "Protean®II xi Cell" sequencing gel apparatus (Bio-Rad cat no. 165-1815) with 0.5mm spacers and comb was used for mutation detection of PCR products.

The 20cm X 20cm glass back plate and 16cm X 20cm glass front plate were cleaned by detergent, then washed and dried with ethanol. Dust and dirt free plates were assembled according to the manufacturer's instructions. The bottom of the plates were sealed using the sealing apparatus.

The gel was prepared using MDE™ gel solution (FMC Bioproducts, cat no. 50620) as follows: 10ml MDE™ gel solution, 4.8ml 5X TBE buffer, 2ml glycerol and 23.2ml distilled water were mixed. Then $50\mu l$ TEMED (N,N,N',N'-Tetramethyl-ethylenediamine, Sigma cat no. T-8133) and freshly prepared 10% ammonium per sulphate (APS) were added to the mixture and mixed well. The mixture was then poured immediately between two glass plates and the 20 well comb was inserted. The gel was allowed to polymerase for one

hour. The sealing apparatus and the well forming comb were removed after the gel was set.

2.7.1.2 Preparation of the samples

$4\mu l$ of PCR was added to $6\mu l$ of SSCP loading mix (95% formamide, 10mM sodium hydroxide, 0.5% bromophenol blue and 0.05% xylene cyanol) and heated for 5 minutes at 95°C. The sample was then cooled on ice for at least 2 minutes and $5\mu l$ of sample was loaded on to the MDE$^{\text{TM}}$ gel.

2.7.1.3 Running conditions of the gel

The gel was placed into the gel tank and sealed. The buffer chambers were filled with 0.6X TBE buffer and the wells were washed with buffer. The samples were then loaded on to the gel and electrophoresis was carried out at 4°C for 16-20 hours at a constant 140 volts.

2.7.1.4 Silver staining of the gel

Following electrophoresis, the gel was removed from the glass plates and placed in a staining dish. The gel was then fixed in a fixation solution (10% ethanol, 0.5% acetic acid) for 10 minutes at room temperature. The fixation solution was poured out and the gel was stained with cold 0.1% silver nitrate solution for 15 minutes (Sigma cat no. S-6506) followed by twice rinsing with distilled water. The gel was then developed with a solution of 1.5% NaOH and 0.1% formaldehyde (both made from 10X stock) for 20 minutes (or until bands were clearly visible) at room temperature. After pouring the developing solution, the

gel was fixed with a solution of 0.75% $NaCO_3$ (made from 10X stock) for 10 minutes. The gel was then covered by a sheet of plastic paper and its image recorded.

2.7.2 Mutation Detection by Protein Truncation Test

The protein truncation test (PTT) is method for detecting nonsense or frameshift mutations that create a stop codon and eventually truncated proteins. In this study, PTT has been performed for exon 11 by 3 overlapping segments because only genomic DNA was available. In this protocol, PCR was performed using forward primers containing the T7 promoter and a eukaryotic translation initiation sequence (Table2.5). Protein products were sysnthesised by using the TNT$^®$ Lysate Coupled Transcription/Translation System (Promega cat no. L4610).

2.7.2.1 Preparation of the forward primer with T7 promoter and target DNA

Because of the absence of T7 promoter and translation initiation sequences for the target DNA segments, they had to be added to the 5' end of the forward primer to generate PCR products suitable for PTT analysis. The T7 promoter and initiation sequences, published by Hogervorst *et al.*(Hogervorst *et al.*, 1995) are presented below:

GCTAATACGACTCACTATAGGAACAGACCACCATGG

As previously described, a 50μl PCR reaction was performed using a forward primer containing the T7 promoter and translation initiation sequences at the 5' end. After confirming

the size and the quality of the product produced, PCR products were purified using isopropanol precipitation as described section 2.6.2.

2.7.2.2 TNT® Lysate Coupled Transcription/Translation System

The kit used in this experiment allowed translation and transcription in one Rnase-free reaction tube. All experiments were employed at 1/2 scale of manufacturer's instructions to save material. ^{35}S-methionine was used in this procedure as the radioactive material. The reactions were carried out as follows: 12.5μl TNT® Lysate, 1μl TNT® reaction buffer, 0.5μl TNT® RNA polymerase, 0.5μl amino acid minus methionine, 2μl ^{35}S-methionine, 0.5μl RNasin (ribonuclease inhibitor 40u/μl), and 8μl DNA in DEPC treated water. This mixture was then incubated at 30°C for 60-120 minutes.

2.7.2.3 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using a Protean®II xi Cell kit. The apparatus was assembled according to the manufacturer's instruction. The SDS-PAGE was composed of two different sections, stacking and separation gel. A 16cm separating gel which was 8.5%-17.5% depending on the size of translated product and containing separating gel buffer (18.17g Tris base and 4ml 10% Sodium Dodecyl Sulphate) was made by mixing the reagents shown in table 2.8, without forming air bubbles. The gel was then poured into the space between glass plates, leaving 4cm for the stacking gel.
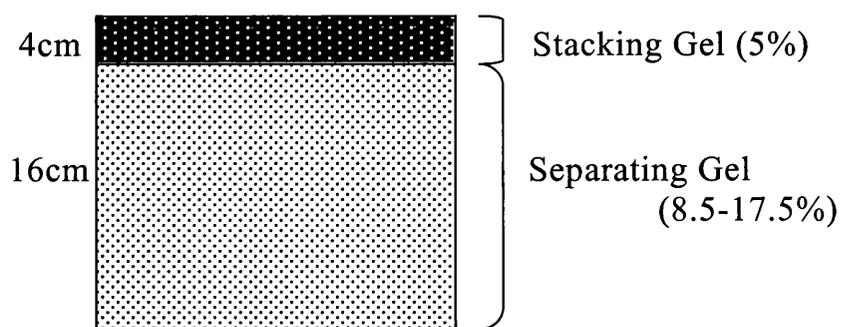
Figure 2.1. Schematic diagram of SDS-PAGE.

The gel was overlaid with 0.1% SDS solution to ensure a flat surface. After the gel was set, the SDS was washed off with water. A 4cm stacking gel which was always 5% in concentration (table 2.9), contained stacking gel buffer (6.06g Tris base and 4ml 10% SDS) was then made by mixing the reagents shown table 2.9, taking care not to form air bubbles.

COMPONENTS	8.5%	10%	12.5%	15%	17.5%
ddH$_2$O	16.85ml	14.95ml	11.85ml	8.7ml	5.6ml
Acrylamide Sol. 30%	10.65ml	12.55ml	15.65ml	18.8ml	21.9ml
Separating gel buffer	9.5ml	9.5ml	9.5ml	9.5ml	9.5ml
10% APS	560μl	560μl	560μl	560μl	560μl
TEMED	25μl	25μl	25μl	25μl	25μl

Table 2.8: Formulation of the SDS-Polyacrylamide separating gel

COMPONENT	VOLUME
ddH$_2$O	5.0ml
Acrylamide Sol. 30%	2.22ml
Stacking gel buffer	1.5ml
10% APS	140μl
TEMED	25μl

Table 2.9: **Formulation of the stacking gel** (5% acrylamide)

The stacking gel was then poured onto the top of the separating gel. A 1mm comb was inserted and the gel allowed to set. The apparatus was assembled and the gel tank was filled with 1X SDS polyacrylamide running buffer (made from 10X stock, 30g Tris base, 144g glycine and 100ml 10% SDS).

2.7.2.4 Sample preparation

A 5μl aliquot from the transcription/translation reaction was diluted with 20μl of SDS sample buffer (2.0ml glycerol, 2.0ml 10% SDS, 0.25mg bromophenol blue, 2.5ml stacking gel 4X buffer, 0.5ml β-mercaptoethanol, and ddH2O to a final volume of 10ml), and mixed well. The mixture was then boiled for 3 minutes to denature the protein. The remainder of the transcription/translation product was stored at -20°C for future usage. The sample was then loaded (5-7μl) on to the gel and run for 5-6 hours at constant 350 volts. In order to determine the

size of the product, *5µl* of the standard protein size marker (Rainbow marker, Amersham) was also loaded on to the gel.

2.7.2.5 Visualising the product

After the electrophoresis was finished, the gel was transferred to a staining tray and fixed for 20 minutes (the fixation solution was isopropanol, water and acetic acid in the proportions of 25:65:10 respectively). The fixing solution was poured out and the gel was soaked in an amplify solution (Amersham cat no. NAMP 100) for 20 minutes. The gel was then transferred to a 3mm Whatman paper, covered with Saran Wrap and dried in the vacuum dryer at 60°C for 45-60 minutes. The gel was then exposed for 2-4 days to X-ray film and the film developed according to the manufacturer's instruction.

2.7.3. Expand Long Template PCR analysis

This analysis was employed to analyse DNA fragment that were too large for conventional PCR. It allows genomic DNA fragments of up to 27 kb to be amplified.

Polymerase chain reaction was performed by using Expand® Long Template PCR system (Roche® cat no: 1-681-834). This system was composed of an enzyme mix, containing a thermostable Taq DNA polymerase and a proofreading polymerase.

Each reaction contained 0.75µl of enzyme mixture, 17.5 mM $MgCl_2$, 350µl, 300nM of each primer and 500ng of genomic DNA (Table 2.11). The forward and reverse primers, 5'-CCATACACATTTGGCTCAGGGTTACCGAAGAGGG-3'

5'TTCGCAGGTCCTCAAGGGCAGAAGAGTCAC-3'

respectively were published by Payne *et al.* (2000). The PCR conditions were set as follows; two minutes at 93°C for one cycle (initial denaturing) followed by ten seconds at 93°C (denaturation), 30 seconds at 65°C (primer annealing) and eight minutes at 68°C (primer extension) for 30 cycles. The final extension was seven minutes at 72°C for one cycle (table 2.10).

INITIAL DEANTURATION	DENATURATION	ANEALING	EXTENTION	FINAL EXENSION
93°C, 2 MIN	93°C, 10 s	65°C, 30 s	68°C, 8 MIN	68°C, 7 MIN
1 CYCLE	30 CYCLES	30 CYCLES	30 CYCLES	1 CYCLE

Table 2.10: PCR conditions for Expand long template PCR analysis.

The products were visualized by 0.7% agarose gel electrophoresis, ethidium bromide staining and transillumination using UV light.

Amp. of Hum. Gen. DNA

Component	0.5-12 kb Volume	System 1 Final concentr.	12-15 kb Volume	System 2 Final Concentr.	>15 kb Volume	System 3 Final Concentr.
ddH₂O		Up to 25 µl		Up to 25 µl		Up to 25 µl
DATP, 10mM	1.75 µl	350 µM	2.5 µl	500 µM	2.5 µl	500 µM
DCTP, 10mM	1.75 µl	350 µM	2.5 µl	500 µM	2.5 µl	500 µM
DGTP, 10mM	1.75 µl	350 µM	2.5 µl	500 µM	2.5 µl	500 µM
DTTP, 10mM	1.75 µl	350 µM	2.5 µl	500 µM	2.5 µl	500 µM
Downstream Primer		300nM		300nM		300nM
Upstream Primer		300nM		300nM		300nM
Template DNA		500ng		500ng		500ng

Master mix 1

Amp. of Hum. Gen. DNA

Component	0.5-12 kb Volume	System 1 Final concentr.	12-15 kb Volume	System 2 Final Concentr.	>15 kb Volume	System 3 Final Concentr.
ddH₂O		Up to 25 µl		Up to 25 µl		Up to 25 µl
PCR buffer with MgCl₂	5 µl	**Buffer 1,** 1.75mM		**Buffer 2,** 2.25mM		**Buffer 3,** 2.25mM
Enzyme mix	0.75 µl		0.75 µl		0.75 µl	

Master mix 2

Table 2.11: Amplification of human genomic DNA, using Expand® Long Template PCR System. Two master mixes were prepared to avoid the contamination between enzyme mix and primers or template without dNTPs (manufacturer's instruction manual). The system 1 was used in this study.

2.7.4. Nucleic Acid Sequencing

The samples, which gave positive screening results, were subjected to sequencing of the PCR products from the corresponding DNA segment(s) to detect any sequence alterations. Automated sequencing of the PCR products was carried out using the "Thermo sequenase II dye terminator cycle sequencing premix kit" (Amersham cat no. US80975).

2.7.4.1. Preparation of sequencing reaction

A $25\mu l$ PCR reaction of DNA was carried out as described in section 2.4.1. $4\mu l$ of PCR product was resolved in a 1% agarose gel to check for quality and quantity of PCR product. It was then purified using the GFX^{TM} DNA purification kit as described in section 2.6.1. The purified PCR product was used for cycle sequencing and carried out as follows:

For the reaction, $15\mu l$ of DNA template (diluted in ddH$_2$O to 80-800fmol) was mixed with $1\mu l$ of one of the initial primers set ($5\mu M$ forward or reverse) and $4\mu l$ of Thermo sequenase II reagent premix. The cycling program for sequencing was carried out as follows:

Two minutes at 96°C for one cycle (initial denaturing) followed by 30 seconds at 96°C (denaturation), 15 seconds at 50°C (primer annealing) and four minute at 60°C (primer extension) for 30 cycles.

2.7.4.2. Purification of the product

At the end of the thermal cycling, the $20\mu l$ of product was removed and $2\mu l$ of Sodium acetate/EDTA buffer and $60\mu l$ of 100% cold (-20°C) ethanol was added. The sample was then vortexed and placed on ice for 15 minutes to precipitate the DNA. The sample was then centrifuged at 12000 r.p.m. for 20 minutes at room temperature. The supernatant was poured off and the pellet was washed with $350\mu l$ of cold (-20°C) 70% ethanol. It was then vortexed and centrifuged again at 12000 r.p.m. for 3 minutes at room temperature. The supernatant was removed and the sample was air-dried.

2.7.4.3. Preparation of the sequencing gel

A 7.5% sequencing gel was prepared as follows:

18g urea was mixed with 18.5ml of ddH$_2$O, 10ml 5X TBE buffer and 7.5 ml 50% long ranger gel solution (Flowgen). The mixture was warmed to 55°C to dissolve the urea. The mixture was then cooled off before the addition of $250\mu l$ of 10% APS and $35\mu l$ TEMED.

Prior to pouring the gel, the glass plates were cleaned thoroughly and assembled as instructed in the ABI manual. The gel was then poured into the glass plates without forming bubbles and the flat comb was inserted. The gel was left for at least two hours to set.

2.7.4.4. The running of the sequencing reaction on the automated sequencer

After the gel was set, the plates were placed in the ABI Prism 373 automated sequencing machine following the instruction manual. A "plate check" procedure was performed to ensure that the plates were dirt free where the laser scanned. If such artefacts were observed, the area was cleaned with a tissue until the scanned area was totally clean. The flat comb was removed and the 24 well comb was inserted. The gel was then pre-run in the 1X TBE buffer for 10 minutes. $4\mu l$ of gel loading mix was added to the air-dried pellet. The sample was heated to 70°C for 3 minutes and placed on ice. The sample was then loaded on to the gel.

2.7.4.5. Analysis of the result

The result obtained from each well were analysed on the Macintosh computer attached to the PRISM 373 DNA system, using the 373A software (ABI). The sequencer, which has a fluorescence detection system, sent the collected data to the computer. The computer processed the data and presented it as a chromatogram with four coloured peaks (G=black, T=red, C=Blue and A=Green). Heterozygosity was represented by two superimposed peaks. Thus, the computer could not call the base and gave it the designation "N".

CHAPTER III

RESULTS

3.1. THE STRATEGY FOR AMPLIFICATION OF THE *BRCA1* CODING SEQUENCE:

The entire *BRCA1* coding sequence was amplified using genomic DNA. For this purpose, 47 sets of primers for SSCP analysis (tables 2.2, 2.3, 2.4) and 3 sets of primers (table 2.5) for PTT were employed for PCR amplification.

3.1.1. Amplification of the *BRCA1* Gene Using Genomic DNA for SSCP Analysis

Because of the nature of the *BRCA1* gene, each exon could be covered by one set of primers giving sizes between 200 bp and 375 bp, except exon 11. Its size is almost 3.4 kb, and it was amplified by 24 sets of primers giving sizes between 200 bp and 400 bp.

The optimisation of each PCR reaction was carried out according to the subject DNA size, primers size and G-C contents. The PCR reaction employed for SSCP is described in section 2.4.1. The amplified product was always electrophoresed in a 1% agarose gel to check the integrity of the PCR product. An example of amplified product from patients DNA using primer set 11N is shown in figure 3.2.

