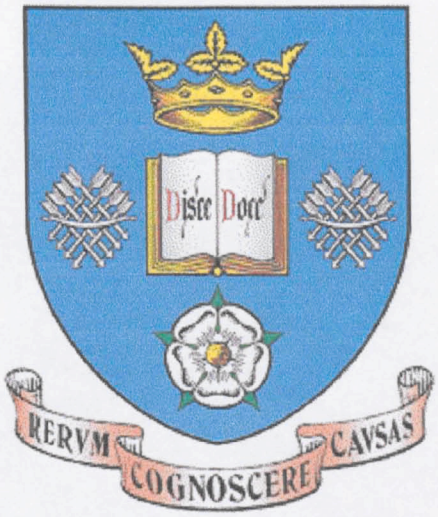
Towards a general strategy for Breast Cancer;
Investigation of germline mutations of BRCA1 and BRCA2 genes in Iranian women with early-onset Breast Cancer



A thesis submitted to the University of Sheffield for the Doctorate degree of Philosophy

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Department of Molecular Biology and Biotechnology
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April 2002

IN THE NAME OF GOD, THE COMPASSIONAL, THE MERCIFUL

To my lovely wife and my mother's soul

ACKNOWLEDGMENTS

I am indebted to the members of the breast cancer families whose participation in the present study that led to precious results to the dedicated Iranian researcher and made millstone for the further study.

I would like to express my deepest gratitude to my supervisors Professor David P. Hornby and Dr Ann Dalton for all their support during my project, and for an inordinate amount of tolerance and patience during the production of the bound thesis.

I am grateful to all my colleagues at the Molecular Genetics Laboratory in Sheffield's Children hospital, especially to Dr Steve Evans for his technical support and to Dr Rob McMahan for his comments. To all my colleagues in D27 and D29 in Department of Molecular Biology and Biotechnology at the University of Sheffield, particularly Dr Maryam Matin, Dr Ahmad Bahrami, Dr Qaiser Sheikh for their comments. To Mrs. Pam Smith for her secretarial help.

To my colleagues in Iran, Dr Iraj Harirchi and his associates at ICBC, Dr Mohammad Ali Mohagheghi and his co-worker at the Cancer Institute, Dr Sirous Zeinali at the Pasture Institute for their participants during this study. To my government, Ministry of Health and Medical Education, which provide me this award. Wish to be able to compensate to my loved country.

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Towards a general strategy for Breast Cancer: Investigation of germline mutations of BRCA1 and BRCA2 genes in Iranian women with early-onset Breast Cancer

Submitted to the University of Sheffield for the Doctorate degree of Philosophy

April 2002

Vahid Reza Yassaee (M.D.)

Summary

Breast cancer is the most common female malignancy and a major cause of death in middle-aged women. It results from genetic and environmental factors leading to the accumulation of mutations in essential genes, BRCA1 and BRCA2. To date, germline mutations in the BRCA1 and BRCA2 genes in patients with early-onset breast and/or ovarian cancer have not been identified within the Iranian population.

This study was set for two main purposes, in first for a cohort study of selected population (Iranian women) with early-onset breast cancer and secondly to evaluate and improve upon existing mutation detection techniques with respect to the BRCA genes.

With the collaboration of two main centres for cancer research and treatment in Tehran-Iran, clinical information, family history and peripheral blood were obtained from 96 unrelated families for scanning of germline mutations in the BRCA1 and BRCA2 genes. These sets of samples consists of 104 women under the age of forty-five, 88 patients affected with early-onset breast cancer or ovarian cancer and 16 unaffected individuals with strong family history of breast and/or ovary cancer.

BRCA1 exons 11 and BRCA2 exons 10 and 11 by the Protein Truncation Test (PTT) and BRCA1 exons 2, 3, 5, 13 and 20 and BRCA2 exons 9, 17, 18 and 23 with the Single Strand Conformation Polymorphism (SSCP) assay were analysed on genomic DNA amplified by polymerase chain reaction.

Ten sequence variants were identified: five are frameshift (putative mutations-four novel); three missense changes of unknown significant and two polymorphisms, one seen [BRCA2 (IVS16-14T>C)] commonly in both Iranian and British population.

Identification of these novel mutations suggests that any given population should develop a mutation database for its breast cancer screening. The pattern of mutations seen in the BRCA genes does not appear to differ from other populations studied. Early-onset breast cancer (less than 45 years) and a limited family history is sufficient to justify mutation screening with a detection rate of over 25% in this group, whereas sporadic early-onset breast cancer (detection rate less than 5%) is unlikely to be cost-effective.

To address the penetrance and mutation spectrum of germline mutation of the contributed genes within Iranian population further studies should be performed.

Meta-PCR technique was evaluated for its implication of BRCA genes scanning. Three distinct sets of BRCA gene fragments were selected to assemble with different approach for downstream analysis: the first set consisted of BRCA1 exons 2, 20 and BRCA2 exon 18 and their subsequent analysis by Protein Truncation Test; the second set comprised BRCA1 exons 2, 20, 23 and 24 and their subsequent analysis by direct sequencing; and the last one contained six key coding regions from the BRCA genes, the 5' and 3'termini of exon 11 from both BRCA1 and BRCA2 genes and

exons 2 and 20 from BRCA1. Downstream analysis of Meta-PCR products by Protein Truncation Test was used rather than direct nucleotide sequencing because the total assembled above fragments size (~2.8kb) is sufficiently big to ignore analysing by the latter approach. PTT and direct sequencing were chosen because of their high sensitivity and specificity. These three trials were performed successfully suggesting that it may be possible to assemble the entire of coding regions of BRCA1 and BRCA2 genes in a multi-step procedure.

ABBREVIATIONS

AMPS	Ammonium persulfate
Bis-acrylamide	N',N', methylene bis-acrylamide
bp	Base pair
BC	Breast Cancer
BRCA1	Breast Cancer susceptibility gene 1
BRCA2	Breast Cancer susceptibility gene 2
cDNA	Complementary deoxyribonucleic acid
CSGE	Conformation Sensitive Gel Electrophoresis
C-terminus	Carboxyl terminus
Del	deletion
DNA	Deoxyribonucleic acid
dH2O	Distilled water
dNTPs	2'-deoxyribonucloside- 5'-triphospate (where N is any nucleotide)
Ds	Double stranded
DS	Direct Sequencing
EDTA	Ethylenediamine tetra-acetic acid
EtBr	Ethidium Bromide
EtOH	Ethanol
Ins	Insertion
Kb	Kilobase pair(s) of nucleotides

kDa	kiloDalton
LOH	Loss of Heterozigosity
ml	Mili litre
mRNA	messenger Ribonucleic Acid
Mu	Mutant
MW	Molecular weight
N-terminus	Amino terminus
°C	Centigrade
OD	Optical Density
Oligo	Oligodeoxynucleotide
pM	Pico mole
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PTT	Protein truncation test
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SSCP	Single strand conformational polymorphism
TAE	Tris-acetic acid-EDTA
TBE	Tris-boric acid-EDTA
TEMED	N',N',N',N'–tetramethyl – 1,2-diaminoethane
Tm	Melting temperature

TS	Tumour suppressor
µl	Micro litre
µM	Micro mole
Wt	Wild type

All other abbreviations are explained in the text, or are the same as the internationally set out rules in "Biochemical Nomenclature and Related document"(1978)

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PRESENTATION AND PUBLICATION

Presentations:

1) Novel mutations in the BRCA1 and BRCA2 gene in Iranian women with early-onset Breast Cancer. 10th International Congress of Human Genetics, 15-19 May 2001, Vienna, Austria

2) Gene assembling, a new approach in mutation detection techniques; an application for BRCA genes scanning. Human Genome Meeting 2002, 14-17 April 2002, Shanghai, China

Publication:

1) Novel mutations in the BRCA1 and BRCA2 gene in Iranian women with early-onset Breast Cancer. Breast Cancer Research Journal. 2002; Vol 4 No.4

INDEX OF APPENDICES

Note: A full detail of appendices is presented as an appended compact disk (CD).

Chapter 1

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Chapter 1

1 Introduction

1.1 Cancer is the natural end-state of multicellular organisms

The evolution from a normal somatic cell to a malignant tumour takes place within the life of an individual, and has to start afresh with each new individual. However, an individual with a sound anti-tumour mechanism transmits it to its offspring, where it continues to evolve. A billion years of evolution have endowed us with sophisticated overlapping mechanisms to protect us against tumours, at least during our reproductive life. Potential tumour cells are either repaired and brought back into line, or made to kill themselves (apoptosis). No single mutation can escape these mechanisms and convert a normal cell into a malignant one. Long ago, studies of the age-dependence of cancer suggested that on average 6-7 successive mutations are needed to convert a normal cell into an invasive carcinoma [1]. In other words, only when half a dozen independent defences are disabled by mutation can a normal cell progress into a malignant tumour.

The chance of a single cell undergoing six independent mutations is negligible, suggesting that cancer should be vanishingly rare. However, two general mechanisms exist that can allow the progression to happen (Box 1.1).

Box 1.1

Two ways of making a series of successive mutations more likely

Turning a normal cell into a malignant cancer cell requires perhaps six specific mutations in the one cell. If a typical mutation rate is 10^{-7} per gene per cell, it is extremely unlikely that any one cell should suffer so many mutations (which is why most of us are alive). The probability of this happening to any one of the 10^{13} cells in a person is $10^{13} \times 10^{-42}$, or 1 in 10^{29}.

Cancer nevertheless happens because of a combination of two mechanisms:

- Some mutations enhance cell proliferation, creating an expanded target population of cells for the next mutation (Figure 1.1)
- Some mutations affect the stability of the entire genome, at either the DNA or the chromosomal level, increasing the overall mutation rate.

Because cancer depend on these two mechanisms, they always develop in stages, starting with tissue hyperplasia or benign growths, while malignant tumour cells usually advertise their genomic instability by their bizarre karyotypes.

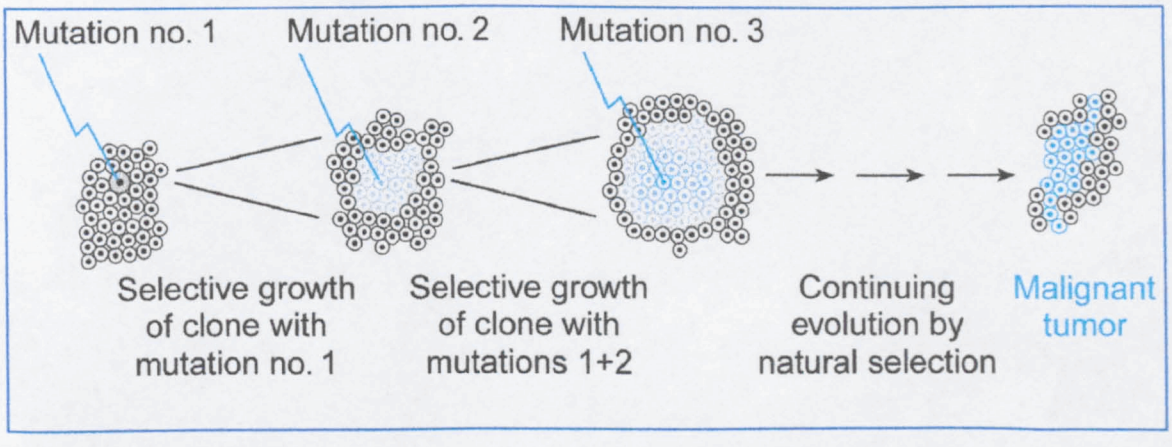


Figure 1.1: Multi stage evolution of cancer
Each successive mutation gives the cell a growth advantage, so that it forms an expanded clone, thus presenting a larger target for the next mutation.

Accumulating all these mutations nevertheless takes time, so that cancer is mainly a disease of post-reproductive life, when there is little selective pressure to improve the defences still further [2].

1.2 Mutations in cancer cells typically affect a limited number of pathways

Cancer is a multifactorial disease involving the interplay of environmental, hormonal, and dietary risks in addition to genetic predisposition.

However, progression of a single cell from a normal to a neoplastic state always involves a series of genetic changes that alter either the regulation or the function of a variety of different genes. Such genes may play roles in a number of overlapping physiologic processes, including genome maintenance, cell cycle control, apoptosis, contact inhibition, invasion and metastasis, or angiogenesis [3]. These cancer genes are often classified in two main categories, oncogenes and tumour suppressor genes.

1.2.1 Oncogenes

Oncogenes are a class of genes that play a role in the growth of cells but when overexpressed by mutation, amplification, translocation, or other means, they can foster the growth of cancer, uncontrolled by the normal signalling pathways of the cell. Oncogenes typically encode for proteins that stimulate mitosis or inhibit apoptosis [4, 5]. The classic definition of an oncogene is a cancer-causing gene carried by an acute transforming retrovirus that has a normal counterpart (homologue) referred to as a proto-oncogene [4]. Cancer-causing oncogenes were identified and generally named based on the virus in which they were originally carried. For example, SRC is an oncogene from the Rous sarcoma virus, SIS from the Simian sarcoma virus, RAS from the Rat sarcoma virus, and so forth [6].

In the 1970s it was discovered that oncogenes and proto-oncogenes encode proteins involved in signal transduction, the orderly and specific transmission of growth-regulatory messages from outside the cell to the machinery controlling replication inside the cell's nucleus [7].

When signalling growth in a normal orderly fashion, oncogenes are termed proto-oncogenes. Mutations and other alterations of the proto-oncogenes turn them into oncogenes, which are unresponsive to outside influences, leading to a permanent activation of the pathway promoting cellular division and growth, thus leading to cancer.

Oncogene mutations are dominant at the cellular level. In other words, they produce an effect when only one allele is altered, regardless of the presence of the corresponding wild-type protein. Most oncogenes are altered in somatic and not germline cells. The notable exception is the RET gene. Mutations in this oncogene can be inherited and can cause multiple endocrine neoplasia type 2, medullary thyroid cancer, and/or Hirschsprung disease [8, 9].

1.2.2 Tumour suppressor genes

Tumour suppressor (TS) gene products inhibit events leading towards cancer. Such genes exert their influence predominantly at or before DNA synthesis in S phase of the cell division cycle. Some TS gene products prevent cell progression, some steer deviant cells into apoptosis, and others (Mutator genes-DNA replication repair genes) keep the genome stable and mutation rates low by ensuring accurate replication, repair and segregation of the cell's DNA in G2 phase for mitosis. By definition, both alleles of a TS gene must be inactivated to change the behaviour of the cell [4, 10].

The distinction between these two categories is that tumour progression is promoted by over-expression or gain of function in oncogenes but by non-expression or loss of function in tumour suppressor genes.

Loss-of-function mutations are much more common than gain-of-function mutations in inherited predisposition, presumably because the loss of function is masked by the remaining normal allele during development

(except in the recessive DNA-repair deficiencies), whereas a gain-of-function cancer-promoting mutation might well be lethal [11].

1.2.2.1 Loss-of-function genetic events

Tumour suppressor mutations are also known as loss of function mutations, because they inactivate the protein and act in a recessive manner, i.e., they are silent in the presence of the corresponding wild-type protein. The first evidence for loss-of-function genetic changes came from studies of children's cancers, in particular retinoblastoma. Like many cancers, retinoblastoma occurs in an inherited and a sporadic form. Knudson [12] described the distribution of age at diagnosis in inherited and sporadic cases. In inherited cases the distribution was consistent with a requirement for one further event for tumour formation. This event occurred with constant probability over time. In sporadic cases, the age distribution was more complex, and consistent with a need for two events. The inference was that in either case, two rate-limiting events were needed to form the tumour, and that in inherited cases one of these was already present in the germline. Comings et al, 1973 [13] suggested that the two events might affect the two alleles of a single gene, implying that their effects would be recessive at the cellular level. Subsequently, in some inherited cases, a germline deletion was found on chromosome 13, implying that loss of a gene in that region might be the first event. This led to biochemical and molecular studies, which showed that tumour development did indeed require loss of both copies of that region of chromosome 13 [14]; using the chromosomal deletions as signposts, the Rb gene was ultimately cloned and found to be mutated in both copies in the tumours. Rb is thus the prototype of the class of tumour-suppressor genes [10] where, in contrast to oncogenes, loss of function is required for tumourigenesis.

For some tumour suppressor genes, loss of function of the first allele is usually a somatic event; for others, a deleterious predisposing allele is usually inherited [15]. BRCA1 gene falls into the second category.

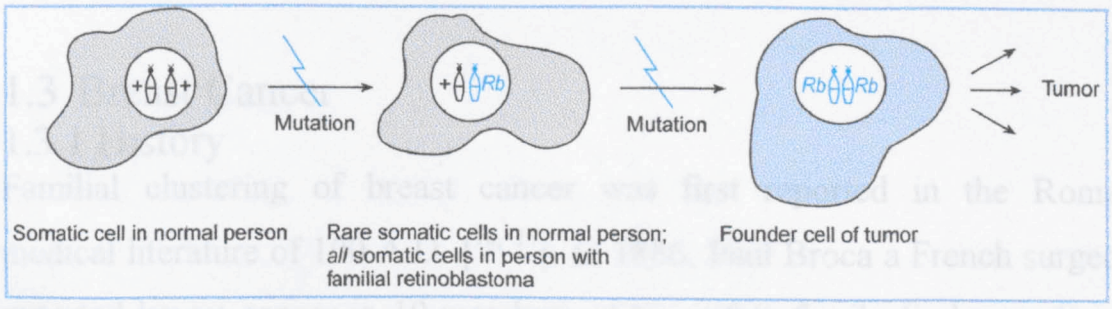


Figure 1.2: Knudson's two-hit hypothesis (Diagram adapted from Ref.2)
Suppose there are one million target cells and the probability of mutation is 10^{-5} per cell. Sporadic retinoblastoma requires two hits and will affect 1 person in 10000 ($10^6 \times 10^{-5} \times 10^{-5} = 10^{-4}$), while the familial form requires only one hit and will be quit highly penetrant ($10^6 \times 10^{-5} = > 1$).

1.2.3 Gatekeeper and caretaker genes

The 'classical' tumour suppressors, termed 'gatekeepers' by Kinzler and Vogelstein [3, 15], which subsequently have been called 'caretakers' genes involved in DNA repair and genome integrity and that are responsible for the fidelity of information transfer. Loss of function mutations in these genes lead to a general genetic instability that has long been recognised as a feature of cancer cells. Gatekeepers are genes that directly regulate tumour growth by inhibition or by promoting cell death. Both copies of the gene must be altered to develop cancer. In contrast, inactivation of a caretaker gene does not initiate a tumour directly. Cancer occurs indirectly; caretaker gene inactivation leads to genetic instability that results in increase in the likelihood that surviving mutations in all genes, including the gatekeeper class. Mutation in caretaker genes would not be expected to lead to sporadic cancers very often, as four mutations would be required (two caretaker alleles and two gatekeeper alleles).

Kinzler and Vogelstein (1997) proposed that the BRCA1 and BRCA2 genes should be added to the list of caretaker genes. Consistent with this hypothesis, mutations in BRCA1 and BRCA2 gene are rarely found in sporadic cancers [15].

1.3 Breast Cancer

1.3.1 History

Familial clustering of breast cancer was first reported in the Roman medical literature of 100 A.D. [16, 17]. In 1886, Paul Broca a French surgeon reported breast cancer in 10 members of his wife's family (in her pedigree at 4 generations)[18]. Since then, many studies have shown an increased risk of breast cancer in relatives of breast cancer cases, compared to the general population. It has been revealed that risk of breast cancer in relatives' increases with the decreasing age of the index case. The risk is also higher for women with several affected relatives in the family than those with one affected relative [19, 20].

1.3.2 Breast cancer definition

Breast cancer is a disease in which breast cells proliferate abnormally. The diagnosis of breast cancer is established histologically. Breast cancer may present as a breast lump, thickening, or skin change. Non-palpable cancers may be detected by mammography. A biopsy is necessary to confirm the diagnosis and determine the type of cancer present. When breast cancer cells metastasise from the original tumour and enter the blood stream or lymphatic system, they can form secondary tumours in other parts of the body. Bilateral cancer is diagnosed when separate primary breast cancers arise in each breast; multifocal breast cancer is diagnosed when breast cancer is present in more than one site in the same breast. Breast cancer is staged from 0 to IV, where 0 is a non-invasive tumour, Stage I is a small locally invasive tumour without lymph node involvement, Stage II is a

medium-sized tumour with or without nodal metastases, Stage III cancer is a locally advanced cancer, usually with axillary node metastases, and Stage IV cancer has already metastasised to distant sites [21]. The survival rate is dependent upon the stage at which breast cancer is diagnosed.

Approximately 5% of benign breast biopsies reveal both excessive cell growth (hyperplasia) and cells that are abnormal (atypia). A diagnosis of atypical hyperplasia increases the risk for future breast cancer.

1.3.3 Breast cancer classification

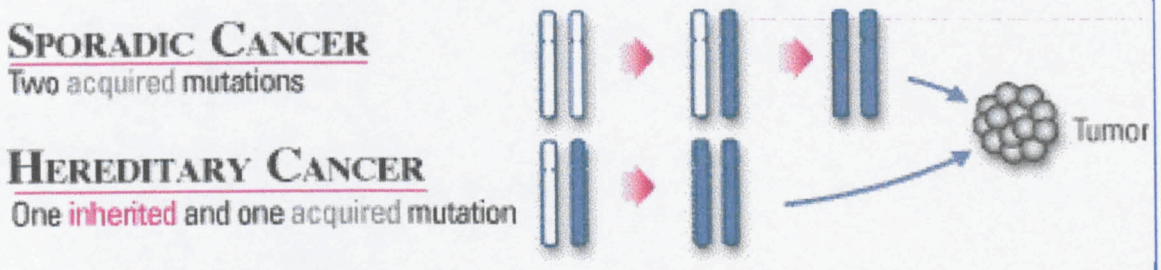
Breast cancer has been subdivided into two main types, early-onset and late-onset, a division that is based on an inflection in the age-specific incidence curve around age 50. Mutation of one gene, BRCA1, is thought to account for approximately 45% of families with significantly high breast cancer incidence and at least 80% of families with increased incidence of both early-onset breast cancer and ovarian cancer [22] Figure1.3.

Late-onset breast cancer is often familial in origin, although the risks in relatives are not as high as those for early-onset breast cancer [23, 24]. The percentage of such cases that are due to genetic susceptibility is unknown.

Breast cancer is considered a multifactorial disorder caused by both non-genetic and genetic factors. It has been categorised into three groups: Hereditary, familial and sporadic [16].

Figure 1.3: Breast Cancer mutation types

- Late onset, Sporadic, somatic mutation, ~90%- 95%
- Early onset, Hereditary, germline mutation, ~5%-10%



The majority of Breast & Ovarian cancers are sporadic in nature and occurs after menopause

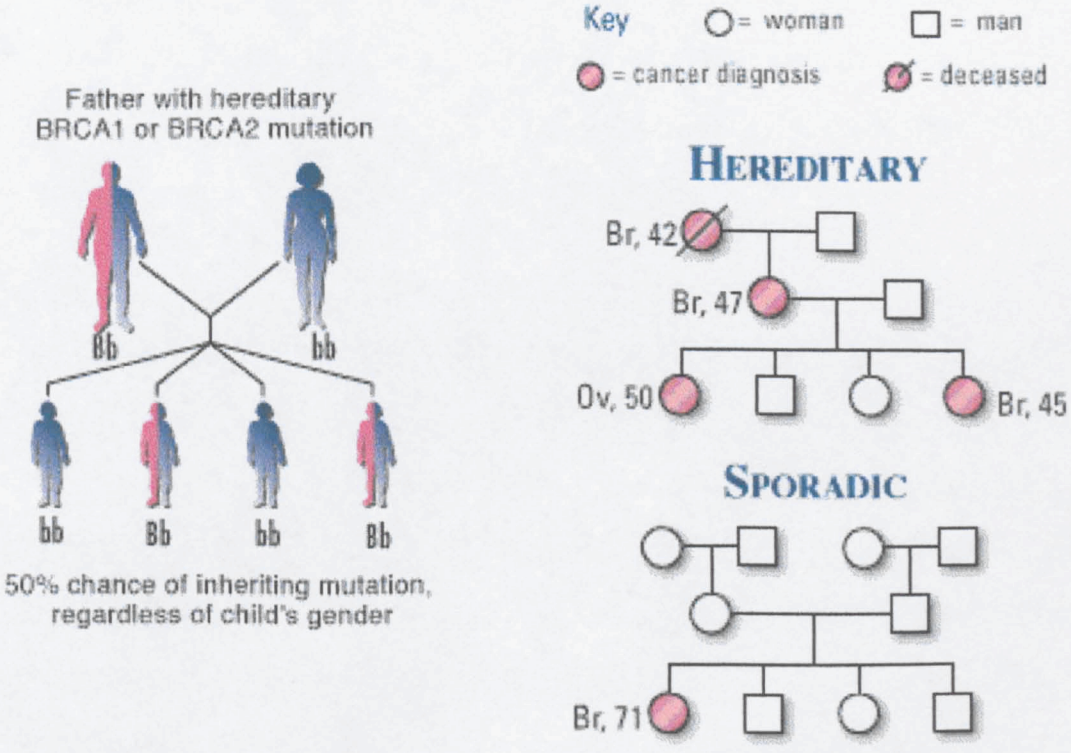
1.3.3.1 Hereditary breast and ovarian cancer

Hereditary breast and ovarian cancers are suspected in an individual who has a family history of breast cancer or breast and ovarian cancer consistent with autosomal dominant inheritance [25, 26, 27]. Other features include an early age of onset (*diagnosis before menopause*) that is considered a cardinal of inherited susceptibility, increased incidence of bilateral disease, and, occasionally, the occurrence of male breast cancer. Hereditary breast cancer is believed to account for 5-9% of all breast cancers [16, 28, 29].