3.1.2. Amplification of the *BRCA1* Gene Using Genomic DNA for PTT

Amplification of exon 11 of the *BRCA1* gene was carried out by three overlapping fragments, using primers described in table 2.5 and genomic DNA as template.
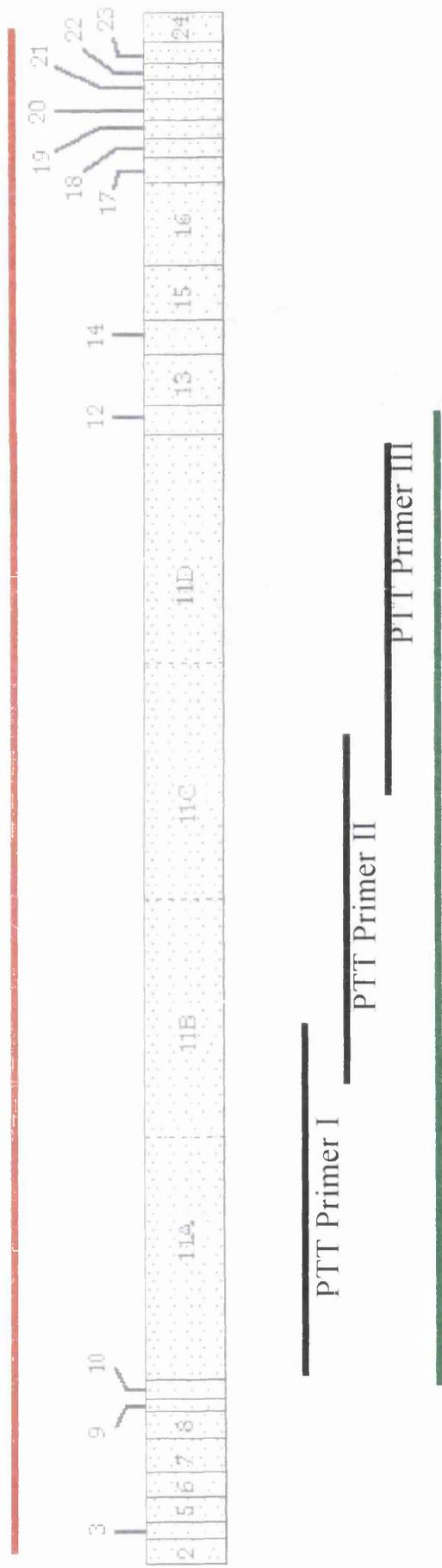
Figure 3.1: Diagrammatic representation of the *BRCA1* sequence. The filled boxes with numbers represent the coding exons from 2 to 24. Red line represents the regions amplified SSCP analysis using primer sets shown table 2.2-4. Three black lines represent the regions amplified for PTT from genomic DNA. Green line represents the region which amplified for Expand Long Template PCR analysis amplifying exon 11 and 12 and Intron 11 (http://www.nhgri.nih.gov/intramural_research/Lab_transfer/BIC). Exon 11 was divided 4 sections to simplify to database search by BIC.

All forward primers contained a T7 promoter and eukaryotic translation initiation sequence in order to generate a PCR product suitable for PTT analysis. The amplified product was always electrophoresed in a 1% agarose gel to check the integrity of the PCR product and examples are shown in figures 3.3-5.
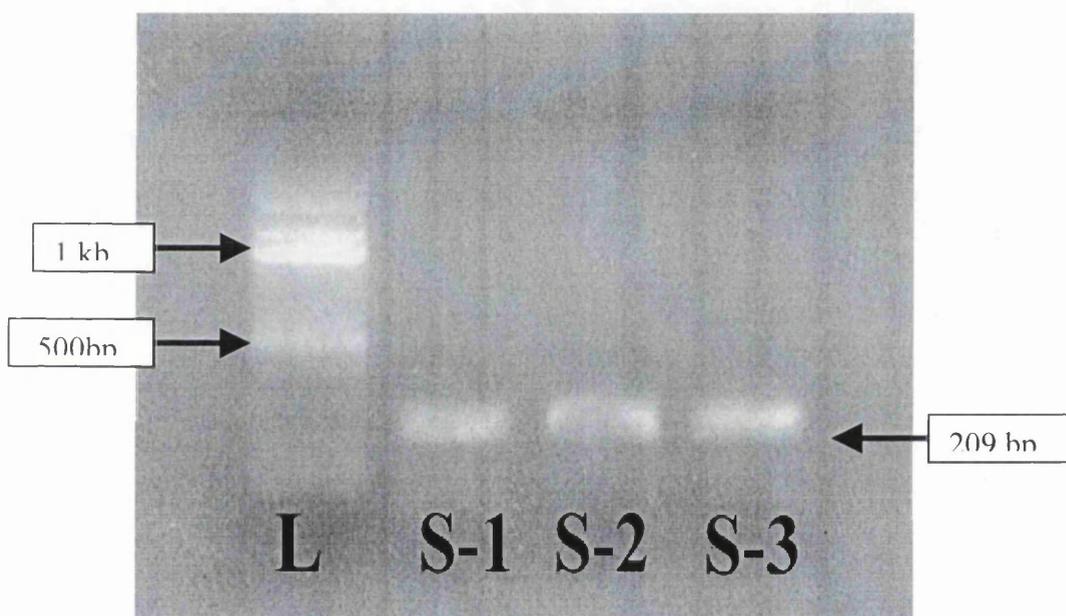
Figure 3.2: PCR-amplified product for SSCP analysis, using genomic DNA as a template. L= 100 bp DNA marker (1 kb and 500 bp bands are indicated), S-1, 2,3 are patients DNA.
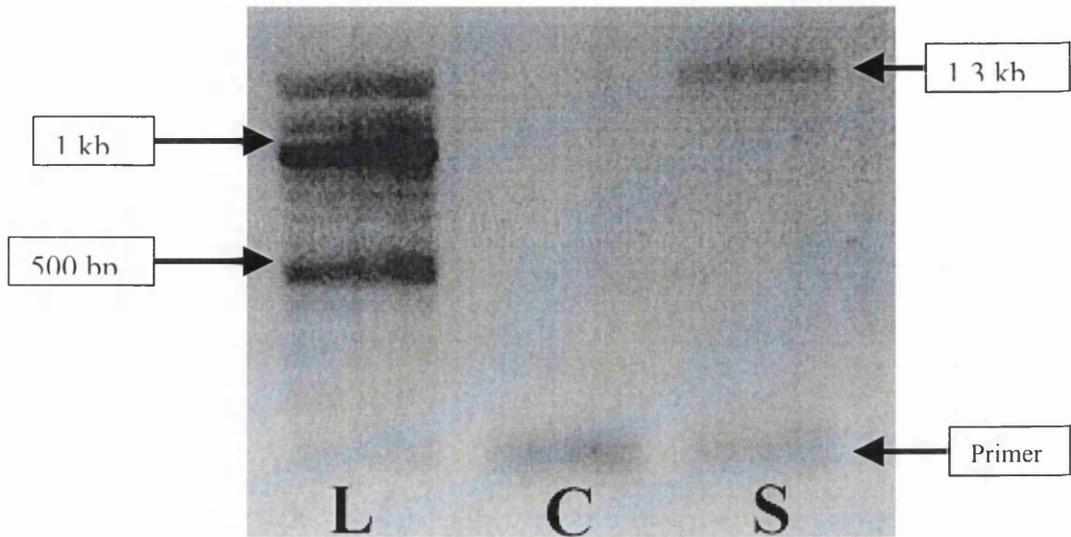
Figure 3.3: PCR-amplified product for PTT analysis from fragment-1 using genomic DNA as a template. L= 100 bp DNA marker (1 kb and 500 bp bands are indicated), C= negative control and S= PCR product from patient (1333 bp)
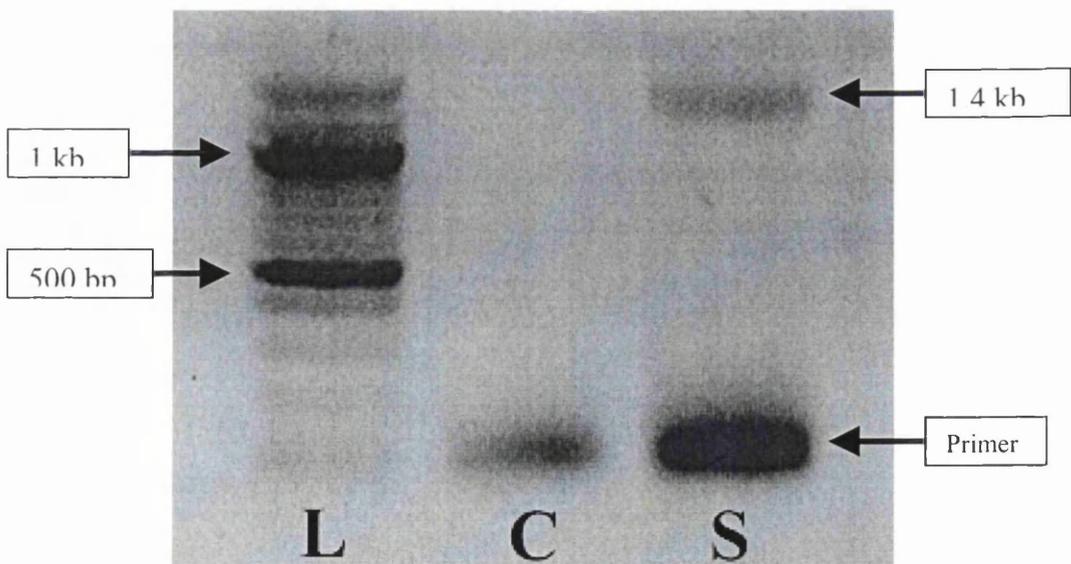
Figure 3.4: PCR-amplified product for PTT analysis from fragment-2 using genomic DNA as a template. L= 100 bp DNA marker (1 kb and 500 bp bands are indicated), C= negative control and S= PCR product from patient (1463 bp)
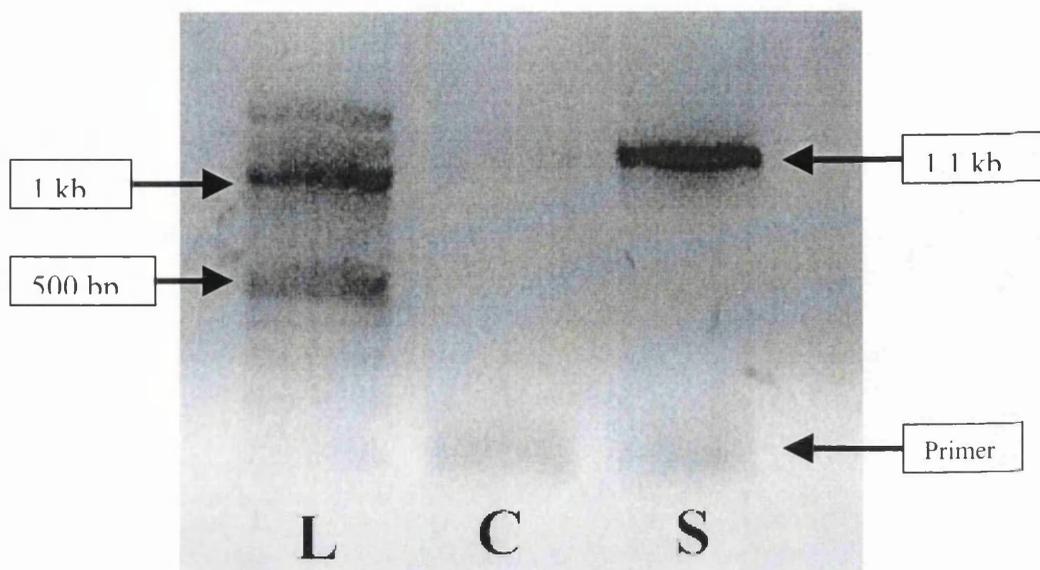
Figure 3.5: PCR-amplified product for PTT analysis from fragment-3 using genomic DNA as a template. L= 100 bp DNA marker (1 kb and 500 bp bands are indicated), C= negative control and S= PCR product from patient (1123 bp).

3.2. THE STRATEGY FOR DETECTION OF GERMLINE MUTATIONS IN THE *BRCA1* GENE

The following strategies used for detection of the mutations in the *BRCA1* gene:

- Genomic DNA was used for this study.
- The entire *BRCA1* gene was screened by SSCP analysis
- The exon 11, which contains almost two-thirds of the *BRCA1* coding sequence, was also amplified for PTT analysis.

In the presence of any abnormal band, direct sequencing was carried out to characterise the nature of that extra band.

Each amplified fragment in the expected size range was subject to SSCP analysis and any change found from it was sequenced to detect and locate the position and the nature of that particular mutation.

For exon 11, PTT analysis was also employed to detect any mutations, which could create a truncated protein.

Automated sequencing was carried out using an ABI Prism 373 automated sequencer on those samples where changes were found by SSCP.

A summary of sequence changes and the methods by which they were detected and characterised are shown in table 3.1. Each mutation will be discussed in detail.

3.3. MUTATION DETECTION USING SSCP

As RNA samples were not available in this study, SSCP analysis was employed to screen all samples for the presence of mutations using the primers listed in tables 2.2, 2.3, and 2.4. The fragments in exon 11 were named after the primers used to amplify them. The findings are as follows:

PATIENT SAMPLE NO.	SEQUENCE CHANGE	LOCATION	AMINO ACID CHANGES	MUTATION TYPE	DETECTION METHODS
13,15,24,28	g.3667 A>G	Exon 11	Lys>Arg	Polymorphism	SSCP, Sequencing
13,28	g.3771 A>T	Exon 11	Ser>Cys	Missense Mutation	SSCP, Sequencing
1,4,19,24,36,62	g.4427 T>C	Exon 13	Ser>Ser	Polymorphism	SSCP, Sequencing
30,36	g.1380 G>A	Exon 11	Glu>Lys	Missense Mutation	SSCP, Sequencing
2	g.5396+60 ins12bp	Intron 20	Unknown	Unclasified Variant	PCR, Sequencing

Table 3.1: The summary of the mutations in the *BRCA1* gene and the methods by which they were detected in this study. All alterations have been previously reported except for alterations g.3771 A>T and g.1380 G>A.

3.3.1. Positive Result from Fragment Exon 11N

SSCP analysis of fragment exon 11N (table 2.3) in patient 24 showed a different band pattern in comparison to all the others patients in this study excluding patients sample no 13, 15, 28 (figure 3.6). In order to understand the basis of this change, the sample was analysed by automated sequencing.
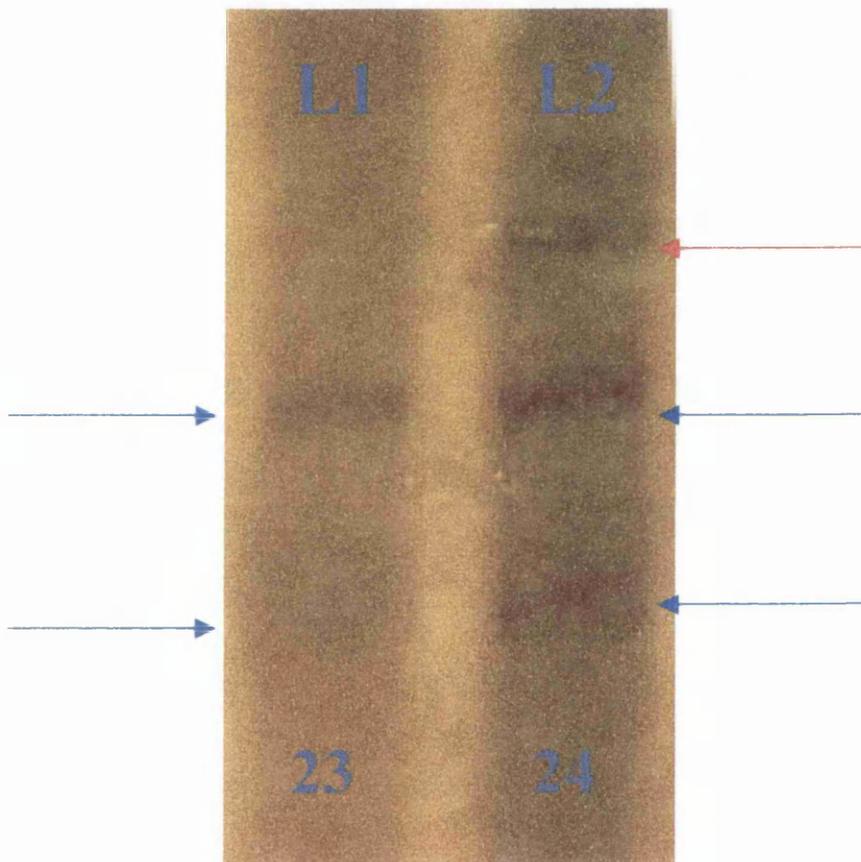
Figure 3.6: SSCP analysis of Exon 11 (fragment 11N) in *BRCA1* gene. Silver stained MDE gel of SSCP analysis of *BRCA1* exon 11, showing a different single strand band in lane two (marked by an red arrow). Lane one was sample 23 showing two single stranded bands, which co-migrated with 2 bands in L2. The results seen in L1 were the same as these found for all patients.