Women and men normally possess two copies (alleles) of BRCA1, one on each copy of chromosome17. Women who inherit one mutated copy of the BRCA1 gene from either parent are at a much greater risk of developing breast and ovarian cancer. This is because a cell with one mutated allele does not have a "backup" if the other allele is damaged or otherwise lost. That is, mutations in these genes can be passed down from either mother or father, and each male or female offspring has a 50% chance of inheriting susceptibility (Figure 1.4).

Half of all women who carry mutations therefore have inherited susceptibility from their fathers. That means a woman can carry a BRCA mutation even if there is little or no history of breast or ovarian cancer on her mother's side of the family. For this reason, it is important to assess both the paternal and maternal sides of the family when evaluating a woman's risk of hereditary breast and ovarian cancer.

Figure 1.4: Mendelian pedigree patterns of BRCA1 and BRCA2 gene inheritance



Diagrams adapted from http://www.myriad.com/med/brac

1.3.3.2 Familial breast cancer

The terms familial breast cancer and hereditary breast cancer are frequently interchanged. The latter refers to cancer that occurs when the family history suggests a highly penetrant major gene for breast cancer, characterized by early age of onset, high incidence of bilateral disease and association with other tumours. This is distinct from familial breast cancer, which implies

that one or more first-degree relatives have had breast cancer and some familial factors are thought to underlie development of the disease and dose not fit to the hereditary breast cancer definition [29].

1.3.3.3 Sporadic breast cancer

In contrast to the above, sporadic breast cancer is associated only with acquired or somatic mutations that occur during a person's lifetime. An individual is the only one with breast cancer among her family and has no family history of breast cancer in her two generations [26, 27]. Since 30-70% of sporadic breast and ovarian cancers exhibit LOH on chromosome 17q12-q21, it was expected that somatic BRCA1 mutations would exist in breast and ovarian tumours [30]. However no BRCA1 somatic mutations have been described in sporadic breast cancer.

1.3.4 Genetic syndromes

Less than 1% of all breast cancer is associated with the genetic syndromes: Cowden syndrome (adenomas and follicular cell carcinomas of the thyroid gland, polyps and adenocarcinomas of the gastrointestinal tract, and ovarian carcinoma), Li-Fraumeni syndrome (childhood leukaemias, brain tumours, adrenal carcinomas and soft tissue sarcomas), Peutz-Jeghers syndrome (characterized by hamartomatous polyps in the small bowel and pigmented macules of the buccal mucosa, lips, fingers and toes), Werner's syndrome, and xeroderma pigmentosum [31]. However, patients with these disorders may have a high breast cancer risk.

1.3.5 Breast cancer formation

Cancer results from the accumulation of mutations in genes that regulate cellular proliferation. These mutations can occur early in the process of malignant transformation or later, during progression to an invasive carcinoma. The earliest mutations occur in the germline, as in the case of

cancer-prone families. In these instances, the inheritance of a mutated allele is commonly followed by the loss of the second allele from a somatic cell, leading to the inactivation of a tumour-suppressor gene and triggering malignant transformation. A classic example is hereditary retinoblastoma, in which there is inheritance of a mutant germline RB1 allele (a tumour-suppressor gene) followed by somatic mutation of the normal RB1 allele [12, 32, 33]. Genes important to the development of cancer regulate diverse cellular pathways, including the progression of cells through the cell cycle, resistance to programmed cell death (apoptosis), and the response to signals that direct cellular differentiation [3]. Moreover, the inactivation of genes that contribute to the stability of the genome itself can favour the acquisition of errors in other genes that regulate proliferation [34, 35]. Errors in DNA that arise during normal replication of the molecule (nucleotide mismatches) or that are induced by ionising radiation or genotoxic drugs can cause mutations in coding sequences or breaks in double-stranded chromosomal DNA. If the nucleotide mismatch is not repaired before a round of DNA replication occurs, that mutation is transmitted to daughter cells. An unrepaired break in double-stranded DNA can cause a mitotic catastrophe when the cell attempts to segregate broken chromosomes. Genes that sense damaged DNA and cause the arrest of the cell cycle allow time for the molecular defect to be repaired. These genes operate at several specific "checkpoints" in the cell cycle as a means of ensuring genomic integrity before DNA is synthesized. The most critical checkpoint gene yet identified that is related to cancer in humans is the tumour-suppressor p53. This gene is not essential for cell viability, but it is critical for monitoring damage to DNA. Inactivation of p53 is an early step in the development of many kinds of tumours [36, 37] (Figure 1.8 and 1.9).

1.3.6 Predisposition Genes

1.3.6.1 Breast Cancer 1 gene (BRCA1- OMIM 113705)

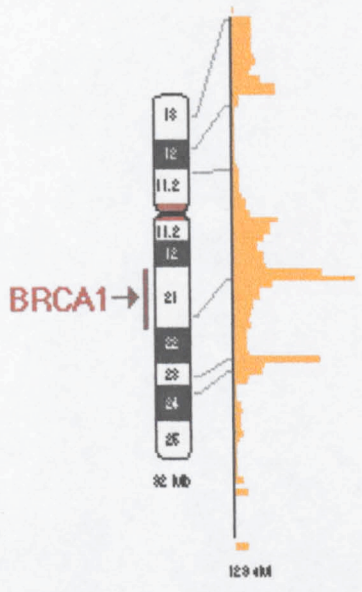


Figure 1.5: The BRCA1 gene maps to chromosome 17

In 1990, the first breast cancer susceptibility gene, BRCA1, was localised using linkage analysis to chromosome 17q21 [38] Figure 1.5.

The following year, it was demonstrated that several ovarian cancer families exhibited linkage to the same locus. In 1994, Miki and associates [39] described a large gene named BRCA1, consisting of 22 coding exons distribute over approximately 100 kb of genomic DNA. BRCA1 contains 24 exons, the first of which is non-coding; exon 4 consists of a repetitive sequence of the Alu family that is omitted from most transcripts; and exon 11 contains 3.5-kb of the entire 5.6-kb coding sequence. The transcript is abundant in testis, thymus and is also present in breast and ovary tissue [39].

BRCA1 codes for a protein of 1863 amino acids, producing a protein of about 220 kilo Dalton (kDa). It is normally located in the nucleus and contains phosphorylated residues [40]. It contains only two recognizable protein motifs, a RING finger domain near the N-terminus (exons 2-5) comprising residues 1-112 of BRCA1 [39], and a BRCT domain at the carboxyl terminus, two tandem copies of a motif (designated the BRCT domain), which are located at residues 1699-1736 and 1818-1855 [41].

1.3.6.2 Breast Cancer 2 gene (BRCA2- OMIM 600185)

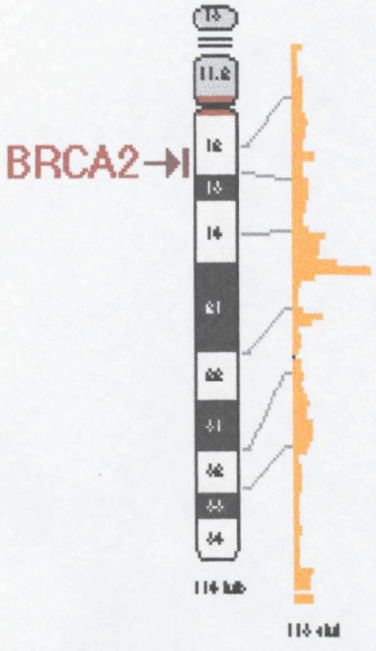


Figure 1.6:The BRCA2 gene maps to chromosome 13

Mutations in BRCA1 were estimated to be responsible for approximately 45% of breast cancer families and for the majority of breast/ovarian cancer families [22]. Therefore in 1994 Stratton et al excluded a major role of BRCA1 in male breast cancer susceptibility.

It was postulated that the majority of remaining breast cancer families would also be due to mutations in other gene and led to the identification of BRCA2 gene on chromosome 13q12-13 (Figure 1.6).

The new gene, BRCA2, was localised to a region of approximately 6 cM between D13S289 and D13S267 on chromosome13 [42, 43, 44].

BRCA2 is a large gene consisting of 26 coding exons distributed over 70kb genomic DNA, encoding a protein of 3418 amino acids making a 384 kDa protein. Both BRCA1 and BRCA2 genes have large exons 11 (3426 and 4932 base pairs, respectively) and a translational start site is located in exon 2 [42]. The BRCA2 protein is normally located in the nucleus and contains phosphorylated residues [45]. The BRCA2 protein has no recognizable structural motifs and no apparent relation to the BRCA1 protein. Nonetheless, the BRCA1 and BRCA2 proteins appear to share a number of functional similarities that may suggest why mutations in these genes lead to a specific hereditary predisposition to breast and ovarian cancer.

Like BRCA1, BRCA2 is expressed in most tissues, in breast and thymus, but the highest level of transcript was found in testis [44]. The higher

contribution of BRCA2 gene to male breast cancer suggests that these two genes may have different functions.

1.3.7 BRCA 1 and BRCA2 gene functions

The breast cancer susceptibility genes BRCA1 and BRCA2 encode multifunctional proteins, the mutant phenotypes of which predispose both to breast and to ovarian cancer [38, 39, 43]. Tumourigenesis in individuals with germline BRCA mutations requires somatic inactivation of the remaining wild-type allele [46]. Thus far, the following important biological roles for BRCA1 and BRCA2 have been demonstrated.

1.3.7.1 Role in genomic maintenance and DNA repair

A variety of lines of evidence now point to BRCA1 and BRCA2 genes as being directly involved in the DNA repair process. The involvements of BRCA1 and BRCA2 gene in complexes that activate double-strand break repair and initiate homologous recombination links the maintenance of genomic integrity to tumour suppression [47, 48, 49]. Therefore, mutations that result in loss of BRCA1 or BRCA2 function might lead to an overall decrease in a cell's ability to repair damaged DNA, resulting in increased somatic mutation, abnormal chromosomal segregation and aneuploidy.

BRCA2 and RAD51 interact and co-localize in a BRCA1-BRCA2-RAD51 complex [47, 50]. Eukaryotic RAD51 proteins are homologues of bacterial RecA and are required for recombination during mitosis and meiosis and for recombinational repair of double-strand DNA breaks [51].

Multiple changes occur in BRCA1 in response to DNA damage. It undergoes distinctive phosphorylation that differs from the phosphorylation during the G1–S transition [52]. RAD51, BRCA1 and BARD1 collectively relocate to chromosomal regions of DNA replication, marked by the

proliferating cell nuclear antigen, PCNA [52]. Phosphorylation of BRCA1 in response to DNA damage is dependent on ATM [34].

Several experiments suggest a role for p53 (TP53) in the development of BRCA1 and BRCA2 tumours in mice. BRCA1 or/and BRCA2 knockout mice can be partially rescued by crossing with a p53 knockout strain suggesting that these genes interact with the p53 mediated DNA damage checkpoint [53]. Therefore, the available evidence indicates that BRCA genes serve as a "gatekeeper," like p53, helping to maintain genomic integrity [54]. When this function is lost, it probably allows for the accumulation of other genetic defects that are themselves directly responsible for cancer formation. Possible models for the involvement of BRCA1 and BRCA2 in response to DNA damage are shown in Figure 1.8.

1.3.7.2 Role in development and normal cell proliferation

BRCA1 and BRCA2 are required for a normal proliferative burst in early embryogenesis, as revealed by the phenotypes of knockout mice [55, 56], and are upregulated with proliferation of breast epithelial cells during puberty, pregnancy and lactation [57]. In order to study the function of BRCA1, homozygous knockout mice have been developed. In most cases, the complete loss of function of BRCA1 results in embryonic lethality characterized by a lack of cell proliferation [58, 59, 60]. Cells derived from mouse embryos lacking BRCA genes are defective in their repair of DNA damage [56, 61, 62].