3.3.2. Positive Result from Exon 13

SSCP analysis of Exon 13 showed that six out of the forty-eight patient samples (Table 3.1) had a unique single strand band pattern (Figure 3.7). These samples were subsequently sequenced.
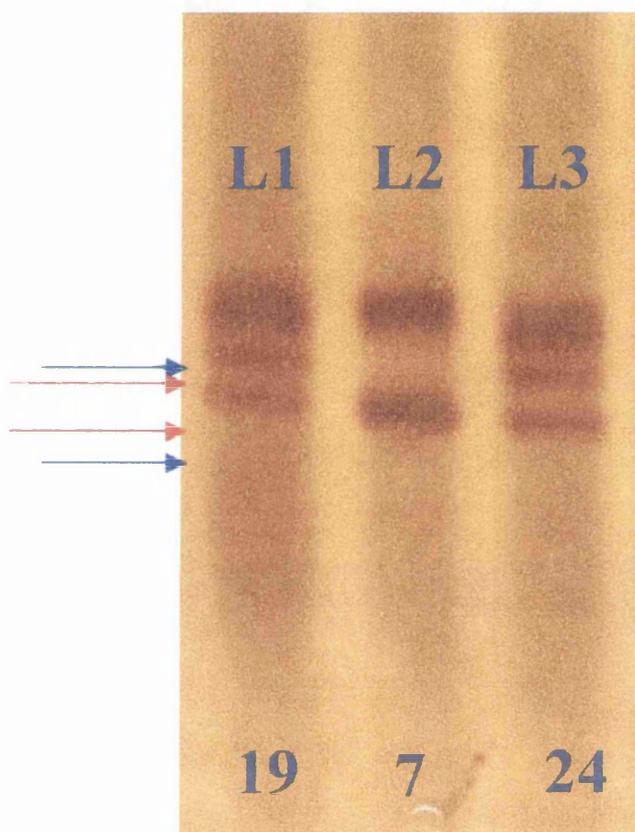
Figure 3.7: SSCP analysis of Exon 13 in *BRCA1* gene. Sample no: 19,24. Silver stained MDE gel of SSCP analysis of *BRCA1* exon 13, showing different single strand band pattern in lanes one and three (marked by red arrows). Lane two was sample no 7, showing two single stranded bands, which were co-migrated. Samples 19 and 24 show the band pattern found in 6 patients (figure 3.1). Sample 7 shows the band pattern found in the other 42 patients.

3.3.3. Positive Result from Exon Fragment 11C

The silver-stained MDE gel of SSCP analysis of *BRCA1* gene fragment exon 11C. The sample from patient no 30 pattern showed a shifted band compared to other samples tested. The fragment exon 11C was subsequently sequenced from patient no 30.
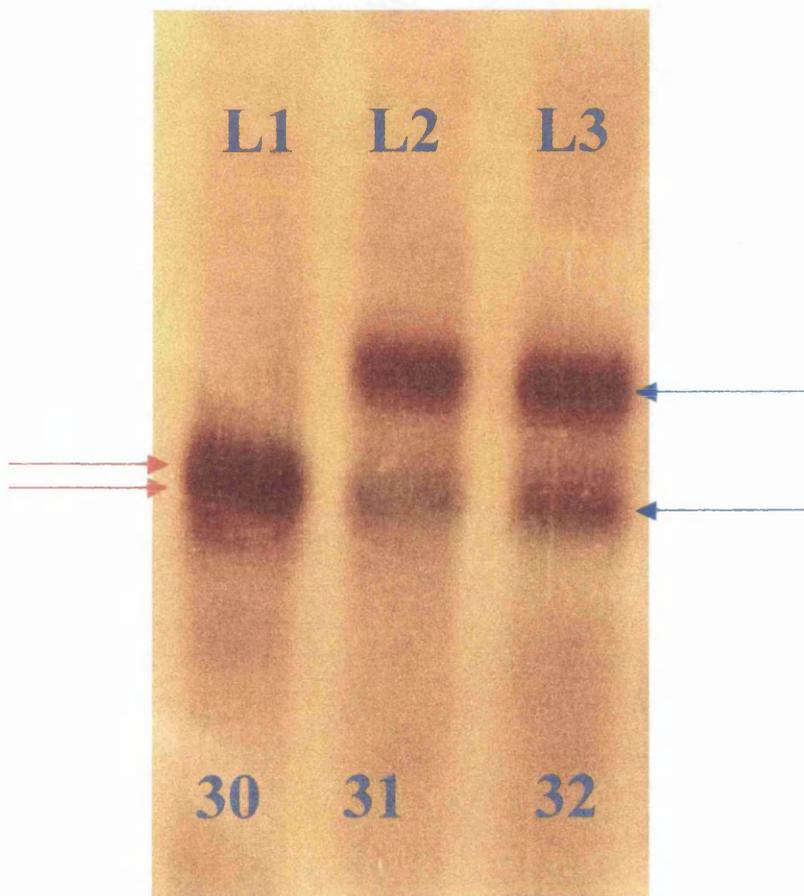
Figure 3.8: SSCP analysis of Exon 11 (fragment 11C) in *BRCA1* gene.. Silver stained MDE gel of SSCP analysis of *BRCA1* exon 11, showing a shifted band from sample no: 30 in lane one (marked by red arrows). There are two co-migrated band at the level of single strands in lanes two and three (sample no: 31,32).

3.3.4. Positive Result from Exon Fragment 11N

SSCP analysis from fragment exon 11N (table 2.3) a revealed different single strand band pattern in patient 13 compared to all other samples. This clearly indicated a possible mutation in this position. The fragment was subsequently sequenced using an ABI Prism 373 automated sequencer.
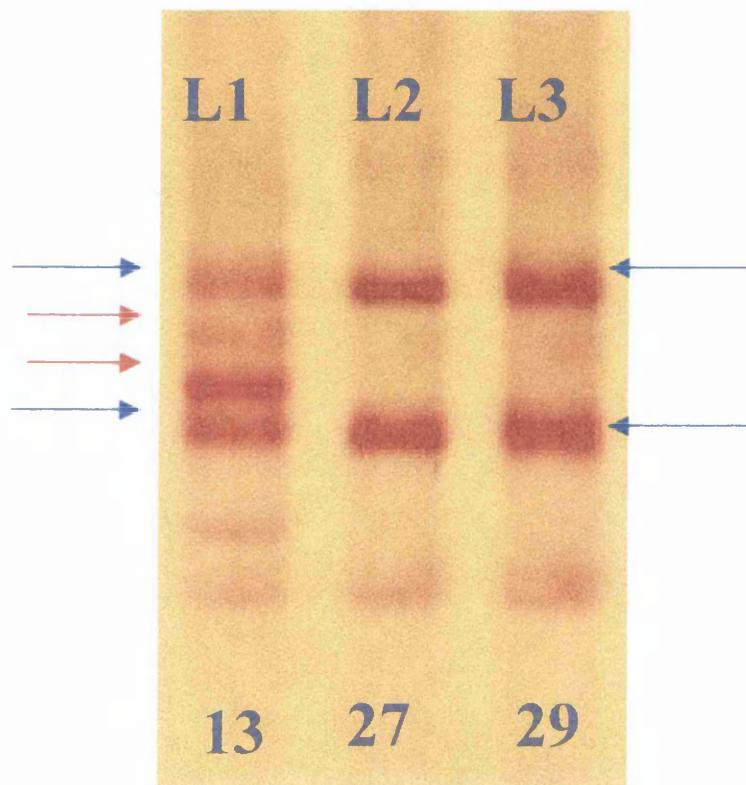
Figure 3.9: SSCP analysis of Exon 11 (fragment 11N) in *BRCA1* gene. Silver stained MDE gel of SSCP analysis of *BRCA1* exon 11N, showing a different single strand band pattern from sample no: 13 in lane one (marked by red arrows). Lanes two and three show two co-migrated single strands (sample no: 27,29) typical of all other samples analysed.

3.3.5. Positive Result from Exon 20

The PCR amplification of exon 20 in patient no 2 resulted in an extra band when analysed by agarose gel electrophoresis (Figure 3.11). The fragment was also analysed by SSCP and an altered band pattern detected compared to all other samples analysed (Figure 3.10).
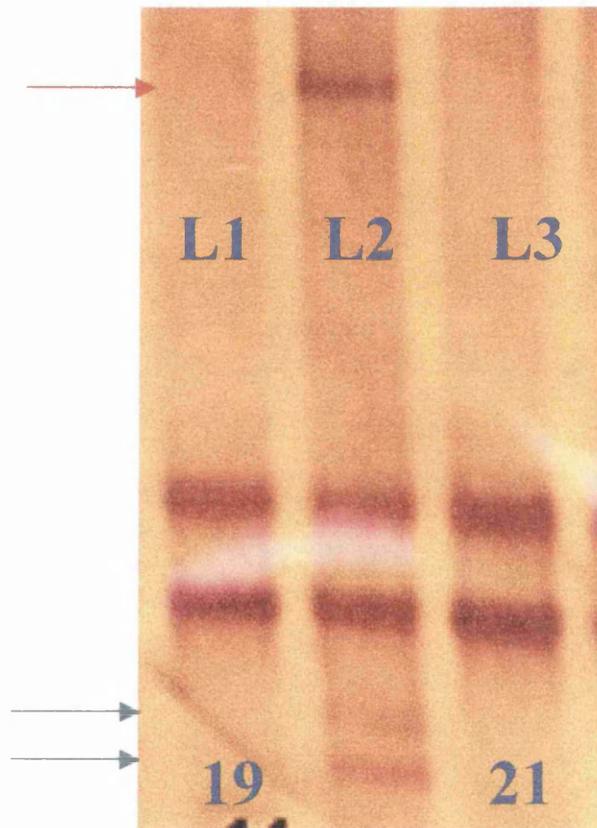
Figure 3.10: SSCP analysis of Exon 20 in *BRCA1* gene. Sample no: 2. Silver stained MDE gel of SSCP analysis of *BRCA1* exon 20, showing heteroduplex bands in lane two (marked by red arrow). There is a clear difference in single strand band pattern in lane two (marked by green arrows). Lane one and three show no heteroduplexes and two co-migrated single stranded band (sample no: 19,21).
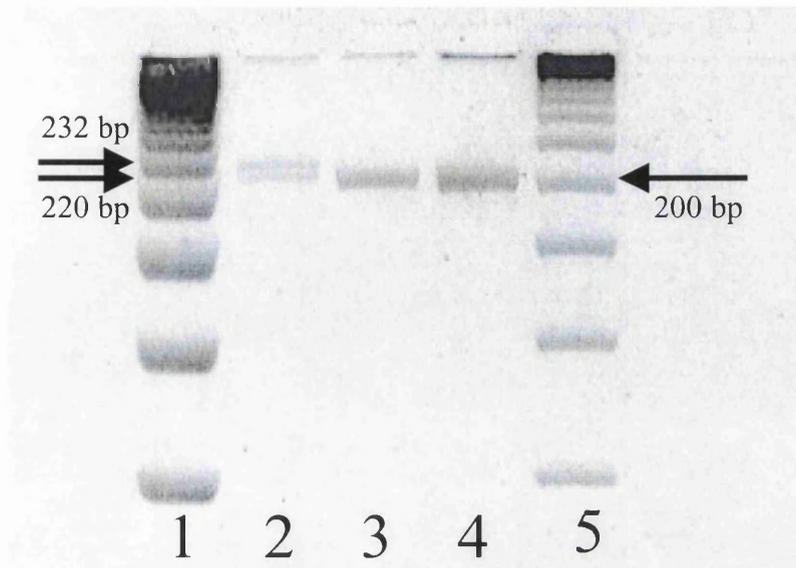
Figure 3.11: The PCR amplification of exon 20 from *BRCA1* gene. The PCR amplified products of exon 20 were analysed in a 1% agarose gel. The target DNA length is 220 bp. Lanes one and five are 50 bp DNA markers. Lane two shows an extra DNA band in patient no 2 suggesting that there is an insertion in one allele on this sample. Lanes three and four are patients no 3 and 4 and are typical of all samples analysed.

3.4. MUTATION DETECTION USING PTT

As RNA samples were not available in this study, PTT analysis could only be applied to exon 11, which was amplified with three overlapping sets of primers (Table 2.5). It was carried out by adding $8\mu l$ DNA in DEPC treated water to the TNT® Lysate Coupled Transcription/Translation System (Promega cat no. L4610) and following the manufacturer instruction (Section

II.7.2.2). A positive control translation reaction using Luciferase DNA (supplied by the manufacturer) and a negative control translation reaction were carried out by performing transcription/translation without adding DNA to analyse the efficiency of the system. The synthesised protein products were separated by 12.5% SDS-polyacrylamide gel electrophoresis. The gel was then autoradiographed for 48-96 hours. The three optimised PTT results are shown in figures 3.12-14.

However, the efficiency of PTT analysis was not always satisfactory. In order to improve the efficiency of PTT analysis, the following parameters were altered.

1. PCR amplified products were purified using a GFX^{TM} DNA purification kit (Pharmacia Biotech) and the concentration was obtained by spectrophotometry. The intensity of the band produced from the product purified was also compared with the intensity of the band from the control DNA supplied with the kit.

2. The translation reaction time was increased up to 100 minutes.

3. In order to increase the sensitivity of detection of ^{35}S, the gel was treated with Amplify™ solution (Amersham) for 20 minutes.
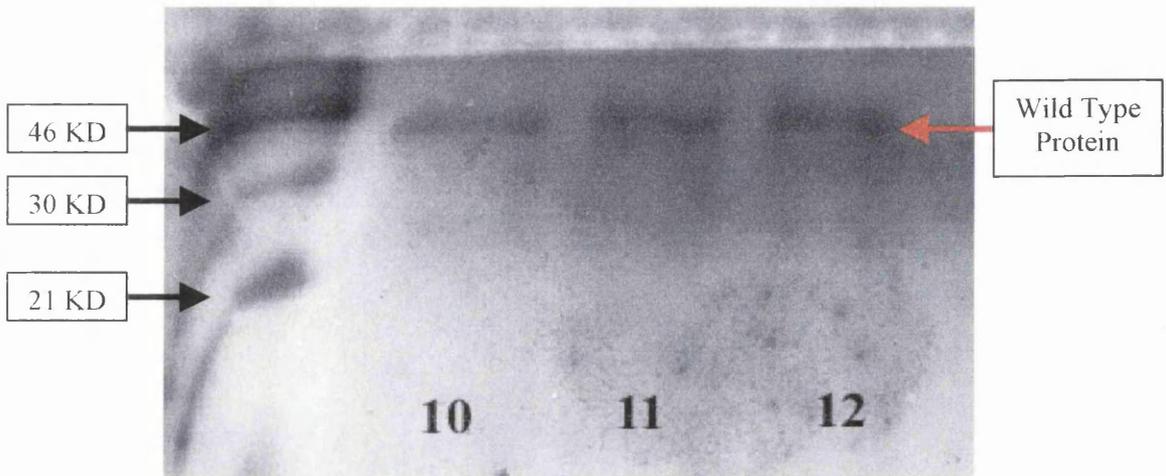
Figure 3.12: The PTT analysis result from fragment I in exon 11. Using an 12.5% SDS polyacrylamide gel. A red arrow indicates the wild type protein produced from patient sample nos 10, 11, 12. Nonetheless, a truncated protein could not be found in this fragment. The first lane is a rainbow protein marker ranging from 21 to 46 KD.
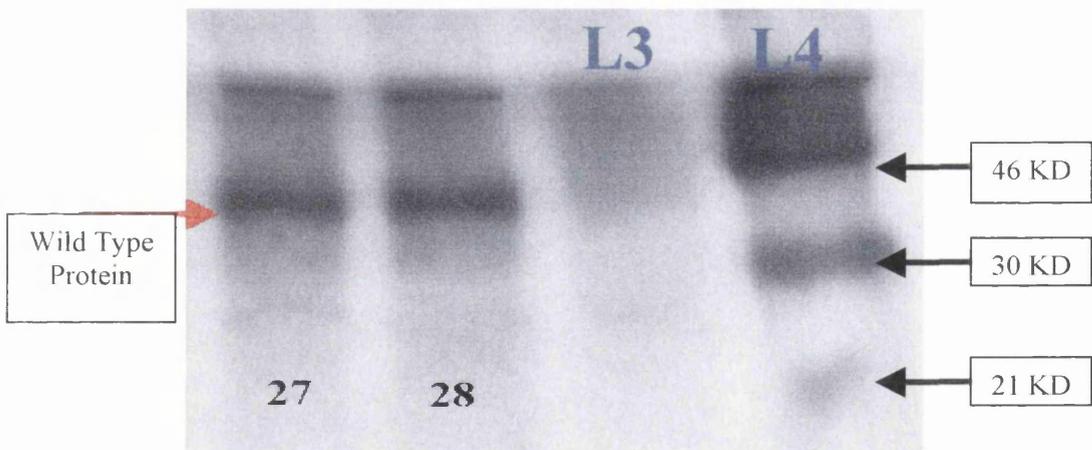
Figure 3.13: The PTT analysis result from fragment II in exon 11. 12.5% SDS polyacrylamide gel. A red arrow indicates the wild type protein produced from patient sample nos 27, 28. Nonetheless, a truncated protein could not be found in this fragment. Lane 4 is a rainbow protein marker. L3 is the weakly produced wild type protein.
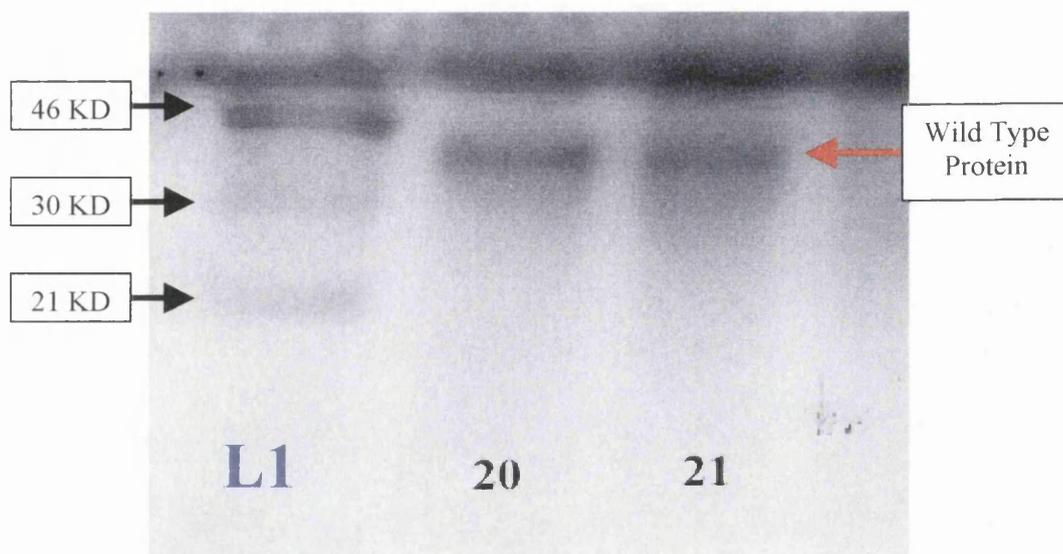
Figure 3.14: The PTT analysis result from fragment III in exon 11. 12.5% SDS polyacrylamide gel. A red arrow indicates the wild type protein produced from patient sample nos 20, 21. Nonetheless, a truncated protein could not be found in this fragment. The first lane is rainbow protein marker.

3.5. CHARACTERISATION OF THE MUTATIONS FOUND BY AUTOMATED SEQUENCING

Automated sequencing of the PCR products was carried out using the "Thermo sequenase II dye terminator cycle sequencing premix kit" (Amersham cat no. US80975). The samples that were screened and gave positive results by SSCP analysis were subjected to sequencing of the PCR products from the corresponding DNA segment(s) which were reproduced for automated sequencing analysis. $4\mu l$ double-stranded DNA (40-400 fmol) template in ddH_2O was used according to the manufacturer's instruction. In order to purify the product, the

GFX^{TM} DNA purification kit and the purification solutions provided with the sequencing kit were employed as described in section 2.6.1 and 2.7.3.2. The sequencing was carried out with the air-dried pellet in $4\mu l$ of gel loading mix provided with the kit.

The analysis of the sequence was carried out using the Macintosh computer attached to the PRISM 373 DNA system, using the 373A software (ABI). The computer processed the data and presented it as a chromatogram with four coloured peaks (G=black, T=red, C=Blue and A=Green). Heterozygosity is represented by two superimposed peaks.

The positive results discussed under the section 3.3 will be discussed in further detail.

3.5.1. Polymorphism at Nucleotide Position g.3667 A>G

The automated sequencing from the positive sample mentioned in section 3.3.1 showed a base change from A to G at the nucleotide position 3667 (figure 3.15). This base change is predicted to produced the conversion of the amino acid Lysine to Arginine (amino acid position: 1183). It was published and categorised as a polymorphism in the Breast cancer Information Core database (http://www.nhgri.nih.gov/). In this study, four out of forty-eight patients (samples no, 13, 15, 24, 28) were found to have this polymorphism (table 3.1). These patients were registered in the database mentioned above and the registration information was detailed in table 3.2.
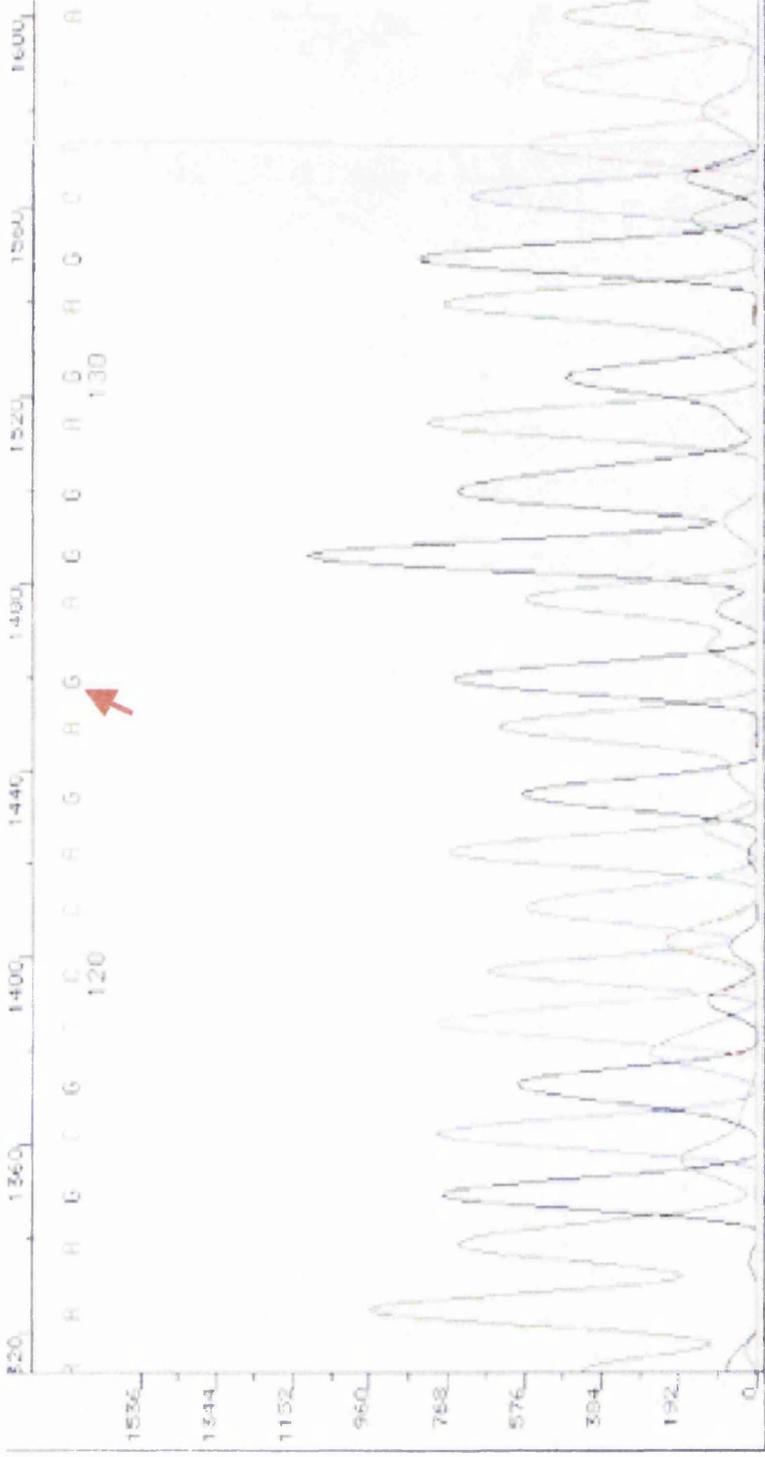
Figure 3.15: Automated sequencing analysis of the fragment exon 11N. Automated sequencing analysis of *BRCA1* fragment exon 11N showed a homozygous base change from A to G at nucleotide position 3667 (marked by a red arrow). This change is predicted to cause to conversion of the amino acid Lysine to Arginine. This change was found in sample nos, 13, 15, 24 and 28.

3.5.2. Polymorphism at Nucleotide Position g.4427 T>C

The automated sequencing of the positive sample mentioned in section 3.3.2 showed a base change from T to C at the nucleotide position 4427 (figure 3.16). This was the most frequently published polymorphism according to the Breast cancer Information Core database (BIC) (http://www.nhgri.nih.gov/). It results in a silent mutation at a serine amino acid, position 1436. In this study, six out of forty-eight patients (sample nos, 1, 4, 19, 24, 36, 62) were found to have this mutation (table 3.1). These patients were registered in the database mentioned above and the registration information was detailed in table 3.2.

3.5.3. Point Mutation at Nucleotide Position g.3771 A>T

The automated sequencing of the positive sample mentioned in section 3.3.3 showed a base change from A to T at the nucleotide position 3771 (figure 3.17). This mutation found in *BRCA1* fragment exon 11N was first published in the Breast cancer Information Core database (BIC) (http://www.nhgri.nih.gov/) as a novel mutation. This mutation is predicted to cause an amino acid change from Serine to Cysteine (amino acid position: 1218). In this study, two out of forty-eight patients (sample nos, 13, 28) were found to have this mutation (table 3.1). These patients were registered to the database mentioned above and the registration information was detailed in table 3.2.
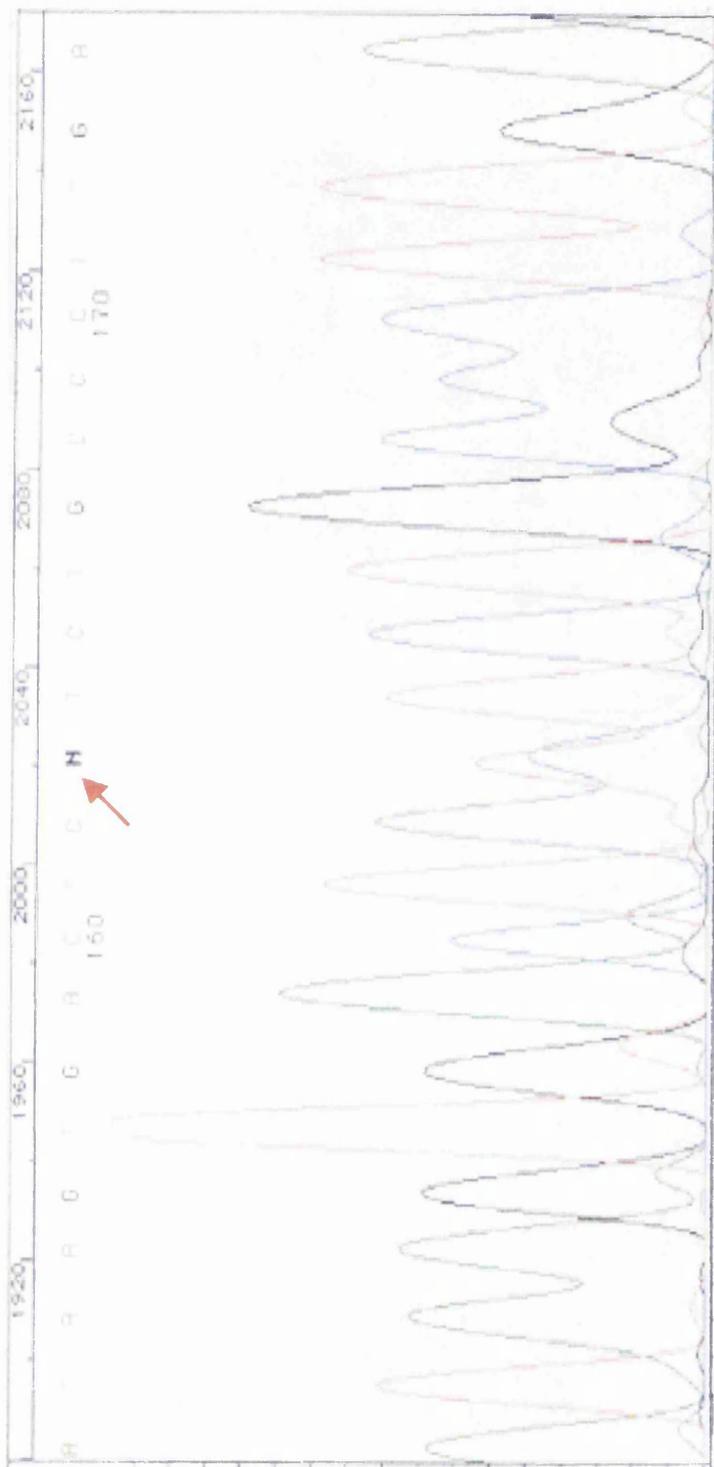
Figure 3.16: Automated sequencing analysis of the exon 13. Automated sequencing analysis of *BRCA1* exon 13 showed a heterozygous base change in nucleotide position 4427 (marked by a red arrow). This was a silent mutation in the amino acid position: 1436 involving Serine. This change was found in sample nos, 1, 4, 19, 24, 36 and 62.

Figure 3.17: Automated sequencing analysis of the fragment exon 11N. Automated sequencing analysis of *BRCA1* fragment exon 11N showed a heterozygous base change from A to T at nucleotide position 3771 (marked by a red arrow). This novel change is predicted to cause to conversion of the amino acid Serine to Cysteine. This change was found in sample nos, 13 and 28.