Embryos deficient for BRCA1 exhibit phenotypic variation and die between 5.5 and 13.5 days of gestation [55, 58, 59]. BRCA2-deficient embryos die between 8.5 and 9.5 days of gestation [58, 60]. Homozygous mice harbouring a mutation truncating BRCA2 in exon 11 die prenatally or perinatally; however, the few that survive to adulthood develop thymic

lymphomas at 12–14 weeks of age [63]. Heterozygous BRCA1 and BRCA2 mice develop normally, are fertile, and do not appear more susceptible to mammary tumours.

1.3.7.3 Role in cell cycle regulation

BRCA1 gene contains only two recognizable protein motifs, a RING finger domain near the N-terminus, (exons 2-5) comprising residues 1-112 [39] and a BRCT (BRCA1 C-terminal) domain at the C-terminus [64].

RING fingers are cysteine-rich sequences that coordinate the binding of two zinc ions and are found in a number of diverse proteins. This type of domain may facilitate both protein-protein and protein-DNA interactions [65]. The RING finger in BRCA1 appears to specifically interact with another similar RING finger protein known as BARD1 (BRCA1-associated RING domain 1) that was identified based on this interaction [66]. Both BARD1 and BRCA1 also share another conserved sequence known as the BRCT domain, a phylogenetically conserved sequence found in proteins involved in DNA repair and cell cycle regulation [67, 68].

Exon 11 of BRCA2 includes BRC repeats, consisting of eight copies of a 30–80 amino acid sequence, which are conserved across mammalian BRCA2 proteins and which interact with RAD51 [49]. Their conservation and interaction with RAD51 suggest that these repeats might be important for BRCA2 function (Figure 1.7).

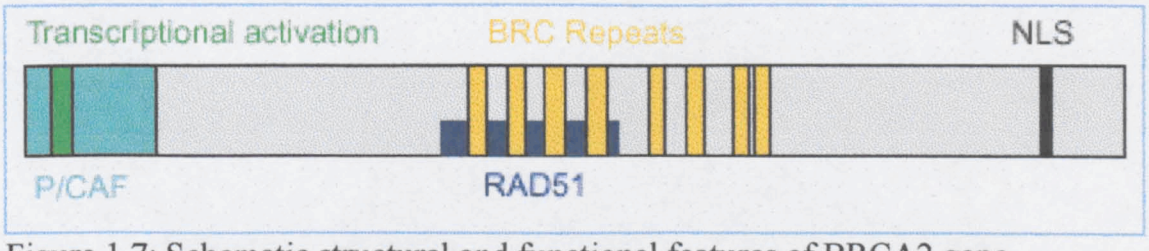


Figure 1.7: Schematic structural and functional features of BRCA2 gene
A domain within BRCA1 amino acids 758–1064 interacts with RAD51. Four of the eight BRC repeats of BRCA2 interact with RAD51. (Diagram adapted from Piri L. Welcsh. Insights into the functions of BRCA1 and BRCA2. Trend in Genetics. 2000, vol 16, No. 2).

1.3.7.4 Roles in transcriptional regulation

BRCA1 and BRCA2 play a role in transcriptional regulation, in that BRCA1 complexes with RNA polymerase II and both BRCA1 and BRCA2 interact with transcriptional regulators [69, 70, 71, 72].

Disease-associated mutations that truncate the BRCA1 C-terminus are inactive in transcriptional activation assays. Full-length BRCA1 is also an activator of transcription: when co-transfected with p53, it induces transcription of reporter genes cloned down-stream of the promoters of p21 and MDM2, an oncogene that serves as a negative regulator of p53 [73, 74].

BRCA1 protein-truncating mutations that lack the extreme C-terminal portion of the transactivation domain do not induce transcription of reporters, suggesting a relationship between transcriptional regulation and tumour suppression. It is not yet clear whether BRCA1 and/or BRCA2 interact directly with p53 to regulate transcription. When BRCA1 and p53 are co-transfected into cells, their interaction modulates p53-mediated transcription [74]. BRCA1 also appears to stimulate p53-dependent transcriptional activation from the p21 promoter [73, 74].

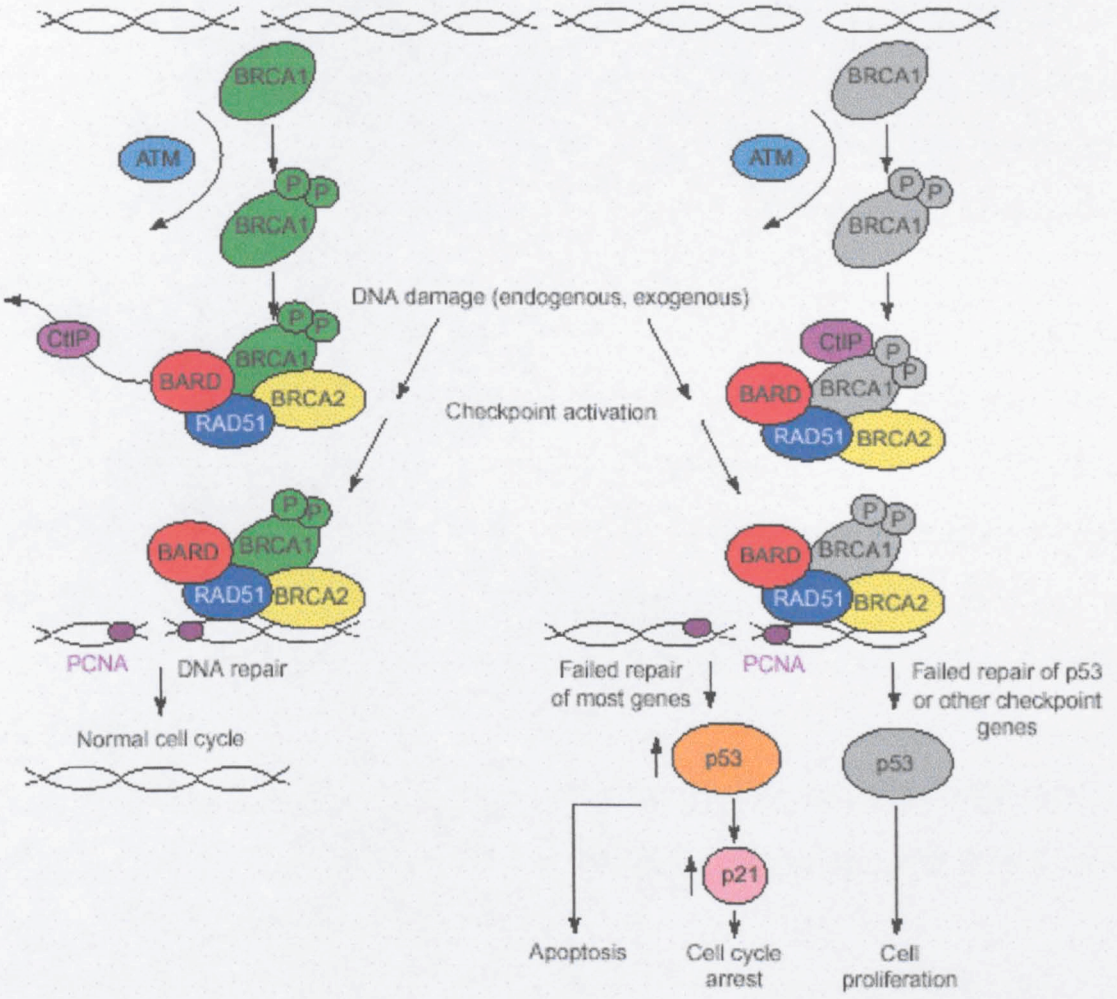


Figure 1.8: Role of BRCA1 and BRCA2 gene in DNA repair

This model suggests that a macromolecular complex consisting of BRCA1, BRCA2, BARD1 and RAD51 functions to repair damaged DNA [53]. In response to DNA damage, the complex relocates to chromosomal regions undergoing DNA replication marked by proliferating cell nuclear antigen (PCNA). DNA damage results in distinctive phosphorylation of BRCA1 and dissociation of BRCA1 and CtIP. The complex might be involved in an S-phase, DNA-damage cell cycle checkpoint. Inactivating mutations (grey) in BRCA1 or BRCA2 would lead to accumulation of DNA damage and checkpoint activation, including activation of p53 and upregulation of p21.

Inhibition of cyclin-dependent kinases by increased levels of p21 would lead to cell cycle arrest. Other genes regulated in response to p53 activation are believed to participate in p53-mediated apoptosis. In addition, loss of BRCA1 or BRCA2 function leads to inability to repair damaged DNA. When damage occurs to critical checkpoint genes, such as p53, checkpoints cannot be activated and cells proliferate[75]. (Diagram adapted from Piri L. Welcsh. Insights into the functions of BRCA1 and BRCA2. Trend in Genetics. 2000; Vol. 16, No. 2).

1.3.8 Future directions

As Figure. 1.8 suggests, genetic instability caused by loss of BRCA1 or BRCA2 could trigger mutation, including mutations in checkpoint genes such as p53 [53]. In this scheme, mutant p53 would overcome incomplete proliferation and lead, instead, to uncontrolled proliferation and invasive growth.

Rapid proliferation of breast epithelial cells during puberty and pregnancy offer the ideal opportunity for somatic mutation, including loss of a BRCA1 or BRCA2 allele. In this context, somatic mutation of BRCA1 or BRCA2 virtually always involves the loss of a large genomic region, whether the loss is of the remaining wild-type allele in a BRCA1 or BRCA2 tumour, or the somatic loss of BRCA1 and BRCA2 in sporadic breast cancer.

But why do some breast and ovarian epithelial cells with complete loss of BRCA1 or BRCA2 function survive, whereas embryonic cells with loss of BRCA1 or BRCA2 function die?

It might be that BRCA1 –/– or BRCA2 –/– breast epithelial cells are protected by their hormonal environment, especially during puberty, pregnancy and lactation. That is, the growth stimulatory effects of estrogenic hormones might tip the balance in favour of growth rather than death of some mutant cells. However, the rare surviving BRCA1 –/– BRCA2 –/– cell would be defective in DNA repair, and hence accumulate mutations at many sites, including in checkpoint genes. As expected, BRCA1 and BRCA2 tumours harbour multiple aberrations and mutations in many genes [76].

These new insights raise important questions about the genetic events that lead to breast cancer. Why should breast epithelial cells be more susceptible than other types of cells to the consequences of the genomic

instability caused by loss of function of the BRCA1 or/and BRCA2 genes? In addition, why should breast cancer develop in carriers of a mutant germ-line BRCA1 allele after the somatic loss of the second BRCA1 allele, whereas BRCA1 is rarely if ever inactivated in patients with sporadic breast cancers?

This argument suggests that tissue specificity of BRCA1 and BRCA2 tumourigenesis might be attributable both to the hormonal environment of breast and ovarian cells, especially during proliferative bursts, as well as to tissue-specific expression of as-yet-unknown genes.

1.3.9 Cellular expression of BRCA1 and BRCA2 gene

In normal cells, BRCA1 and BRCA2 are nuclear proteins [45, 69]. BRCA1 protein expression is reduced or absent in most sporadic, advanced (grade III) ductal breast carcinomas [77]. BRCA1 and BRCA2 mRNA and protein are preferentially expressed during the late G1–early S phase of the cell cycle [78, 79]. BRCA1 function is regulated by phosphorylation: it is hyperphosphorylated during late G1 and S phases by an endogenous kinase activity with dephosphorylation occurring at M phase [80].

A function of BRCA1 at the G1–S transition might be to arrest cell cycle progression by binding hypophosphorylated retinoblastoma protein (RB)[81]. In addition to a role at the G1–S transition, BRCA1 might regulate the G2–M checkpoint by controlling the assembly of mitotic spindles and the appropriate segregation of chromosomes to daughter cells [82]. In a significant fraction of cells, centrosomes were amplified, resulting in abnormal chromosomal segregation and aneuploidy.

Proteins that regulate the G2–M checkpoint, including p53 and RB, localize to the centrosome (Figure 1.9).

BRCA1 also localizes to centrosomes during mitosis [83]. Mutant BRCA1 might induce genetic instability by disrupting regulation of centrosome

duplication. Mutant BRCA2 might also lead to disruption of mitotic checkpoints [84].

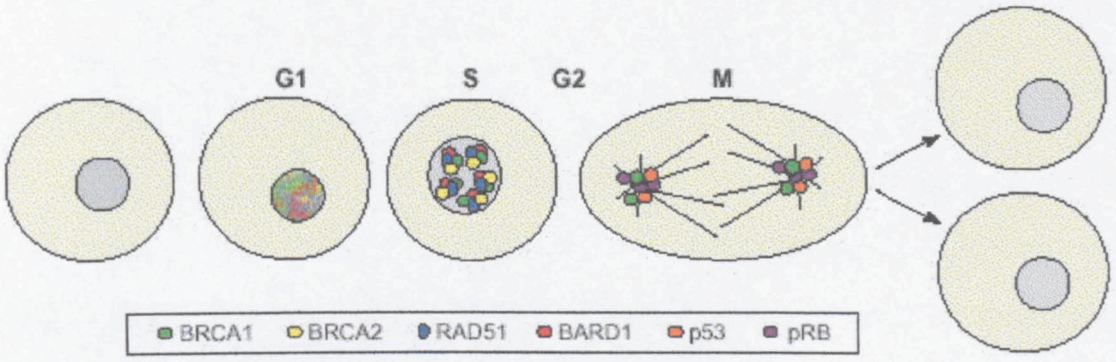


Figure 1.9: Expression of BRCA1 and BRCA2 gene
Cellular levels of BRCA1 and BRCA2 proteins increase as cells progress through G1, peak during S phase, and remain elevated during G2–M transition. BRCA1, BRCA2, RAD51 and BARD1 co-localize during S phase, forming punctate nuclear foci. During M phase, BRCA1, p53 and retinoblastoma protein (RB) localize to centrosomes. BRCA1 is hyperphosphorylated during late G1 and S phases, and dephosphorylated during M phase. (Diagram adapted from Piri L. Welcsh. Insights into the functions of BRCA1 and BRCA2. Trend in Genetics. 2000, vol 16, No. 2).

1.3.10 Loss Of Heterozygosity (LOH)

Loss of heterozygosity (LOH) is a term that refers to the loss of one allele. When the first allele in a given gene is lost, that cell is said to be heterozygous, ie, having two different forms or alleles. When the second copy is lost through mutation, deletion, or other mechanism, this is referred to as "loss of heterozygosity," (commonly abbreviated as LOH). This allele would normally function to suppress the development of a cancer. Loss of this allele, also known as a tumour suppressor gene, allows unchecked cell proliferation. Identifying loss of heterozygosity is an approach to identifying the location of the tumour suppressor gene. Many cancer genetic studies report LOH in tumour tissue when compared to normal tissue, ie, places where the second copy of a relevant cancer susceptibility gene has been lost. The significance is that sites of LOH indicate potential TSG locations. Loss of heterozygosity in the region of BRCA1 in hereditary breast cancers suggests that BRCA1 is a tumour suppressor gene

[85]. Loss of function of both alleles of a tumour suppressor gene is usually required for expression of the associated tumourigenic phenotype.

The first mutation may occur as a somatic event, or in an inherited form in the germline from a parent. In either case, the second mutation occurs somatically and is usually extended over large genetic distances (loss of all or part of chromosome). This is detectable as a loss of heterozygosity of polymorphic DNA markers located at or close to the gene. When the first inactivating mutation is present in the germline, the second hit deletes the allele of the susceptible gene (plus nearby polymorphic markers) that has been inherited from the normal parent. This model has been substantiated in breast and ovarian cancers developing in individuals carrying BRCA1 [46, 86] and BRCA2 gene mutations [87, 88].

To date, however, only a handful of somatic mutations have been described in sporadic breast and ovarian cancers [89, 90]. Such studies of mutational inactivation of BRCA1 and BRCA2 in sporadic disease suggest that familial and sporadic breast and ovarian cancer may be fundamentally different diseases at a molecular genetic level.

There is growing evidence that epigenetic silencing rather than mutation is a common mechanism for loss of suppressor gene function.

1.3.11 Epigenetic pathway events

Epigenetic regulation of gene expression by methylation is an important mechanism of the determination of cell fate in embryogenesis. It has been shown that methylation of regions rich in cytosine–guanine doublets ('CpG islands') in the promoter region in somatic cells is a common mechanism of epigenetic silencing of one or sometimes both alleles of tumour-suppressor genes such as BRCA1 [91, 92]. It is unclear what determines whether a particular gene will lose function by an epigenetic or a mutational mechanism [91].

Inheritance of methylation patterns in human DNA has also been described [93]. It is possible that susceptibility to cancer may be influenced by inherited variation in genes that regulate epigenetic silencing.

Hypermethylation in the BRCA1 promoter region has been associated with 11% of sporadic breast cancer and 5% of ovarian tumours [92].

1.3.12 Germline mutations in BRCA1 and BRCA2 gene

At present over 878 and 900 distinct mutations, polymorphisms and variants throughout the BRCA1 and BRCA2 gene have been identified respectively [94] (Figure 1.10). These variants are distributed along the entire coding region of the BRCA1 and BRCA2 gene and more than 58% and 68% of them have been identified only once respectively [94] (See also Appendix III, IV). BRCA1 somatic mutations have never been reported in sporadic breast cancer [30] although they have occasionally been found in sporadic ovarian cancers [89, 90].

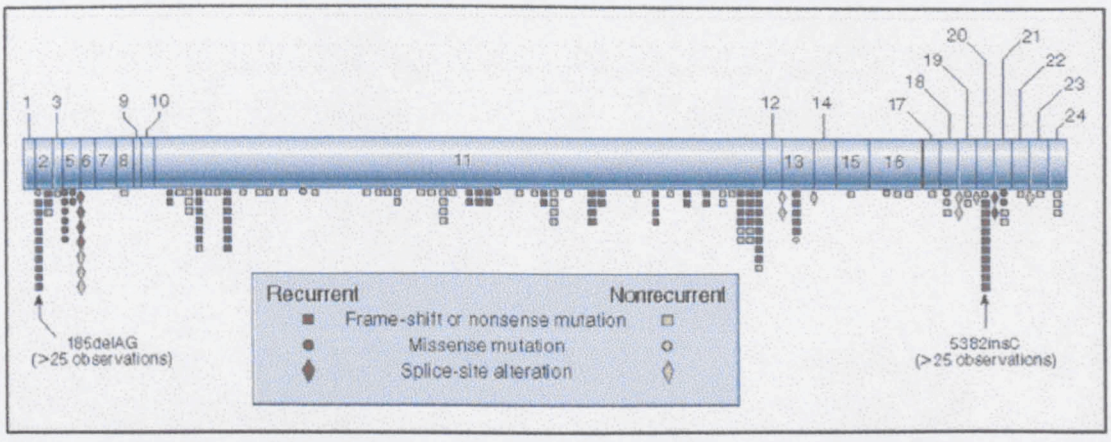


Figure 1.10: Germline mutations that have been reported in the BRCA1 gene mostly in families with a high incidence of Breast Cancer, Ovarian Cancer, or both. The numbers refer to the exons of the gene; there is no exon 4. Information is provided courtesy of the Breast Cancer Information Core database, which is accessible on the World Wide Web at http://www.nchgr.nih.gov/dir/lab/transfer/bic/

1.3.13 Mutation types

Mutations in BRCA genes result from different types of DNA alteration. Two classes of mutations result from a single nucleotide substitution. If the

substitution changes a single amino acid but does not affect the remainder of the protein open reading frame, it is termed a *missense mutation. Nonsense mutations* occur when the nucleotide substitution produces a stop codon (TGA, TAA, or TAG), and translation of the protein is terminated at that point. *Frameshift mutations* occur when one or more nucleotides are either inserted or deleted. If the number of bases inserted or deleted is not divisible by three, a change in the reading frame alters the remainder of the translation of the protein being altered; most often a stop signal is encountered prematurely. Another class of mutation, intron/exon *splice-site mutations*, can result from either single base changes or the insertion or deletion of one or more nucleotides in the intronic sequence, leading to the presence of a stop codon at a splice site. Splice-site mutations cause abnormal inclusion or exclusion of DNA in the coding sequence, resulting in an aberrant protein. A final class of mutation occurs when a mutation in a gene's regulatory region causes reduction or loss of protein synthesis from the mutant chromosome through a direct effect on mRNA biosynthesis. Such *regulatory mutations* usually occur outside of the coding sequence of a gene and can be either nucleotide substitutions or insertion/deletion events.

1.3.14 The frequency of BRCA1 and BRCA2 mutation in familial breast cancer

The frequency of BRCA1 was estimated to be 0.051% (95% CI: 0.021-0.125%) and of BRCA2 0.068% (95% CI: 0.033-0.141%). The breast cancer risk by age 70 years, based on the average incidence was estimated to be 35.3% for BRCA1 and 50.3% for BRCA2. The corresponding ovarian cancer risk was estimated 25.9% for BRCA1 and 9.1% for BRCA2. This low association of BRCA1 and BRCA2 mutation to breast

cancer suggest that other low penetrance gene may contribute to the most breast cancer families [95].

1.3.15 BRCA1 and BRCA2 mutation rate in early-onset breast cancer

Peto et al (1999) reported that only 5.9% of women who diagnosed with breast cancer before age 36 years carried BRCA1 or BRCA2 mutations. For women with breast cancer between age 36 and 45 years the rate was even lower (4.1%). The corrected percentage (due to limitation of sensitivity of screening methods) was 9.4% and 6.6% for BRCA1 and BRCA2 respectively [96]. These rates are much lower than predicted for probability of mutation in rare autosomal dominant gene (36% in individuals aged 20-29 years) that was estimated earlier [27].

Two independent population based studies using young women with breast cancer in West Washington [97] and North Carolina [98] also reported result consistent with these data [96].

1.3.16 Double heterozygotes mutation in both BRCA1 and BRCA2 gene

The mutation 185delAG in BRCA1 and 6174delT in BRCA2 has been reported in Hungarian patients who developed breast cancer at age 48 and ovarian cancer at age of 50 years [99].

The mutation G2508T in BRCA1 and 3295insA in BRCA2 has been reported in Canadian patients who developed breast cancer and ovarian cancer at age of 35 years. The probability of finding a double heterozygote in this population was estimated at 1 in 250000 [100].

1.3.17 Significance of mutations

Perhaps the most difficult aspect of sequence-based BRCA1 predisposition diagnostic testing is interpretation of sequence variants other than chain-terminating mutations. These fall primarily into two categories: potential

splice alterations and rare missense polymorphisms. From the present data relating to allele frequencies of common polymorphisms in BRCA1, it is estimated that about 60% of people carry at least one polymorphism within the expressed exons of the gene that can be used to distinguish between transcripts originating from each of their two alleles [101]. For individuals who carry rare nucleotide substitutions near a splice junction or an insertion/deletion polymorphism in intron sequences proximal to a splice junction, the exonic polymorphisms can often be used to test for proper/improper splicing of both alleles. This may be carried out by comparing the genotype of a genomic DNA PCR products that contains the polymorphism to the genotype of a cDNA PCR products that crosses the splice junction in question and should contain the polymorphism.

1.3.18 Mutation-specific variation in cancer risk

The expression of BRCA1 and BRCA2 mutations is variable. Mutation carriers may develop breast cancer, ovarian cancer, other types of cancer, or no cancer at all. Some of the variation in expression is due to chance and some may be attributable to risk modifiers. However, a further component appears to be due to mutation-specific differences in cancer risks.

Understanding these differences is potentially important in refining the clinical management of susceptible individuals.

1.3.18.1 BRCA1 genotype-phenotype correlation

Mutations at the 3' end of the BRCA1 gene that cause truncated proteins have been correlated with a larger increase in ovarian cancer risk than mutations in the 5' end [102, 103, 104]. The junction between the two areas was best characterized as a sharp demarcation point (rather than a gradual transition region) located between codons 1435 and 1443.

Relatives of carriers of BRCA1 5382insC (which is close to the 3' end of the gene) are reported to have a higher risk of ovarian cancer than relatives

of carriers of BRCA1 185delAG (which is close to the 5' end of the gene), although the difference was not statistically significant [105].

Overall, the balance of evidence remains in favour of heterogeneity of risk of different BRCA1 mutations. There is also reasonable support for a higher risk of ovarian cancer associated with mutations at the 5' end compared with the 3' end of the gene, although the difference is modest. This pattern is most consistent with the existence of a domain in the vicinity of the transition point that specifically confers protection against ovarian cancer. Truncating mutations 5' to the transition point, which disrupt critical functions of the domain, lead to a high risk of ovarian cancer, whereas mutations 3' to the transition point, which leave the domain intact, confer lower risks of ovarian cancer.