3.5.4. Point Mutation at Nucleotide Position g.1380 G>A

The automated sequencing of the positive sample mentioned in section 3.3.4 showed a base change from G to A at the nucleotide position 1380 (figure 3.18). This mutation found in *BRCA1* fragment exon 11C was first published in the Breast cancer Information Core database (BIC) (http://www.nhgri.nih.gov/) as a novel mutation. This mutation is predicted to cause an amino acid change from Glutamine to Lysine (amino acid position: 421). In this study, two out of forty-eight patients (sample nos, 30, 36) were found to have this mutation (table 3.1). These patients were registered on the database mentioned above and the registration information was detailed in table 3.2.
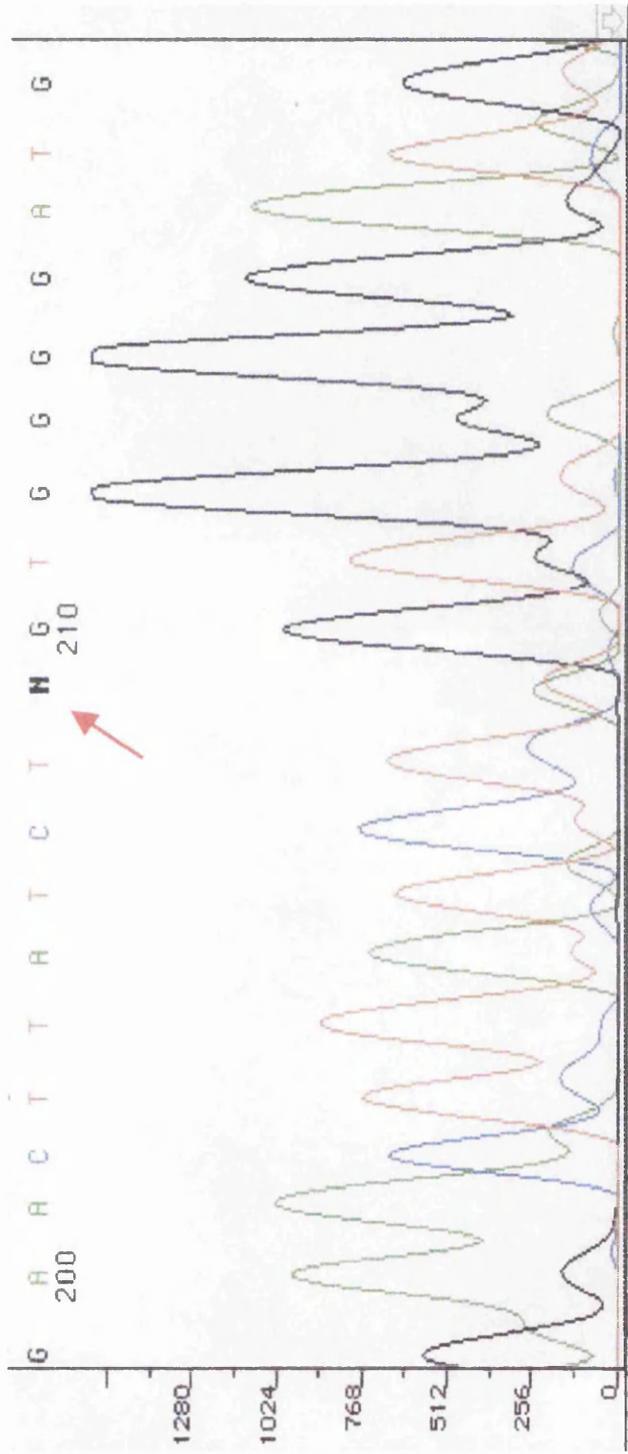
3.5.5. U. Variant at Nucleotide Position g.5396+60 ins12bp

The automated sequencing of the positive sample mentioned in section 3.3.5 showed an insertion of 12bp (insGTATTCCACTTC) at the nucleotide position 5396+60 (figure 3.19). This mutation found in *BRCA1* intron 20 has been published in the Breast cancer Information Core database. In this study, one out of forty-eight patients was found who had this mutation (table 3.1). The nature and effect of this mutation is still not clear. Further details of this mutation will be discussed in the next chapter. This mutation was registered to the database mentioned above and the registration information was detailed in table 3.2.
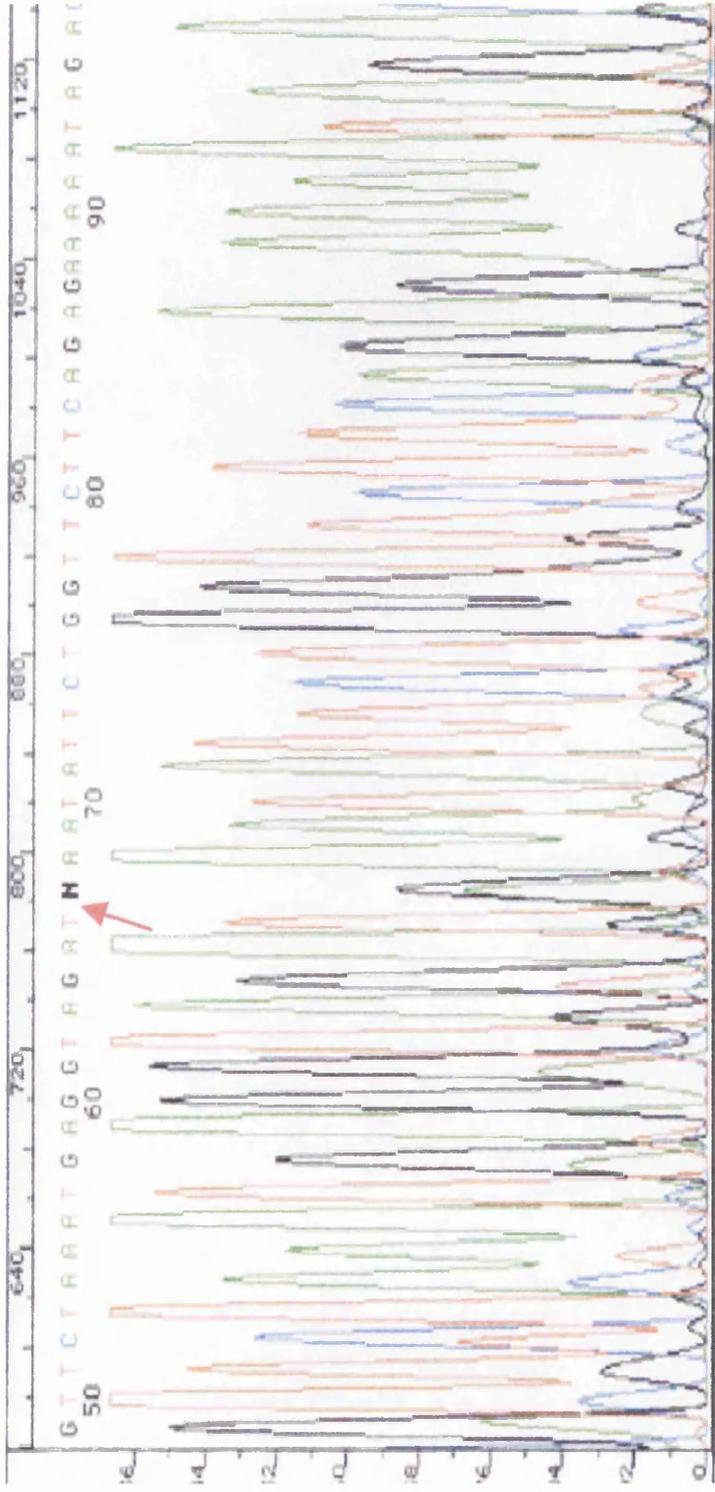
Figure 3.18: Automated sequencing analysis of the fragment exon 11C. Automated sequencing analysis of *BRCA1* fragment exon 11C showed a heterozygous base change from G to A at nucleotide position 1380 (marked by a red arrow). This novel change is predicted to cause to conversion of the amino acid Glutamine to Lysine. This change was found in sample nos, 30 and 36.
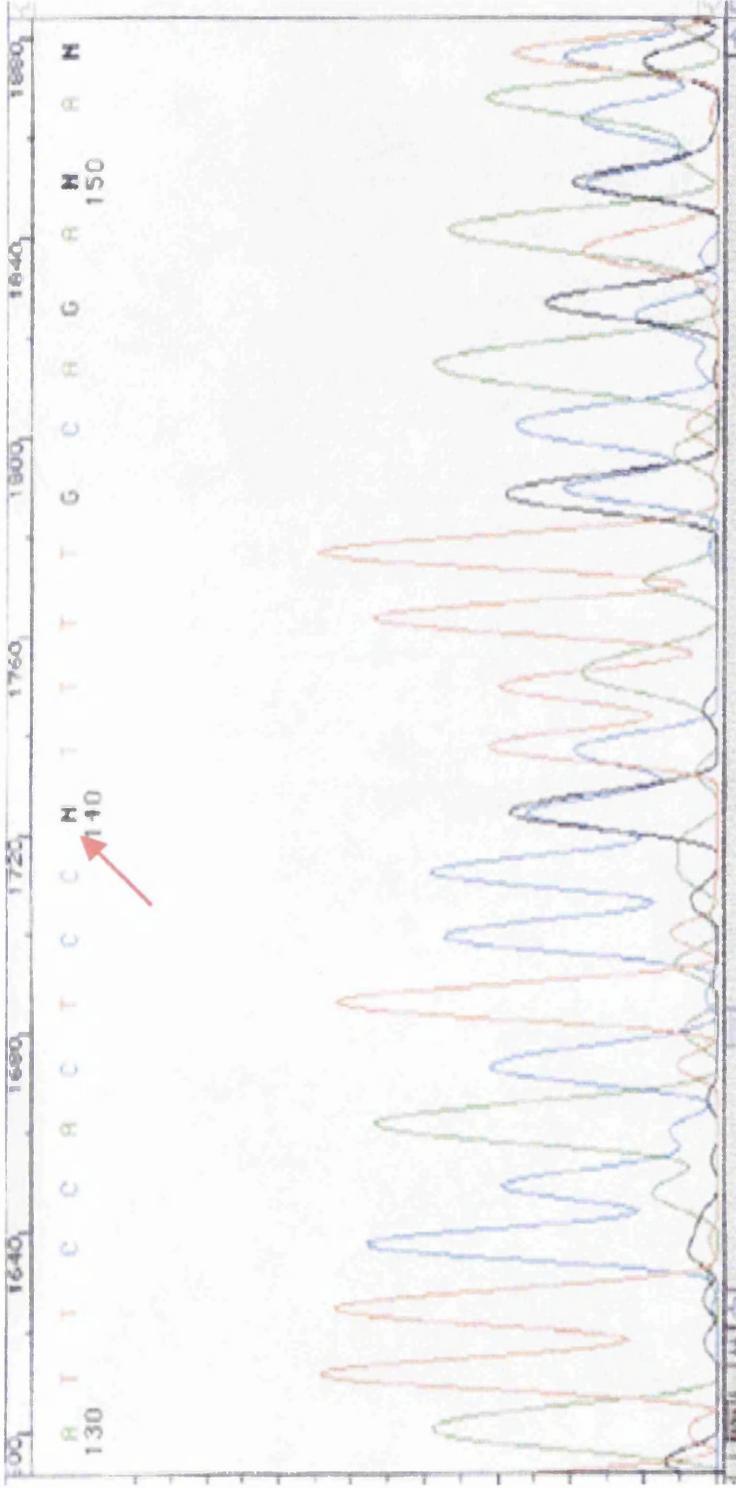
Figure 3.19: Automated sequencing analysis of the intron 20. Automated sequencing analysis of *BRCA1* intron 20 showed 12 bp insertion at nucleotide position 5396+60 (starting point marked by a red arrow). This change was found in sample no, 2.

3.6. POSSIBLE LARGE DELETION IN SAMPLE 28

The sequencing of sample 28 for fragment exon 11N (figure 3.15) showed a G at position 3667. The normal sequence has an A at this position. The result could be due to homozygosity at this base or to the presence of one allele containing the A>G change and the other allele deleted for this region. To identify whether a small deletion within the exon 11 was present, the Expand Long Template PCR analysis was employed (section 2.7.3.). Primers published by Payne *et al.* (2000) were used to amplify the region containing exon 11, 12 and intron 11 (figure 3.1)The analysis of the fragments produced by this amplification are shown in figure 3.20.
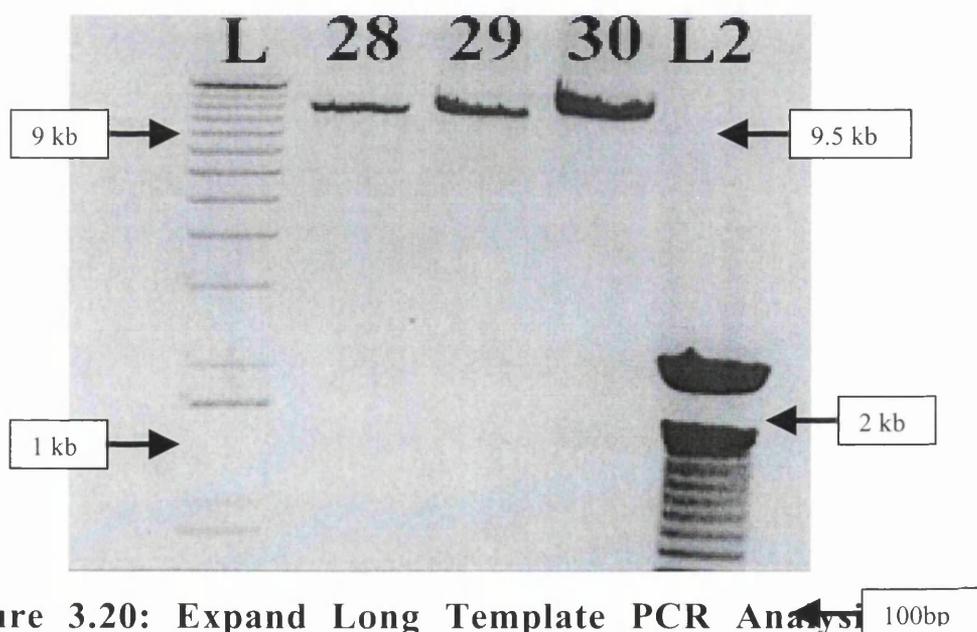
Figure 3.20: Expand Long Template PCR Analysi 100bp product encombassing exon 11, 12 and intron 11 were analysed in a 0.7% agarose gel. Lane one and five are DNA markers (1 kb and 100 bp respectively). Sample 28, showed the same band size as those amplified from patients 29 and 30.

3.7. POLYMORPHISM FREQUENCIES IN A CONTROL GROUP FROM THE WEST OF SCOTLAND

Two widely published polymorphisms (g.4427 T>C, g.3667 A>G) and one intronic insertion of 12 bp (g.5396+60 ins12bp) were found in this study. The frequency of these mutations in a normal population from the West of Scotland was unknown. In order to establish the frequency of these mutations in a normal population, 100 DNA samples were obtained from the medical genetics department at the Duncan Guitre Institute of Medical genetics, Glasgow. These samples were anonymatised control samples from a consented normal West of Scotland population. SSCP analysis was employed to find out the frequency of two single base-pair polymorphisms and positive results were subsequently sequenced. The 12 bp insertion in intron 20 was analysed by PCR. Size differences between two alleles in each sample was investigated by analysing products in a 1% agarose gel.

3.7.1. Analysing Polymorphism g.5396+60 ins12bp in the Control Group

One hundred control samples were analysed by PCR using the primer set for exon 20 (section 2.4.1.). Samples were size separated by 1% agarose gel electrophoresis and visualised using ethidium bromide. None of the control samples were found to have this insertion in their intron 20 of BRCA1 gene (figure 3.21)
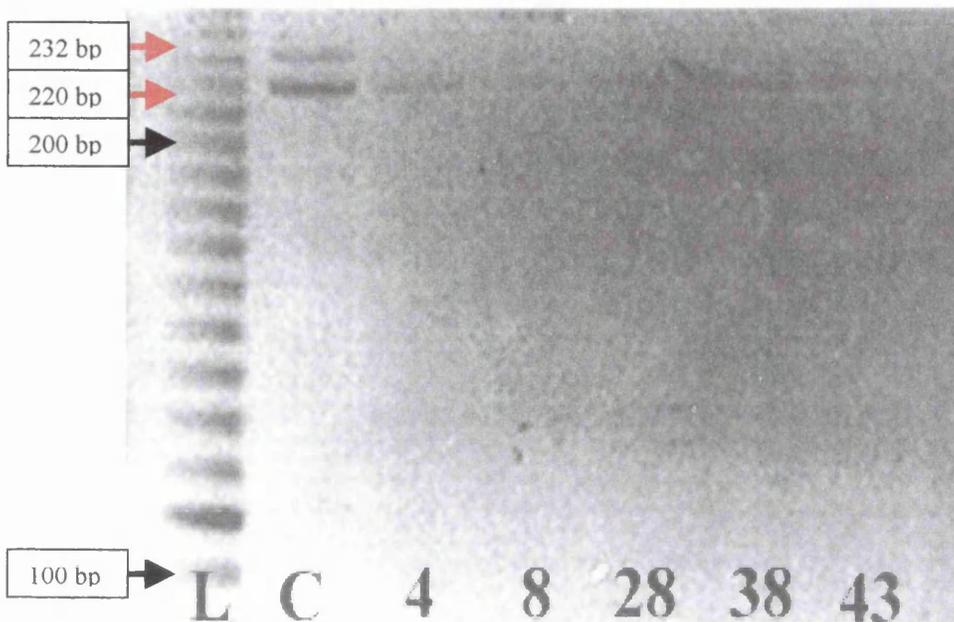
Figure3.21: The PCR amplification of exon 20 from *BRCA1* gene. The PCR amplified products of exon 20 were analysed in a 1% agarose gel. Lane one is a DNA marker (10 bp) and 100, 200 bp are indicated by black arrows. Samples no 4, 8, 28, 38, 43 showed no extra band in comparison to the positive control (C) suggesting that there was not an insertion in intron 20.