1.3.18.2 BRCA2 genotype-phenotype correlation

There is strong evidence for heterogeneity in cancer risks conferred by different BRCA2 mutations. Families with truncating mutations located approximately between codons 1000 and 2000 are characterized by a high ratio of ovarian cancer to breast cancer compared with mutations located 50 or 30 to this region (which has been designated the Ovarian Cancer Cluster Region or OCCR) [106]. In principle, this difference in phenotype between BRCA2 mutations is attributable to an increased risk of ovarian cancer within the OCCR and/or a decreased risk of breast cancer.

1.3.19 Population genetics of BRCA1 and BRCA2 gene

Early linkage studies based on large families, especially those with ovarian cancer indicated that BRCA1 contributed approximately 45% of hereditary breast cancer [22], BRCA2 adds a further 35%, including large families with male breast cancers [107]. More recently analyses of BRCA1 and BRCA2 genes in many populations revealed that only 30%-60% of breast cancer families are attributable to BRCA1 and BRCA2 mutations.

Szabo and King (1997) have been conducted a combined data analysis on multiple population-based studies for comparison of frequencies of BRCA1 and BRCA2 mutations in high-risk families and in other series of patients from most populations represented in the literature [**[108]**] (Table 1.1). They have suggested several conclusions:

First, the proportion of high-risk families with breast or ovarian cancer attributable to BRCA1 mutations varies widely among populations.

Second, in the most populations, BRCA1 and BRCA2 together explain 6%-10% of breast and ovarian cancer unselected for family history.

Third, about 30% of high-risk families have no detected mutations in either BRCA1 or BRCA2 gene.

Fourth, in families with male breast cancer, BRCA2 mutations are more common than BRCA1 mutations, as has been apparent since BRCA2 was mapped (Wooster et al. 1994).

Finally, in all regions other then Iceland, the frequency of BRCA1 mutations is 1.5-2.0 fold higher than the frequency of BRCA2 mutations.

Table 1.1: Frequency of germline mutations in BRCA1 and BRCA2 genes in families screened from various populations. (Adapted from Szabo et al. 1997)

Population	No. of families or patients with mutations/No. of families or patients screened		Reference(s)
	BRCA1	BRCA2	
A) Families with three or more cases of female breast and/or ovarian cancer:			
Britain	71/339(21%)	25/290 (9%)	Gayther et al. (1995, 1996, 1997b); Xu et al. (1997)
Canada	12/30 (40%)	8/49 (16%)	Simrad et al. (1994); Phelan et al. (1996)
Finland		8/100 (8%)	Vehmanen et al. (1997)
France	38/160 (24%)	14/77(18%)	Serova-Sinilkova et al. (1997); Stoppa-lynonet et al. (1997)
Germany	9/49 (18%)		Jandrig et al. (1996); Hamann et al. (1997)
Holland and Belgium	71/517 (14%)		Hogervorst et al. (1995); Peelen et al. (1997)
Hungry	7/32 (22%)	4/32 (13%)	Ramus et al. (1997)
Iceland	1/11 (9%)	7/11 (64%)	Thoracius et al. (1996)
Israel	16/34 (47%)	8/34 (24%)	Levy-Lahad et al. (1997)
Italy	21/73 (29%)		Caligo et al. (1996); Montagna et al. (1996); De Benedetti et al. (1996)
Japan	2/20 (10%)		Inoue et al. (1995)
Norway	3/25 (12%)		Anderson et al. (1996)
Russia	15/19 (79%)		Gayther et al. (1997a)
Sweden and Denmark	24/106 (23%)	12/106 (11%)	Johannsson et al. (1996); Hakansson et al. (1997)
United States	69/179 (39%)	24/94 (25%)	Castilla et al. (1994); Friedman et al. (1994, 1995); Struewing et al. (1995)
B) Families with male and female breast cancer:			
United States	2/24 (8%)	12/64 (19%)	Couch et al. (1996); Friedman et al. (1997); Serova et al. (1997)
Hungry	0/6 (0%)	2/6 (33%)	Ramus et al. (1997)
Iceland	0/10 (0%)	9/10 (90%)	Thorlacius et al. (1996)
C) Breast and/or ovarian cancer patients not selected for family history:			
Iceland		42/497 (8%)	Johannesdottir et al. (1996)
Italy	4/49 (8%)		De Benedetti et al. (1996)
Israel	23/243 (9%)	14/243 (6%)	Abeliovich et al. (1997)
Japan	8/179 (4%)	2/103 (2%)	Matsushima et al. (1995); Katagiri et al. (1996); Miki et al. (1996)

1.3.20 The prevalence, incidence and prognosis of breast cancer

Breast cancer is the most common cancer among women, excluding skin cancers [109, 110] (Figure 1.11) with a lifetime risk of 10% in the general population [111]. In spite of earlier detection and better treatment, largely due to recent technological advances it is still the second leading cause of cancer death in women, exceeded only by lung cancer [112]. Global common cancer incidence rate in developed and developing countries is shown in Figure 1.12 [113].

The risk of breast cancer increases with age, with the majority of sporadic breast cancers occurring after menopause. In addition, women who carry BRCA1 or BRCA2 mutations have a significantly increased chance of developing breast cancer before the age of 50 [114, 115].

Both BRCA1 and BRCA2 are considered to be responsible for a similar proportion (40%) of inherited breast cancer [116] but the majority (81%) of the breast-ovarian cancer families are due to BRCA1 whereas BRCA2 is found in the majority (76%) of families with male and female breast cancer [117]. Carriers of BRCA1 or BRCA2 mutations from families with high cancer risk have been estimated to have an 85% lifetime risk of developing breast cancer [116]. In addition, BRCA1 mutations' carriers have a 65% lifetime risk of developing ovarian cancer [102].

Studies in various populations report the risk of breast cancer in women with BRCA1 or BRCA2 mutations as 56% to 87% by age 70 [105, 118]. In comparison, women in the general population have approximately a 10% lifetime risk for breast cancer and a 1%-2% lifetime risk for ovarian cancer.

Penetrance estimates for breast cancer risk in women with germline BRCA1 mutations vary depending on the population used for analysis [22, 119, 120].

Figure 1.11: Incidence of female cancers (all ages) World 2001 -IARC

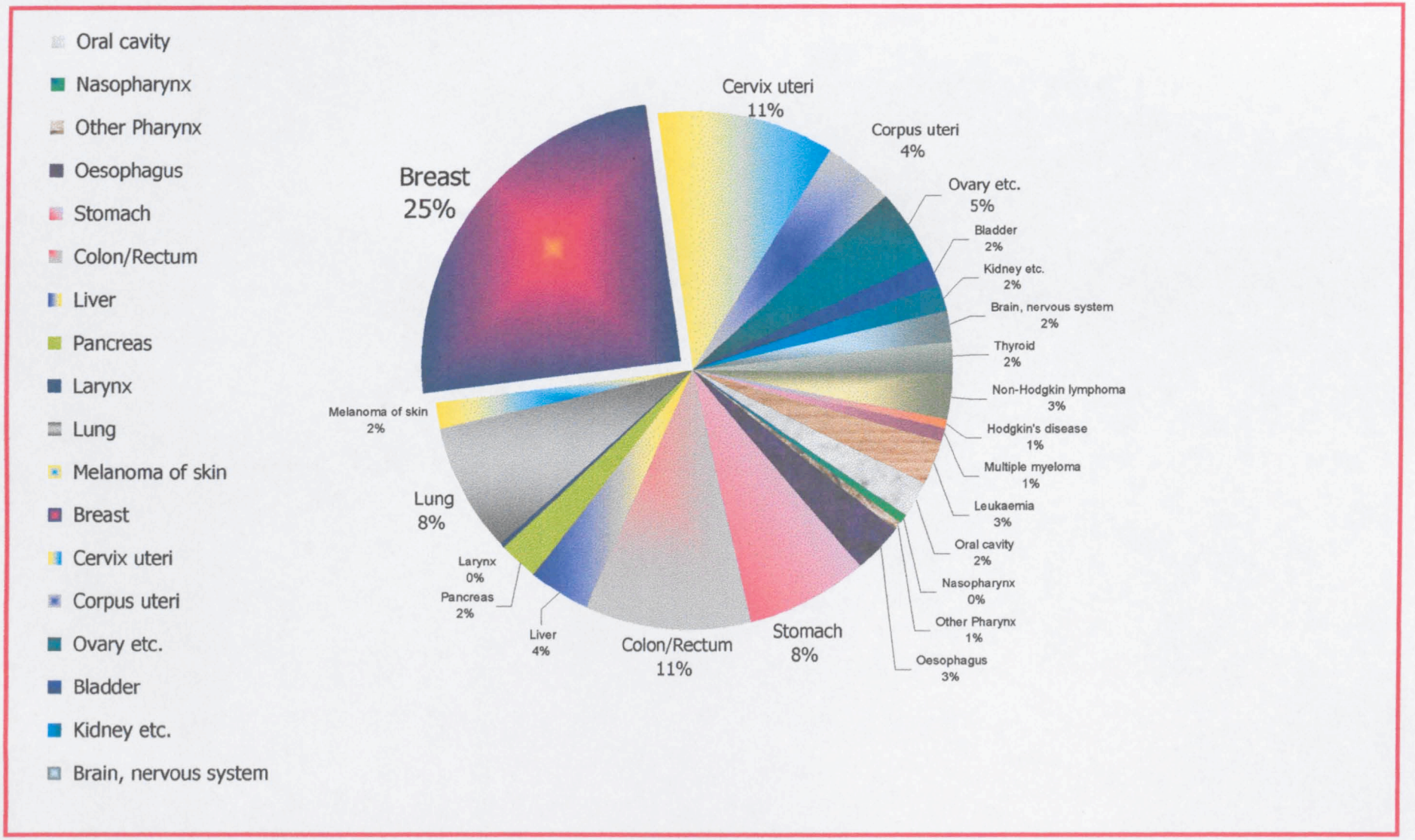
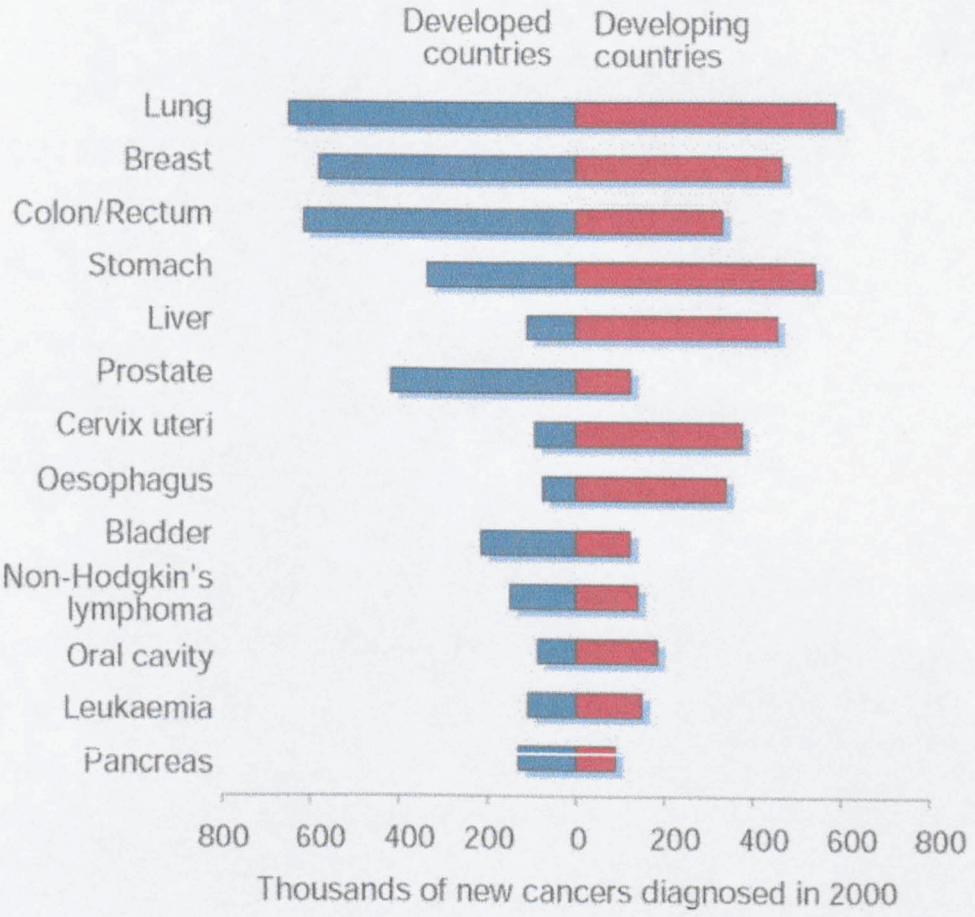


Figure 1.12: Global cancer incidence in developed and developing countries
(Sites contributing over 2% of the 10 million cancers worldwide)



Despite the high penetrance of the mutant gene, not all carriers develop cancer. Hormonal, environmental, reproductive, and other genetic factors may influence penetrance [111]. In spite of obvious differences in hormonal condition between male and female, progression of breast tumour is almost identical. BRCA2 expression effect in tumour progression pathway is similar in male and female [121].

Most studies on prognosis of breast cancer have not found a significant difference in survival between individuals with BRCA1 or BRCA2 cancer-predisposing mutations and controls and it depends upon the stage at which breast cancer is diagnosed [122, 123, 124, 125, 126].

The National Cancer Institute (NCI) estimates that about 1 in 50 women will develop breast cancer by age 50 years and about one in ten women in the United States will develop breast cancer by age 80 years [127, 128].

The Western diet is associated both with earlier age at menarche and with post-menopausal obesity, which increases endogenous oestrogen production and hence breast cancer risk [129]. Breast cancer incidence is much higher in most Western countries than in many developing countries, and this is partly (and perhaps largely) accounted for by these dietary effects combined with later first childbirth, lower parity and shorter breastfeeding [130, 131]. Table 1.2 shows this comparison pointing these figures in Iran and UK.

Table 1.2: The new cases and incidence rate of female breast cancer in Iran compared to other countries

Country	New cases	Incidence rate *	
		Crude	ASR (W)
Islamic R. of Iran	3491	10.46	14.76
United Kingdom	34815	116.27	74.93
Less developed countries	471063	19.66	23.07
More developed countries	579285	94.93	63.22
World	1050346	34.94	35.66

1) The column marked with * show the incidence rate (per 10^5 persons) of female breast cancer whilst another depicts the number of new cases (See chapter 7 section 7.6 Glossary).

2) Adapted from GLOBACAN 2000: Cancer Incidence, Mortality and Prevalence Worldwide, Ver. 1.0. IARC CancerBase No.5. Lyon, IARC Press. 2001.

1.3.20.1 The prevalence of BRCA1 and BRCA2 mutations in general population

Mutations of BRCA1 and BRCA2 are rare in the general population. A segregation analysis of a population-based case-control study of 4730 breast cancer cases (aged 20–54 years) and 4688 matched controls (the CASH study) estimated a prevalence of 0.0033 (0.6% heterozygote frequency) for all high-risk dominant breast cancer–susceptibility alleles together [27]. By combining BRCA1 penetrance estimates and population-based figures of cancer mortality in the relatives of British breast cancer patients, an indirect estimate of the prevalence of BRCA1 mutations of 0.0006 (0.12% heterozygote frequency) consistent with the CASH data was obtained [117]. Precise, direct estimates of the frequencies of BRCA1 and BRCA2 mutations are difficult to obtain as they require screening of many individuals and are hampered by the large sizes of the genes, the diversity of mutations, and the incomplete sensitivity of current mutation screening methods.

The true incidence of germline BRCA1 mutations in the general population remains unknown [132], but it is estimated that 1 in 200 to 1 in 400 women may be carriers of the BRCA1 mutation [133, 134].

Some BRCA1 and BRCA2 mutations occur with greater frequency in certain ethnic groups because of the founder effect. The *founder effect* occurs when a relatively small group is genetically isolated from the rest of the population. If an individual in that isolated group carries a rare mutation, later generations will have a higher frequency of that mutation, as the allele becomes dispersed throughout the group.

Three specific mutations in BRCA1 and BRCA2 are more common in the Ashkenazi Jewish population. A large-scale population study has been conducted [135] to investigate the prevalence of specific BRCA1 and BRCA2 mutations in Ashkenazi Jewish individuals who were unselected

for breast cancer. BRCA1 mutation screening on approximately 3,000 Ashkenazi Jewish samples determined a carrier frequency of 1.09% for the 185delAG mutation and 0.13% for the 5382insC mutation. BRCA2 analysis on 3,085 individuals from the same population showed a carrier frequency of 1.52% for the 6174delT mutation. This expanded population-based study confirms that the BRCA1 185delAG mutation and the BRCA2 6174delT mutation constitute the two most frequent mutation alleles predisposing to hereditary breast cancer among the Ashkenazim, and suggests a relatively lower penetrance for the 6174delT mutation in BRCA2. There was no evidence for differences between them in penetrance [105].

In Iceland, the single BRCA2 999del5 mutation has a heterozygote frequency of approximately 0.5% [136, 137, 138]. While these results have important implications for the management of breast cancer susceptibility in the Ashkenazi and Icelandic populations, they are not directly relevant to other populations. The BRCA2 mutations 982del4 in American and French, 2041insA in Dutch, Canadian and American, 3034del4 in Belgium, Canadian, Spanish, French, Switzerland, Italian and American families have also been reported [98, 139].

Knowledge of the carrier frequency of specific mutations in every population will greatly influence the counselling of those women regarding breast cancer risk and genetic testing, as well as the approach to mutation detection taken in the laboratory.

1.3.21 Breast cancer screening and prevention

Breast cancer is not a systemic disease at its inception, but is a progressive disease and screening can arrest its development; treatment of advanced breast cancer is often futile and disfiguring [140].

Screening reduces mortality from breast cancer largely because,

1-Breast cancer is a progressive disease.

2-The progression of breast cancer can be halted by early detection and treatment.

3-By halting disease progression, screening for breast cancer can prevent a significant proportion of deaths from the disease.

4-The basic mechanism for the prevention of deaths by screening is the reduction in the incidence rate of advanced tumours.

Recommendations for cancer screening of individuals with a BRCA1 or BRCA2 gene mutation have been made by a task force convened by the Cancer Genetics Consortium (CGSC), an NIH-sponsored consortium of researchers assessing the ethical, legal, and social implications of genetic testing for cancer risk [141]. The CGSC statement emphasized that recommendations are based on presumed benefit and may change as new evidence becomes available; therefore, patients should be counselled regarding the limited knowledge about strategies to reduce risk and patient preference should be taken into account in decisions about follow-up. The CGSC recommendations for individuals with a BRCA1 or BRCA2 gene mutation are:

- Monthly breast self-examination starting in early adulthood
- Annual or semi-annual clinical breast examination beginning at age 25-35 years
- Annual mammography beginning at age 25-35 years

No studies have evaluated the outcome of early breast cancer screening. The breast cancer screening recommendations are based on data from families with cancer-predisposing BRCA1 or BRCA2 mutations, which indicate that elevated breast cancer risk begins in the late 20s or early 30s [141]. Men with BRCA2 cancer-predisposing mutations may also be at increased risk for breast cancer, and evaluation of any breast mass or

change is advisable; however, there are insufficient data to recommend a formal program of surveillance [141].

1.3.22 Risk estimation to family members

Virtually all individuals with a cancer-predisposing mutation in BRCA1 or BRCA2 have inherited it from a parent. The parent may or may not have had a cancer diagnosis depending upon the penetrance of the mutation, the gender of the parent with the mutation, the age of the parent with the mutation, and other variables. It is appropriate to offer mutation analysis to both parents of an individual with a BRCA1 or BRCA2 mutation. Occasionally neither parent will be identified as having the BRCA1 or BRCA2 mutation. Reasons for this include a *de novo* mutation in the proband, alternate paternity or adoption.

In breast cancer, the risk to close relatives of a case, averaged across all ages, is about twofold. Most of this familial risk is probably genetic in origin [142]. The risk is about the same for the mother, sisters or daughters of a case, suggesting dominant rather than recessive effects. Large population-based studies indicate that only 15–20% of overall familial risk is attributable to mutations in BRCA1 and BRCA2 [143]. The possibilities for the remaining 80% are some combination of a small number of moderately strong genes, and a larger number (possibly a hundred or more) of weaker genes [142] Figure 1.13.

The variant alleles are associated with risks of around 1.5-fold and are predicted to account for only a few per cent of breast cancer incidence. Collectively they account for only a very small fraction of the familial risk.

There are many almost certainly more genes to be identified, which together will account for a much higher fraction of cancer incidence than the genes in the inherited cancer syndromes.

The identification of these genes will be greatly accelerated by the data that have emerged from the Human Genome Project [144].

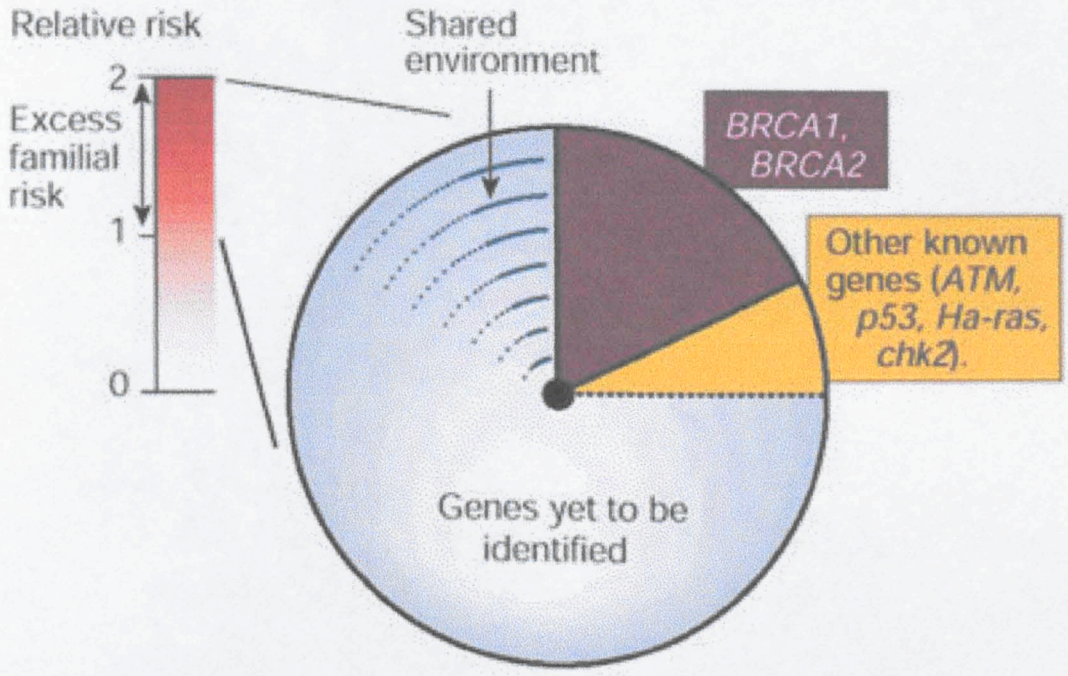


Figure 1.13: The risk of breast cancer to the first-degree relatives
Average across all ages, the risk of breast cancer to the sister, mother or daughter of a case is increased about twofold. (Diagram adapted from Ponder BAJ. Cancer genetics. Nature.2001; Vol.411, no. 6835.)

1.3.23 The comparison of breast tumour type in women carrying mutations in BRCA1/2 genes

It has been shown that the histological characteristics of breast cancers due to BRCA1 and BRCA2 mutations differ from those of sporadic breast cancers [145]. Moreover, cancers associated with BRCA1 and BRCA2 mutations differ from each other [146, 147].

The occurrence of invasive lobular carcinoma and invasive ductal carcinoma was not significantly different between carriers of BRCA1 or BRCA2 mutations and sporadic breast cancers. However, Medullary carcinoma was found more often in BRCA1 than in BRCA2-mutation carriers or sporadic cases [145]. In a histological review of the population-based series of 4071 breast cancers diagnosed between ages 20 and 54

years in the Cancer and Steroid Hormone Study, [148] a higher familial risk was reported for lobular carcinoma in situ than for other subtypes.

The tumour grade of both BRCA1 and BRCA2 breast cancers was significantly higher than that of sporadic breast cancers [145]. High grade as an independent index of prognosis may lead to a poorer outcome [149]. However, some reports suggest a better prognosis in familial breast cancer [146, 147, 150].

These observations may also have implications for the management of women with a raised risk of breast cancer.

1.3.24 Genetic testing strategies

Genetic testing for risk is now part of the standard of clinical care for families, although its value may be controversial when the practical benefits of the actions open to someone at risk are not clear [151].