3.7.2 Analysing Polymorphism g.4427T>C in Control Group

One hundred control samples were analysed by SSCP analysis (section 2.7.1.) using an MDE gel and silver staining to visualise the samples (figure 3.22). Ten control samples out of 100 were found carrying polymorphism g.4427 T>C. the presence of this polymorphism was subsequently proved by automated sequencing (section 2.7.4)(figure 3.23). Control no 11 was found to only have C at bp 4427. This could indicate homozygosity for this polymorphism. (figure 3.24).

Figure 3.22: SSCP analysis of Exon 13 in *BRCA1* gene. Silver stained MDE gel of SSCP analysis of *BRCA1* exon 13 from control samples no 1-19. First lane is a positive control (C). Samples no 5, 10, 17 show heteroduplex bands (marked by red arrows). There is clear difference in single strand band pattern in comparison to positive control (marked by green arrows). Sample no 14 shows a different single band pattern suggesting homozygosity for this polymorphism.
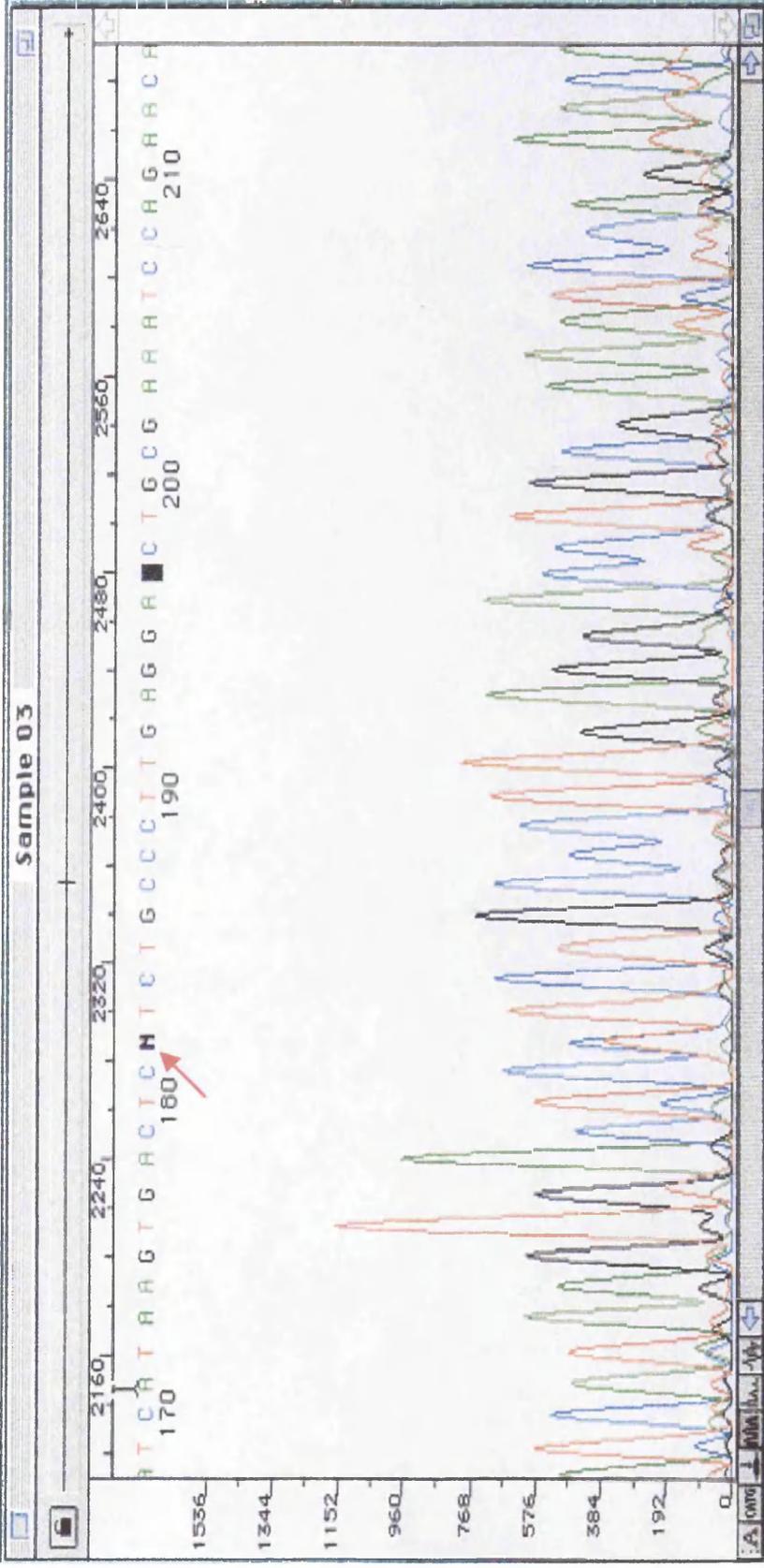
Figure 3.23: Automated sequencing analysis of the exon 13. Automated sequencing analysis of *BRCA1* exon 13 showed a heterozygous base change from T to C at nucleotide position 4427 (marked by a red arrow). This change was found in 10 control samples.
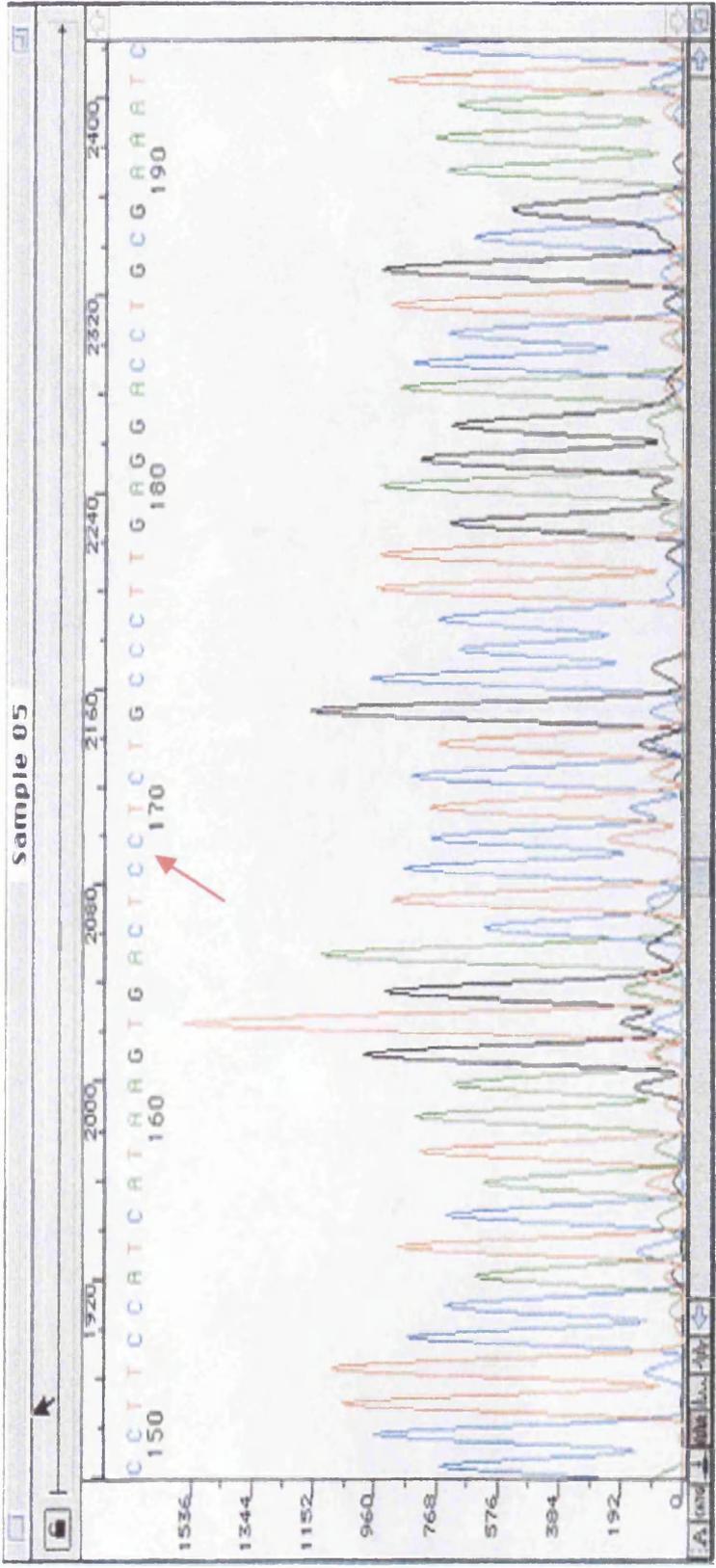
Figure 3.24: Automated sequencing analysis of the exon 13. Automated sequencing analysis of control sample no 14 in *BRCA1* exon 13 showed a homozygous base change from T to C at nucleotide position 4427 (marked by a red arrow).

3.7.3 Analysing Polymorphism g.3667A>G in Control Group

One hundred control samples were analysed by SSCP analysis using primer set 11N (table 2.3). Using MDE gel and silver staining, samples were visualised (figure 3.25). Six control samples out of 100 were found carrying polymorphism g.4427 T>C and this was subsequently confirmed by automated sequencing (section 2.7.4)(figure 3.26).
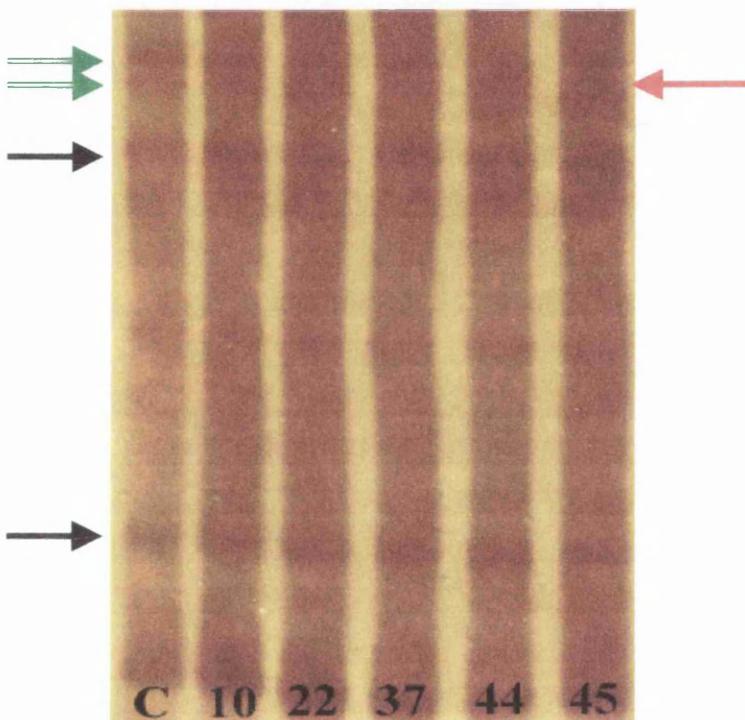
Figure 3.25: SSCP analysis of the fragment Exon 11N. Silver stained MDE gel of SSCP analysis of *BRCA1* gene, the fragment of exon 11N. First lane is a positive control (C). Samples no 10, 22, 37, 44 showed a clear difference in the single strand band pattern in comparison to the positive control (marked by green arrows). The single strands in sample no 45 (marked by red arrow) is co-migrating with the band in polymorphism samples and is related to the normal sequence for this fragment.
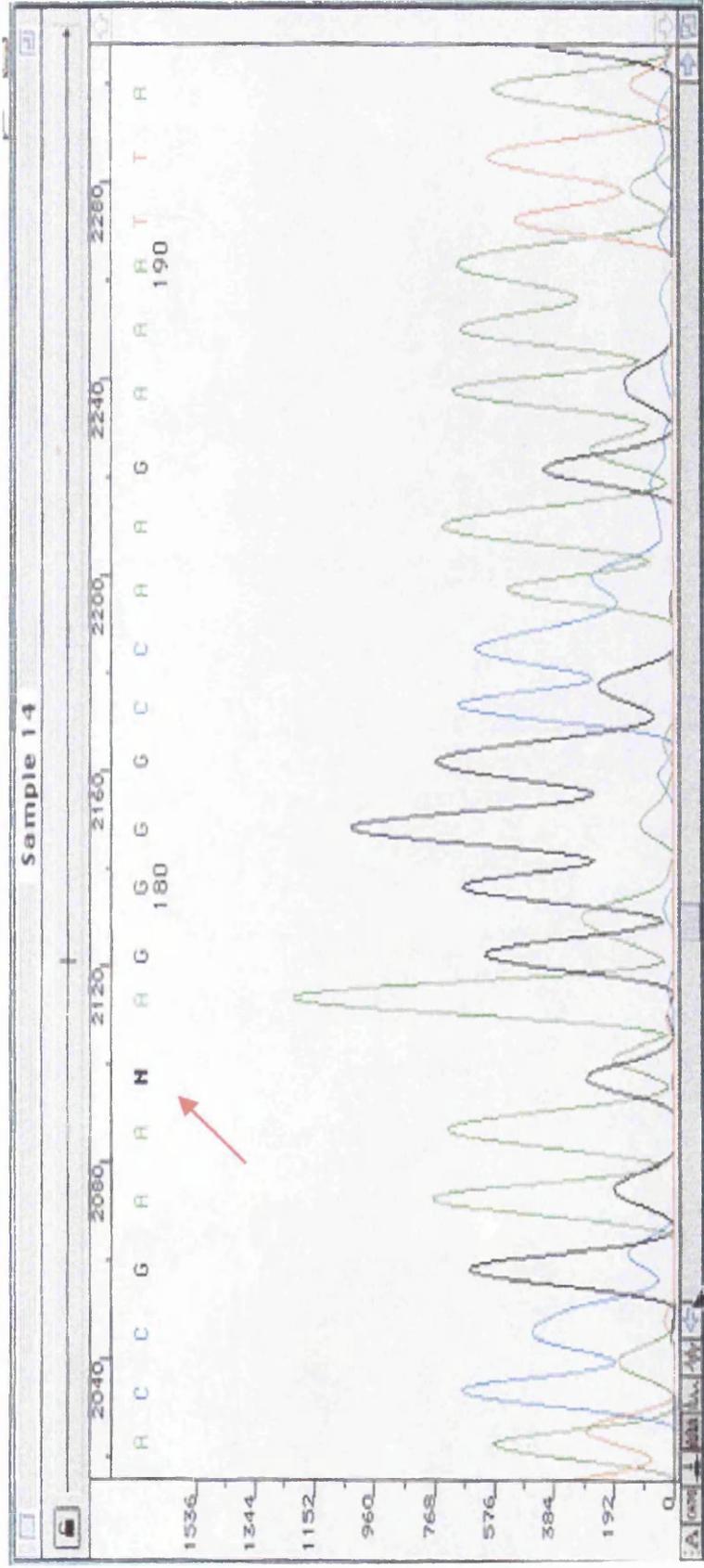
Figure 3.26: Automated sequencing analysis of the fragment exon 11N. Automated sequencing analysis of the fragment exon 11N showed a heterozygous base change from A to G at nucleotide position 3667 (marked by a red arrow). This change was found in 6 control samples.

PATIENT ID NO	SAMPLE ID	DATABASE ENTRY NO	DESIGNATION	MUTATION TYPE
14830	13	4147	g.3667 A>G	Polymorphism
14107	28	4150	g.3667 A>G	Polymorphism
13928	15	4148	g.3667 A>G	Polymorphism
12945	24	4149	g.3667 A>G	Polymorphism
10645	1	4174	g.4427 T>C	Polymorphism
11246	4	4175	g.4427 T>C	Polymorphism
11500	19	4177	g.4427 T>C	Polymorphism
12945	24	4178	g.4427 T>C	Polymorphism
11243	36	4176	g.4427 T>C	Polymorphism
16529	48	4179	g.4427 T>C	Polymorphism
13632	36	4082	g.1380 G>A	Missense Mutation
14213	30	4081	g.1380 G>A	Missense Mutation
14830	13	4152	g.3771 A>T	Missense Mutation
14107	28	4153	g.3771 A>T	Missense Mutation
12418	2	Pending	g.5396+60 ins 12bp	Unclassified Variant

Table 3.2: Mutations registered with the Breast Cancer Information Core Database.

CHAPTER 4

DISCUSSION

4.1 MUTATION SPECTRUM IN THE *BRCA1* GENE

Mutations in the *BRCA1* gene are thought to account for most of familial ovarian cancer. These mutations include nonsense mutations, small deletions and insertions causing frameshift mutations, missense mutations occurring at crucial aminoacid positions within well-conserved domains and mutations affecting the splice sites.

According to the Breast Cancer Information Core database (BIC), more than 850 different mutations in the *BRCA1* gene have been reported out of nearly 4000 entries. In addition,
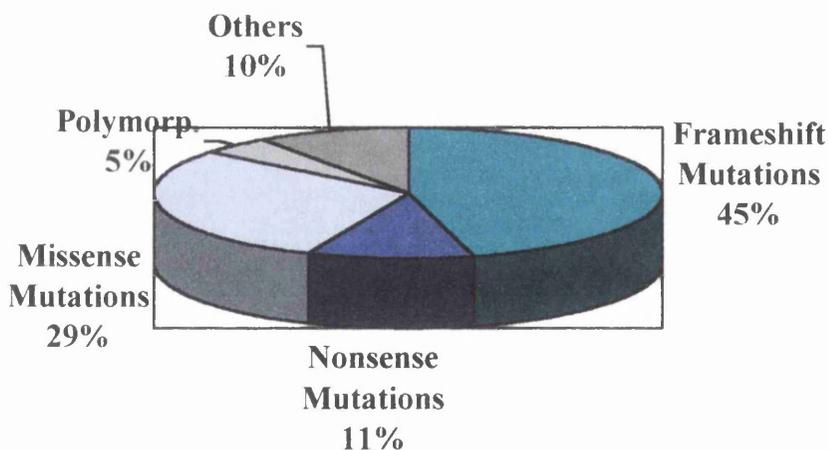
Figure 4.1: Distribution of *BRCA1* mutations.

more than 500 (57%) of them were reported only once. (http://www.nhgri.nih.gov/intramural_research/Lab_transfer/). Almost 2250 (56%) of the mutations are nonsense mutations and frameshift mutations including, small insertions, deletions, splice variants and regulatory mutations that result in truncation or absence of the BRCA1 protein (figure 4.1).

More than 1150 (29%) of those entries are missense mutations that are predicted to result in an amino acid changes in the coding sequence and more than 240 (5%) polymorphisms and nearly 500 (10%) of those entries are silent mutations and unclassified variants have been reported to the database.

The most common mutations are 185 delAG and 5382 insC which account for 490 (12%) and 270 (7%) respectively, of all mutations reported so far. The mutations are spread over the entire length of the coding sequence (figure 4.2).

The frequency of mutations varies greatly depending on the racial or ethnic group. In general, it is lower than expected from data based on families identified by linkage analysis (Szabo & King MC., 1997). In a recent report from the Breast Cancer Linkage Consortium, it was estimated that only 63% of all families linked to BRCA1 have a mutation detectable by standart screening methods (Ford et al., 1998), thus suggesting that other types of gene activation might account for some of the predisposed kindreds. To date, an inhibition of BRCA1 transcription by regulatory mutations has been rarely reported

(Miki *et al.*, 1994; Gayther *et al.*, 1995; Xu *et al.*, 1997a; Serova *et al.*, 1996).

Major genomic rearrangements of the BRCA1 gene including deletions, which escape most of the current PCR-based mutation detection methods, are likely to account for at least some of the cases without apparent BRCA1 mutations (Petrij-Bosch *et al.*, 1997; Swensen *et al.*, 1997; Puget *et al.*, 1997). This possibility is also supported by the high density of *Alu* repeats dispersed in the BRCA1 genomic sequence (Smith *et al.*, 1996) and *Alu* sequences can mediate major genomic rearrangements by promoting unequal crossing-over or other types of recombination.

In a study from Holland (Petrij-Bosch *et al.*, 1997) reported that repetitive finding of a 510 bp *Alu*-mediated deletion comprising exon 22 and a 3835 bp *Alu*-mediated deletion comprising exon 13 were responsible for 36% of all BRCA1 mutations found in Dutch breast cancer families to date. These had not been detected by PCR-based mutation screening methods.

BRCA1 mutations which are detectable using commonly employed PCR-based techniques are small insertions, deletions or single base-pair substitutions. These comprise most germ-line mutations in high risk families but rarely occur as somatic mutations (Hosking *et al.*, 1995; Berchuck *et al.*, 1998b; Matsushima *et al.*, 1995; Takahashi *et al.*, 1995). On the other hand, large deletions concerning sizes of mega-bases in the BRCA1 gene identified by loss of heterozygosity (LOH), occur frequently in both inherited and sporadic breast and ovarian

cancer (Devilee & Cornelisse, 1994). It was therefore suggested that large deletions in BRCA1 gene could also be responsible from some of the somatic breast and ovarian cancer cases.

In the absence of suitable assays to study the effects of aminoacid changes on BRCA1 protein function, the identification of a missense mutation with functional consequences relies on indirect evidence. Missense mutations associated with a disease state suggest a functional significance of the aminoacids involved and in their location. Important amino acids would be expected to be conserved across species. Sequenced analysis of BRCA1 reveals several possible protein motifs (figure 1.1). The RING finger motif (aminoacids 24-64) is the most highly conserved. It is 100% preserved among known human RING finger domains and only a single aminoacid substitution (K55R) is found in canine and murine species (Szabo et al., 1996). It has also been reported that a repeatedly published missense mutation, Cys61Gly, which leads to an alteration of the sixth cysteine of the putative RING finger domain, could result in a deleterious effect by cousing the loss of lost wild-type allele in patients tumour tissue (Merajver et al., 1995). It has also been reported that a particular missense mutation (R841W) found in patients with a family history is related to the late age of onset of disease (Janezic et al., 1999).

Some other published missense mutations which result in changes in conserved protein domains are found in patients with a family history. However, until a functional assay is

available, missense mutations and their role in tumorigenesis remain unknown.

Polymorphisms are defined as non-pathogenic variations in the normal gene sequence. It has been also reported that almost none of the aminoacids that reported as polymorphic variation are conserved in murine and canine species (Sharan *et al.*, 1995). However, the effect of polymorphisms on BRCA1 gene function is unknown. Narod *et al.* (1998) suggested that allelic variation of host gene might influence the course of cancer. He also suggested that genetic variations could influence the prognosis of cancer, including grade, stage and other relevant prognostic factors. In addition, some polymorphisms in some genes may carry a functional importance and may create a mutation hot spot. For example, in Ashkenazi Jewish colorectal cancer patients, a polymorphism (I1307K) in the APC gene has been shown to create a mutation hot spot.

Although, polymorphisms role in tumorigenesis is not clear, polymorphic variants could be used as a marker for analysing large deletions. Using patient's both DNA and cDNA for highly frequent polymorphic sites (providing that patient has a polymorphism), heterozygous polymorphisms in genomic DNA located the distal or proximal part of the BRCA1 gene from suspected large deletion might be found homozygous from the individual's cDNA for the same loci (Swensen *et al.*, 1997; Catteau *et al.*, 1999). This

4.2 MUTATION ANALYSIS

In this study, 48 ovarian cancer patients with a family history of breast and/or ovarian cancer have been screened for germline mutations in the *BRCA1* gene. The patients had 1-6 affected family members with onset of disease between 22-78 years of age (table 2.1). The mean age of onset of ovarian cancer with *BRCA1* alterations was 50 years.

PTT and SSCP analysis was employed to detect changes as described in section 2.7.1-2. The characterisation of those changes was achieved by automated sequencing analysis as described in section 2.7.3.
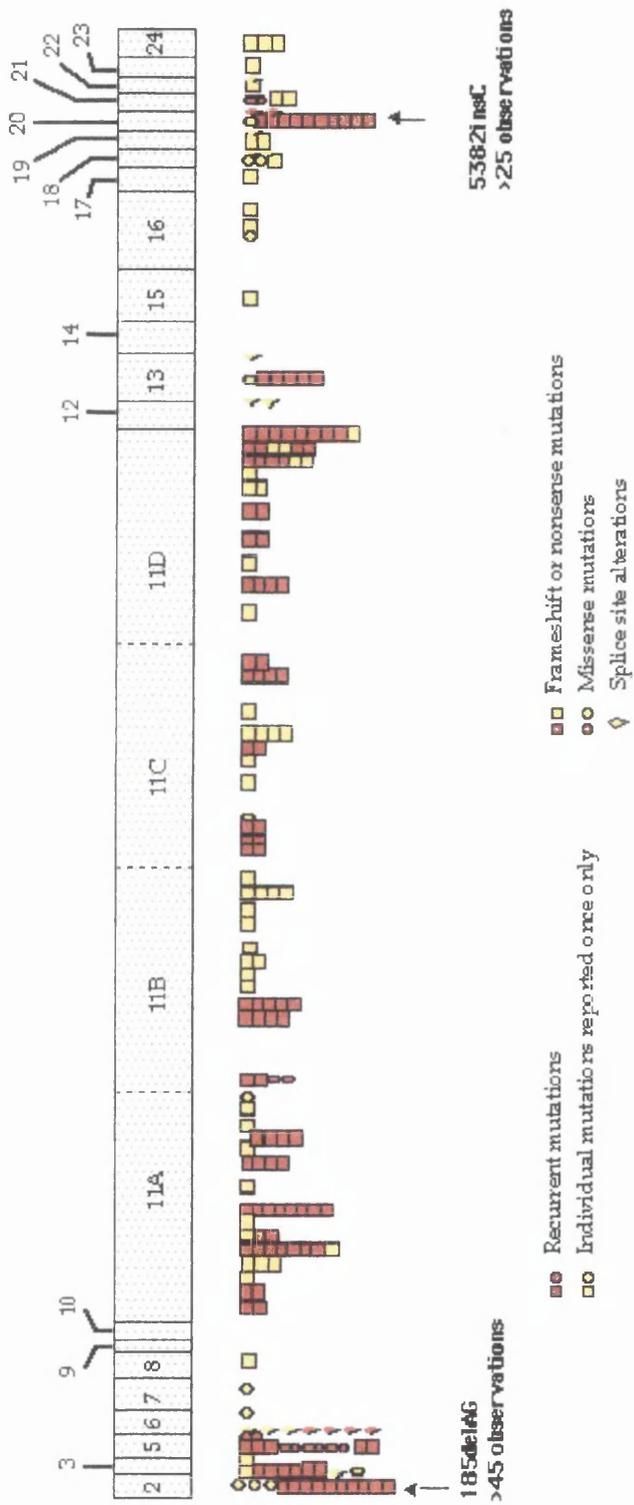
Figure 4.2: Distribution of the reported mutations in the *BRCA1* gene in the BIC database. The mutations 185 delAG and 5382 insC are the most common mutation found so far.

(http://www.nhgri.nih.gov/intramural_research/Lab_transfer/BIC)

4.3 MUTATIONS IN THE *BRCA1* GENE

A total of 5 different alterations were detected in 15 of the 48 ovarian cancer patients analysed (table 3.1). Two of those alterations, g.1380 G>A and g.3771 A>T were novel.

4.3.1 *BRCA1* Gene Polymorphisms

Two polymorphisms were detected in the patient group. The first, g.3667 A>G, was found in four out of forty-eight patients and the second, g.4427 T>C, was found in six out of forty-eight patients (table 4.1).

These two polymorphisms are the most frequently found in ovarian cancer cases and the normal population. In our study, frequency of g.3667 A>G polymorphism in a control population was 6 in 100 and the g.4427 T>C polymorphism in a control population was 10 in 100.

The frequencies found in the study published by Durocher *et al.* for polymorphisms g.3667 A>G were 30% for patient population and 32% for normal population and for polymorphisms g.4427 T>G, they were 30% for patient population and 33% for normal population. One other study published by Janezic *et al.* has revealed the frequencies for polymorphism g.3667 A>G were 49% for patient population and 38% for normal population and for polymorphisms g.4427 T>G, they were 50% for patient population and 41% for normal population (table 4.1). The difference between published frequencies and the frequencies found in this study is noticable. The control and patient population published by Durocher *et al.* were French-Canadians from Quebec, the control and patient population published by Janezic *et al.* were from California, USA. Although,

there is a noticable frequency difference between first two study and the present study, Scottish population is a well-conserved population and frequency for some other genetic mutations can vary even between England and Scotland (for example, Cystic fibrosis mutation R117H is four times higher in Scotland than it is in England, Kazazian *et al.*, 1994). Furthermore, polymorphism g.3667 A>G was also reported 1 out of 7 non-breast cancer patients (15%) (Shen *et al.* 2000) with Hispanic origin and 1 in 15 breast-ovarian cancer patients (7%) from Sweden (Johannsson *et al.* ·1996). In addition, one study has reported that there was 3 times difference for polymorphism Gln356Arg between Breast cancer patients and ovarian cancer patients (Duning *et al.* 1997). Studies published by Durocher *et al.* and Janezic *et al.* mentioned above was patients who had a breast cancer. Therefore, the significant frequency difference might have happened because of population and cancer type difference.

In addition, the 12 bp insertion in intron 20 found in one patient in this study and none of the control samples tested had the same alteration. On the other hand, a study from Poland has reported that 5 out of 122 patients with breast and ovarian cancer and 2 out of 236 control samples had the same chance in their *BRCA1* gene (Sobczak *et al.*, 1997). This may further support that polymorphism frequencies may vary in different ethnicities.

Although the polymorphisms are generally thought to be a benign variation in the gene sequence, histopathological studies suggest that the aggressiveness of tumour progress, the histological type and the survival rate may be related to the presence of these polymorphisms (Narod, 1998).

One study on prostate cancer reported that a polymorphism in the androgen-receptor gene is associated with a doubling of the risk of the cancer spreading beyond the prostate (Giovanucci *et al.*, 1997). However, the polymorphisms and their role in cancer development are still unclear.

BRCA1 Poylmorp.	Predicted effect on coding sequence	Allele frequencies (a) A (b)	Allele frequencies (a) B (b)	Allele frequencies (a) C (b)
g.3667A>G	Lys > Arg	57/182, 50/156 30% 32%	49/100, 38/100 49% 38%	4/48, 6/100 9% 6%
g.4427T>C	Ser > Ser	72/238, 55/166 30% 33%	50/100, 41/100 50% 41%	6/48, 10/100 13% 10%

Simplified figures (%)

Table 4.1: The polymorphisms and their frequencies in ovarian cancer patients (a) and normal population (b). The frequencies in column (A) were published by Durocher *et al.* (1996), column (B) was published by Janezic *et al.* (1999), and column (C) was found in this study.

4.3.2 Two Novel Missense Mutation in *BRCA1* Gene

Two novel missense mutations were detected in the *BRCA1* coding sequence. The mutations, g.3771 A>T, and g.1380 G>A, were found in two out of the forty-eight patients tested.

The mutation, g.3771 A>T, is predicted to result in an amino acid change from serine to cysteine at position 1218. This would alter the granin site (amino acids 1214-1223) and the leucine zipper (amino acids 1209-1230). An indication of the functional importance of a sequence motif, is the conservation of that particular motif across species. The granin consensus sequence is completely conserved in other secreted human proteins including BRCA1, and except for one amino acid (1216), the murine and the canine species are also conserved in the granin motif (Szabo *et al.*, 1996). Biochemical characterization suggests that human BRCA1 shares functional features as well as sequence similarity with the granin family of proteins (Jensen *et al.*, 1996). The same author suggested that BRCA1 is a secreted protein and that its secretion is triggered by activation of cyclic AMP, and induced by estradiol. Further studies have also identified that BRCA1 is also present in the golgi complex. Jensen *et al.* also reported the following points.

- The granins are acidic with isoelectric points between 4.9 and 5.6; the predicted isoelectric point of BRCA1 is 5.2.
- They both have 15-25% acidic residues.
- Both are localised in secretory vesicles.

- The granin was shown to be regulated by estrogens (Fischer-Colbrie, 1991) as was BRCA1 (Marquis *et al.*, 1995).
- BRCA1 and granins are both heat stable.

Like other well-conserved domains in the *BRCA1* gene, granin could be a very important component for the normal functioning of the BRCA1 protein.

The serine to cysteine mutation reported here would be expected to be pathological. It is well established that cysteine has a very important role in stabilising three-dimensional protein structure. Two cysteine residues in different parts of a polypeptide chain but adjacent in the three-dimensional structure of a protein can be oxidised to form a disulfide bridge. Disulfide bridges usually occur in secreted proteins and make the protein less susceptible to degradation (Branden & Tooze, 1991).

The presence of this new cysteine in the BRCA1 protein could destabilise the protein's three-dimensional structure by forming an inappropriate disulfide bridge. It is possible that this change may also lead to molecular degregation and a lower level of BRCA1 expression.

The second missense mutation, g.1380 G>A, is predicted to cause an amino acid change from glutamic acid to lysine at amino acid position 421. The effect of this mutation on the BRCA1 protein is unknown. However, this amino acid is conserved in the murine *BRCA1* sequence (Sharan *et al.*, 1995;

Jensen *et al.*, 1996). Almost all of the missense mutations that are associated with the disease in humans were in amino acids that are conserved between human and murine species. In contrast, most of the amino acids that are involved in polymorphic variations are not conserved between species. This suggests that the interspecies conservation of predicted amino acid sequences could be a functional conservation. These two studies suggest that a certain proportion of uncharacterised variants (missense mutations) may affect BRCA1 function and increase the breast and ovarian cancer risk.

The predicted amino acid change is from glutamic acid to lysine and both amino acids are in the "charged amino acids" group. However, glutamic acid is negatively charged whereas lysine is positively charged. The glutamic acid's isoelectric pH is 3.24 and, lysine's isoelectric pH is 9.12. This amino acid change from acidic amino acid to basic amino acid might therefore affect the net isoelectric charge of the molecule (Ege, 1994).

4.3.3 Unclassified Variant at Nucleotide Position g.5396+60

The insertion of 12 bp (5'-GTATTCCACTCC-3') in intron 20 begins 60 bp downstream of the 3' boundary of exon 20. This variant has been previously described in four cases by Takahashi *et al.* (1995) in five cases by Sobczak *et al.* (1997) and in one case by Robledo *et al.* (1997). In these three studies, the variant was not present in any of the subjects in the control population analysed. It was also reported that this

variant did not modify the correct splicing of exons 19-21 (checked by analysing the cDNA obtained from the patient's peripheral blood).

However, Sobczak *et al.* (1997) reported that splicing errors might be present in other tissues e.g. ovary and breast. It was also suggested that a relatively large insertion in a region of non-repetitive DNA could affect the kinetics of splicing and result in a lower level of mRNA from the mutant allele.

Furthermore, Robledo *et al.* (1997) reported that one case with the 12 bp insertion in intron 20 was also heterozygous for a common polymorphic site in exon 13 (g.4427 C>T). In that study, heterozygosity was also tested using cDNA covering the position of the polymorphism mentioned. Loss of heterozygosity was found suggesting that the intron 20 insertion created an unstable transcript.

Although, all reports suggest that the 12 bp insertion in intron 20 could lead to an unbalanced BRCA1 protein function, drawing a firm conclusion requires further study.

4.3.4 Possible Large Deletion in BRCA1 Gene

The sequencing of sample 28 for fragment exon 11N (figure 3.15) showed a G at position 3667. The normal sequence has an A at this position. This result could be due to homozygosity at this base or to the possibility of a large deletion, which included one or both of the primer sites. For this polymorphism, 100 control samples were selected to find out the frequency in the general population. Applying SSCP analysis and automated sequencing, 6 out of 100 control samples showed this

polymorphism in their BRCA1 gene (discussed under section 4.3.1).

The Expand Long Template PCR Analysis was employed to identify any possible deletion that creates shorter amplified product (figure 3.20). Using a published primer set (section 2.7.3) by Payne *et al.* (2000), 9518 bp of genomic DNA was amplified, including exon 11, 12 and intron 11 (figure 4.3). The result showed that there was no deletion within the amplified region. Southern blotting would be the next choice of analysis to detect any large deletion in this area using the 9518 bp product as a probe.

However, there was no positive control within the region for analysis to compare our possible deletion sample. Southern Blotting technique will be informative when a positive control within the probe region and sample can be tested together for dosage effect.

Not knowing the size of the putative large deletion covering exon 11, increasing the size of probe until the probe includes a known deletion was not feasible. Therefore, The Fluorescence In Situ Hybridisation (FISH) which can detect a large deletions from 40 kb from a genomic DNA sample (Trask, 1991; Grange *et al.*, 1987) might be the next choice to detect any large deletion in the interested area.

| EXON 10 | INTRON 10 | EXON 11 | INTRON 11 | EXON 12 | INTRON 12 |

Expand long Template PCR analysis, 9513 bp

220 bp
11N

11M   11O
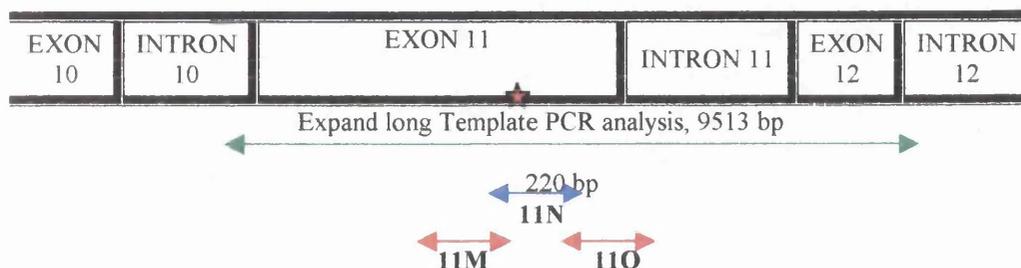
Figure 4.3: Detection strategies for possible deletion in Exon 11 in BRCA1 gene: Possible large deletion that includes Exon 11 has been searched for due to the presence of possible homozygous polymorphisms, g.3667 A>G (indicated by red star).

1. In order to prove that there was no deletion at primer sites for fragment exon 11N, 220 bp, (indicated by blue double arrow) amplified product containing elements from both alleles (if there was no deletion), two other sections of Exon 11 (fragment Exon 11M, 270 bp and 11O, 289 bp)(table 2.3), covering primer sites for fragment 11N was also amplified and SSCP analysis was performed (indicated by two red double arrows).

2. Applying Expand Long Template PCR analysis (section 2.7.3), Exon 11, 12 and Intron 11 have been amplified (the area amplified indicated by green double arrow) and a minimum 100 bp product could be visualised (figure 3.20). A large deletion within described area has not been found.

3. The possibility of a deletion larger than amplified part of BRCA1 gene could be detected by FISH analysis and providing a positive control by Southern analysis.

There is only one large deletion published that included exon 11. This was a 23.8 kb deletion that encompassed exon 8 to 13 (Puget *et al.*, 1999) (table 4.2). Presumably, the large size of exon 11 might not easily allow the *Alu* elements in the BRCA1 intronic regions, which may promote unequal crossing-over, to create intragenic recombination and deletion.

In addition to the argument, the possibility of homozygosity in our sample for polymorphism g.3667 A>G is much higher than the same sample having large deletion that includes exon 11. From control samples, the frequency of this polymorphism was found in 6 out of 100 samples and the ratio that both parents have the same polymorphism is the same as control population. This gives us a 1 in 1102 chance of passing both polymorphic alleles to the next generation (6/100x6/100x1/4). On the other hand, one study from Holland (Petrij-Bosch *et al.*, 1997) has shown no large deletion comprising exon 11 in 170 breast and ovarian cancer patients and considering this published data, the possibility of having one parent a polymorphism and the other parent a large deletion, which includes exon 11, would be much lower.

Without excluding the possibility of a large deletion in BRCA1 gene in sample 28, evidence suggests that a homozygous polymorphism is much more likely.

4.4 DIFFICULTIES AND LIMITATIONS WITH MUTATION SCREENING OF THE *BRCA1* GENE

Mutation screening was found to have difficulties and limitations in this study.

This study was designed to screen for mutations in ovarian cancer patients retrospectively. Therefore, there was no opportunity to collect more blood from these patients for RNA experiments. Only DNA was prepared from the original blood samples.

Two missense mutations found in this study could not be tested for loss of heterozygosity because of the absence of tumour tissues from these patients.

The *BRCA1* gene is a comparatively large gene, spanning 100kb on chromosome 17q21 and contains 24 exons. Screening a gene this size was very time consuming. Forty-seven sets of primers were employed to detect mutations in the *BRCA1* gene using SSCP analysis on 48 patient samples.

Even though the latest and the most advanced analysis kit (TNT® Lysate Coupled Transcription/Translation System) was used to detect any truncated proteins, the success rate of producing a protein was approximately 50%.

The optimisation of PCR conditions was more complicated than for SSCP analysis because of the size differences between forward and reverse primers.

Because of the absence of RNA samples in this study, the mutation found in intron 20 could not be checked to see if it had an impact on splicing between exon 20-21. The most

important limitation was whether or not all *BRCA1* mutations were detected in this study. It has been reported that the success rate for SSCP analysis is nearly 100% if the target DNA size is under 200bp. Almost all the targets in this study were above 300bp.

All samples used in this study had not been screened before and therefore detecting the success rate of SSCP analysis for mutations was not possible. In addition, It has been reported (Ford *et al.*, 1998) that mutation screening techniques might not detect mutations affecting expression or stability of the protein and deletions of large regions of the gene.

On the other hand, the clinical interpretation of the mutations found would be a challenge. The effect of missense mutations and polymorphisms on the *BRCA1* gene is still uncertain because of the absence of a functional assay. Genetic testing based on family history and the age of onset for ovarian cancer may not be as valuable as they are for breast cancer. Therefore, results including missense mutations and polymorphisms found in a patient with a family history and without ovarian cancer could be very difficult to interpret. Although hereditary breast ovarian cancer clinics have been established, the raw genetic data received, concerning missense mutations and polymorphisms, would be further a difficulty for the clinician who might already have problems with medical management of patients who do not have the ovarian cancer.

SIZE OF THE DELETION	AFFECTED AREA OF BRCA1 GENE	FREQUENCY (IF PUBLISHED)	REFERANCE
14 kb	Exons 1,2	1 cases reported	(Swensen *et al.*, 1997)
3 kb	Exon 17	2 /60	(Montagna *et al.*, 1999)
1039 bp	Exon 3	1/406	(Payne *et al.*, 2000)
510 bp	Exon 22	8/170	(Petrij-Bosch *et al.*, 1997)
3835 bp	Exon 13	6/170	(Petrij-Bosch *et al.*, 1997)
~14 kb	Exons 13,14,15,16	1 case reported	(Petrij-Bosch *et al.*, 1997)
7.1 kb	Exons 7,8	2 cases reported	(Rohlfs *et al.*, 2000)
3 kb	Exon 15	1/78	(Puget *et al.*, 1999)
1 kb	Exon 17	1 case reported	(Puget *et al.*, 1997)
510 bp	Exon 22	6 cases reported	Gille et al. 1998 (BIC)
510 bp	Exon 22	4 cases reported	Ligtenberg et al. 1999 (BIC)
510 bp	Exon 22	14 cases reported	Hogervorst et al. 1998 (BIC)

Table 4.2: Most of the published large deletions. Some of the data was also gathered from Breast Cancer Information Core (BIC) (http://www.nhgri.nih.gov/intramural_research/Lab_transfer/BIC).

In addition, the ethical approach to the information obtained from genetic testing is very important. The conflict between respect for autonomy (the right not to know) and the responsibility towards future generations (the duty to know for the sake of one's descendants) ought to be considered. Cancer susceptibility testing should remain confidential because, for example, the possible difficulty in securing employment and life, health or disability insurance. Therefore, genetic testing should also be evaluated for its general and specific ethical implications.

4.5 CONCLUSION

Ovarian cancer is the fifth most common female malignancy and the fourth-leading cause of cancer death in the modern world. An understanding of the molecular pathologies has advanced during the last decade. Screening for the mutations in a candidate gene is still the main strategy to characterize the hereditary alterations. It has been reported that 3-13% of all ovarian cancers are hereditary and the *BRCA1* gene is responsible for 90% of familial ovarian cancers. However, the role of the *BRCA1* gene in tumorigenesis is still not clear. The roles in transcriptional regulation and DNA repair have been proposed so far. In addition to functional uncertainty, localization of BRCA1 protein is also not clear. Because of the absence of a functional assay for BRCA1, the effect of the missense mutations, unclassified variants and polymorphisms found in the *BRCA1* gene remains to be unexplained.

The strategy of using genomic DNA as a template for PCR amplification and the use of SSCP and PTT as the main

screening techniques has proved to be a useful screening strategy for the detection of mutations within the *BRCA1* gene. However, recent studies reported that, large deletions and mutations affecting regulatory sites cannot be detected by these conversional PCR-based screening methods. Therefore, techniques that can detect those mutations such as Expand Long Template PCR analysis, Southern Blotting and even FISH should also be considered.

In this study, using the screening techniques, 5 different alterations were found. Although more than 50% of the mutations reported in the BIC database are frameshift mutations, nonsense mutations, splice variants and regulatory mutations that create premature stop codons leading to truncated proteins, none of them was found in this study. Because of the absence of any functional assay for BRCA1 protein, most of the published data focused on those types of mutations that create truncated protein. On the other hand, nearly one-third of all mutations reported to the BIC database were missense mutations and their importance in BRCA1 function could only be estimated by their localisation and their conservation in species. In addition, polymorphisms to date are accepted as variations in the gene with no effect on the function of the protein.

The mutation frequency in this study is lower than the published data. The reasons for this low mutation frequency may be that:

Some mutated cases may have escaped detection, because of the technical insufficiencies of the methods used.

Mutant *BRCA1* alleles bearing duplications, translocations or large deletions, which affect the target sequence for the PCR primers, are likely to remain undetected.

Testing possible sporadic cases because of their small family size or large numbers of male descendants may give rise to false negative results. Only single individuals from each family were analysed, and it is possible that some of these may have been phenocopies.

Finally, *BRCA1* is not the only gene responsible for tumorigenesis in ovarian cancer. BRCA2 is also responsible for more than 10% of all hereditary cases. As mentioned before, large deletion may not be detected; therefore, some of the unexplained familial cases can also be attributed by the presence of large deletions.

For the reasons mentioned above, it is probably premature to exclude the *BRCA1* gene as a cause in all patients in whom we were not able to find mutations.

The control samples for frequency analysis of polymorphisms g.3667 A>T and g.4427 C>T also showed lower frequencies for polymorphisms mentioned.

The carrier frequency for *BRCA1* gene mutations is estimated to be 1 in 500 in the general population. Using the number of family members affected and the age of onset are the only two main diagnostic parameters used to make an intelligent selection for hereditary cases. Mutation screening and characterisation in the *BRCA1* gene has been and is difficult because of its size. Mutation detection technology has limitations and is another factor adding difficulty to the

screening strategies. Furthermore, the mutations are well spread over the entire length of the coding sequence.

4.10 FUTURE WORK

Although, the *BRCA1* gene has been cloned and a number of frequently encountered inherited mutations are recognized, the function of BRCA1 protein in tumorigenesis is still not clear. Its relation to p53 and Rad51 and its function in transcription regulation and DNA repair mechanisms are so far only hypothesized. In addition, nearly half of the mutations are missense mutations, polymorphisms and unclassified variants. Because of the absence of any functional assay, their functional damage in BRCA1 cannot be established. Therefore, the development of a functional assay will help, greatly, the identification of mutations, which may lead to cancer.

As mentioned before, mutation screening in a gene of this size has many limitations. Time and cost effectiveness will depend on the technique used and the selection of patients for testing. The patients with a family history of breast and ovarian cancer and early age of onset help to establish their hereditary status. Very recently, a mutation-screening project, for breast and ovarian cancer and involving this department, has been funded by the National Services Department Scotland (NSD). The new technology known as "WAVE® HS Nucleic Acid Fragment Analysis System" has been introduced. The accuracy in this system is provided by Temperature Modulated Heteroduplex Analysis (THMA) with fluorescently labeled DNA fragments.

It is believed that this new system will provide much more accurate, sensitive, and rapid results.

Finally, as much as the determination to find mutations in the *BRCA1* gene responsible for ovarian cancer is appreciated, the clinical importance of the molecular findings must be determined. Proper guidelines and procedures for following a patient with a *BRCA1* mutation with or without ovarian cancer should be established.

In addition, an integral part of a mutation screening program should be the counselling of patients about the risks, benefits, likely outcomes and options of testing and treatment. Patients who participate in a molecular screening program for ovarian cancer require substantial pre and post screening counselling.

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