Mutation screening can be carried out on either affected individuals, or unaffected individuals with a very strong family history of breast/ovarian cancer. Testing should be carried out on the following category of patients using genomic DNA/cDNA:

- Mutation screening in affected individuals
- Mutation screening in unaffected individuals with strong family history
- Mutation screening in unaffected individuals for common mutations found in the selected population
- Predictive testing of at risk relatives
- Confirmation of the presence of a known mutation

The type of patient accepted for mutation screening is dependent upon local practices and funding.

1.3.25 Mutation detection methods

The detection of inactivating mutations in tumour suppressor genes is critical to their characterisation, as well as to the development of diagnostic testing. Most approaches for mutational screening of germline specimens are complicated by the fact that mutations are heterozygous and that missense mutations are difficult to interpret in the absence of information about protein function. To date there is no one technique that is ideally suited to a complete analysis of BRCA genes with each method carrying both advantages and disadvantages [117].

For example, Single Strand Conformational Polymorphism (**SSCP**) analysis is simple to perform and rapid but is less sensitive than other techniques. Reproducibility can also be a problem because of the extreme sensitivity of SSCPs to temperature and other gel conditions. (Orita et al 1989). Heteroduplex Analysis (**HA**) can detect small insertions and deletions and not at the detection of single base changes. It can be useful as an initial screen to look for common mutations (Gayther et al 1996).

Fluorescent Conformational Sensitive Gel Electrophoresis **(F-CSGE)** can be used for the detection of all types of mutations by automated heteroduplex analysis). It is reported that the sensitivity is approaching 100%. This technique will pick up polymorphic variants and variants of unknown pathological significance as well as truncating mutations (Ganguly et al 1998).

Unlike all other techniques, the Protein Truncation Test (**PTT**) detects only those mutations, which result in premature termination of the protein product. This technique is particularly suited to analysis of the large exons in BRCA1 and BRCA2 and allows the use of genomic DNA as a test source. The technique is relatively easy to perform and does not require special equipment. It is very sensitive but is expensive and involves a radioactive detection method (Roest et al 1993).

Direct Sequencing (**DS**) analysis is often considered the "gold standard" for mutation detection in terms of sensitivity, but is labour intensive when not automated, time consuming and expensive (Sanger et al 1997).

BRCA1 and BRCA2 are relatively large genes, comprising 22 and 26 coding exons respectively. The majority of these exons are small, often less than 100 bp in size, and there is one large exon in both BRCA1 and BRCA2 gene that comprises 61% and 45% the entire coding region respectively (See Appendix II). These aspects alone present number challenging features for the analysis of mutations in the BRCA1 and BRCA2 genes.

86% of alterations are predicted to lead to a truncated protein product as a result of frameshift, nonsense, splice or regulatory aberrations [[104]]. The remaining 14% are missense mutations of which half are located in regions of putative functional importance, the RING finger motif near the 5' end of the gene and sequence encoding the acidic blob at the 3' end. There appears to be little evidence of mutation clustering with a fairly even distribution of mutations throughout, and there are no specific alterations which occur with a particularly high frequency, although five individual mutations (185delAG; 5382insC; 1294del40; 4184del4; Cys61Gly) represent approximately one third of all those reported to date [[94]].

The utility of any screening test depends on three basic factors: **the prior probability** of having the condition of interest, **the sensitivity of the test** (the chance that a person with the condition will have a positive test result), and **the specificity of the test** (the chance that a person without the condition will have a negative test result).

For BRCA genes, one must consider the distinct possibility that a woman diagnosed with breast cancer at a particular age or with a specific family history harbours a mutation in BRCA1 or BRCA2; that a mutation will be

detected using a given screening technique; and that a benign sequence variant will be interpreted as a deleterious BRCA1 mutation.

1.3.25.1 Factors affecting sensitivity of the test

There are a number of families reported in which the genetic linkage evidence for involvement of BRCA1 is very strong but for which mutations have not been identified [152]. Although these could be due to coding mutations missed by SSCP, similar families have been found among groups using direct sequencing. Such families could be analysed initially for the presence of a single transcript (indicating that on one chromosome, the copy of BRCA1 is improperly transcribed into messenger RNA) through analysis of the polymorphisms described in the region in cDNA and genomic DNA [39] or by a functional assay when available. The frequency of these presumed regulatory mutations lying outside of the regions currently being analysed (exons and intron/exon junctions) will affect the utility of any sequence-based test for BRCA1 mutations.

1.3.25.2 Factors affecting specificity of the test

In the preceding paragraphs, I have focused on factors affecting the sensitivity of an assay for BRCA1 mutations, i.e., the chance that a true, clinically meaningful alteration in the BRCA1 gene will be detected using a particular screening method. However, one must also consider the specificity of such an assay, i.e., the rate of false positives. In practical terms this usually involves the proper interpretation of observed missense mutations. How can one be certain that an observed amino acid substitution found in a patient or relative of a patient is clinically meaningful? Missense mutations, which are recurrent in cancer cases but absent or very rare in the general population, are likely to be clinically meaningful; cosegregation of the missense mutation with the disease in relatives of the proband provides further support that the missense mutation is indeed causal [104].

1.4 Aims

This study was set for the following purposes:

- To report initial experience in identifying germline mutations in the BRCA1 and BRCA2 breast and ovarian cancer susceptibility genes, to assess the spectrum of such mutations, and to determine the frequency of recurrent mutations in a cohort study from the Iranian population.

- To show the implication of the result and the management of early-onset breast cancer within the Iranian District Health Services (DHS) [153]

- To exploit of the result as a basis for further studies on breast cancer in Iran

- To evaluate and improve upon existing mutation detection techniques with respect to the BRCA genes

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2 Methods and Materials

2.1 Subjects and Methods

2.1.1 Subjects (Data collection)

Two main centres for cancer research and treatment in Tehran-IRAN, namely the Iranian Centre for Breast Cancer (ICBC) and the Cancer Institute affiliated to the Medical Sciences of Tehran University were chosen for a cohort study includes 104 individuals from 96 unrelated families with either breast or ovarian cancer for screening of germline mutations in the BRCA1 and BRCA2 genes.

From the ICBC about 4000 medical records of individuals who have been attended a clinic between 1997-1999 for routine breast examination or for breast disease examination were reviewed. Fifty-four women from 46 unrelated families who met the criteria were selected for this study.

In the 12 months after December 1999, a consecutive series of fifty patients from 50 unrelated families who had been diagnosed with either breast or ovarian cancer, attending clinic at Cancer Institute for surgery who met the criteria were also chosen for screening.

All selected women (104 individuals from 96 unrelated families) were under the age of forty-five and were informed that their DNA samples

would be analysed for known genes associated with susceptibility to breast cancer; they were offered the opportunity to receive the results and asked to sign a second consent form if they chose to learn the results (Appendix VII).

Criteria were proposed to address the spectrum of germline mutations associated with onset breast and ovarian cancers in this selected group.

2.1.1.1 Inclusion criteria

Different criteria have been chosen in different studies using pedigree analysis and based on estimation of individual's risk for probability of carrying germline mutation in BRCA1 and/or BRCA2 genes [1, 2, 3, 4, 5, 6]. Family history, age of onset (premenopausal or postmenopausal state), male breast cancer, bilateral breast cancer, ovarian cancer, number of affected relatives and etc were factors that considered to estimate of developing breast cancer and lead to select the participants in these studies. However, the ideal approach for data collection is the way, which capable to covers most of the above factors to obtain greater chance for detection a mutation in an individual at risk.

The present study is performed to obtain initial experience in identifying germline mutations in the BRCA1 and BRCA2 genes in the Iranian population. More recently a survey has been carried out in Esfahan-Iran concerning a common age of menopause in Iranian women (Data in press). This survey suggests that Iranian women may experience menopausal state between the age of 35 and 45 years.

In this study, the age of 45 years was chosen based on the previous population studied [7, 8, 9, 10], the cancer registry data in Iran [11] and because it is more likely to detect germline mutation in breast cancer predisposing genes at the premenopausal state.

Due to time limitation as a PhD course and presence of difficulties for data collecting in a short period, age of onset (premenopausal state) was chosen as a main criterion in high-risk families in this selected group. Other criteria were considered to select participants are as follow:

1. Affected women with breast cancer at the age of less than 45 years.
2. Affected woman with ovarian cancer at any age.
3. Affected women with breast cancer more than 45 years of which at least one first degree relative has breast cancer less than 45 years.
4. Unaffected women who have at least one first degree relative with breast cancer less than 40 years or two second degree relatives more than 40 years.
5. The family contains a male breast cancer patient who is the first or second degree relative.

A total of 104 samples derived from 96 unrelated families who met one of the above criteria were collected. These samples were included six ovarian cancer cases, two bilateral breast cancer cases and sixteen unaffected women who met the above criteria. The reason for collection of samples from unaffected individuals was due to presence of a strong family history among them (Table 2.1, Figures 2.1 and 2.2, find out for more detail in chapter 6 section 6.1.8).

Note: The participant must be a first degree relative (mother, sister, daughter) of an affected member of a high-risk family.

Figure 2.1: File record-146UBF

The pedigree shows a family with affected twins. Probands III:3 and III:4 are seeking a genetic test. Their sisters (twin) probands III:1 and III: 2 who has been diagnosed with breast cancer less than 40 years. Their mother proband II:2 died with breast cancer at the age of 42 years, maternal grandmother I:4 died from uterine cancer at the age of 70 and paternal grandmother I:2 died with breast cancer at the age of 43 years.

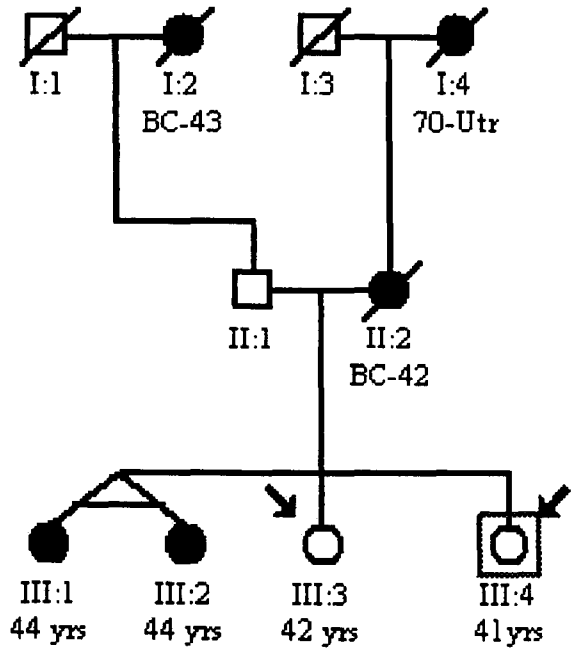


Figure 2.2: File record-104UBF

The pedigree shows a proband IV:3 who is seeking advice.

Her two sisters IV:1 and IV:2 were diagnosed with breast cancer at the age of 31 and 34 respectively. Her paternal grand father and her cousin II:1 and II:2 died from colon cancer.

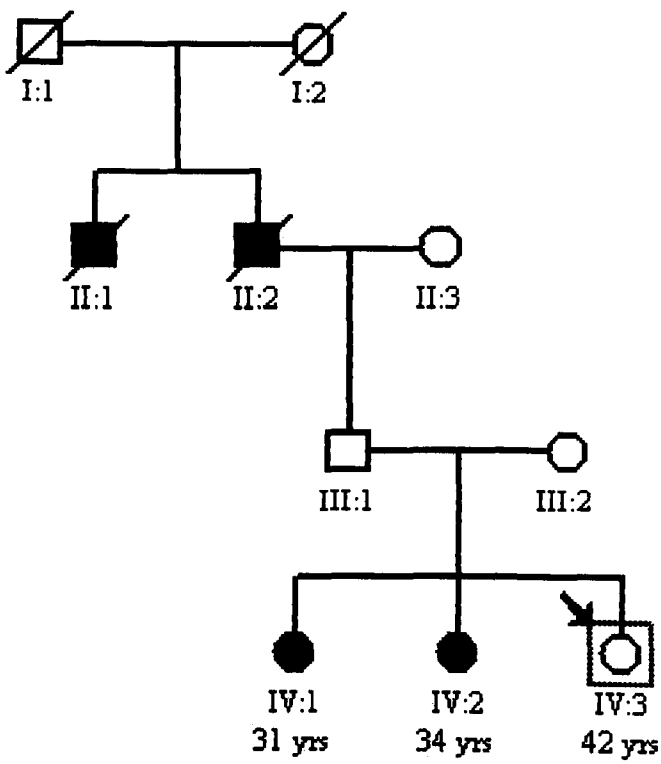
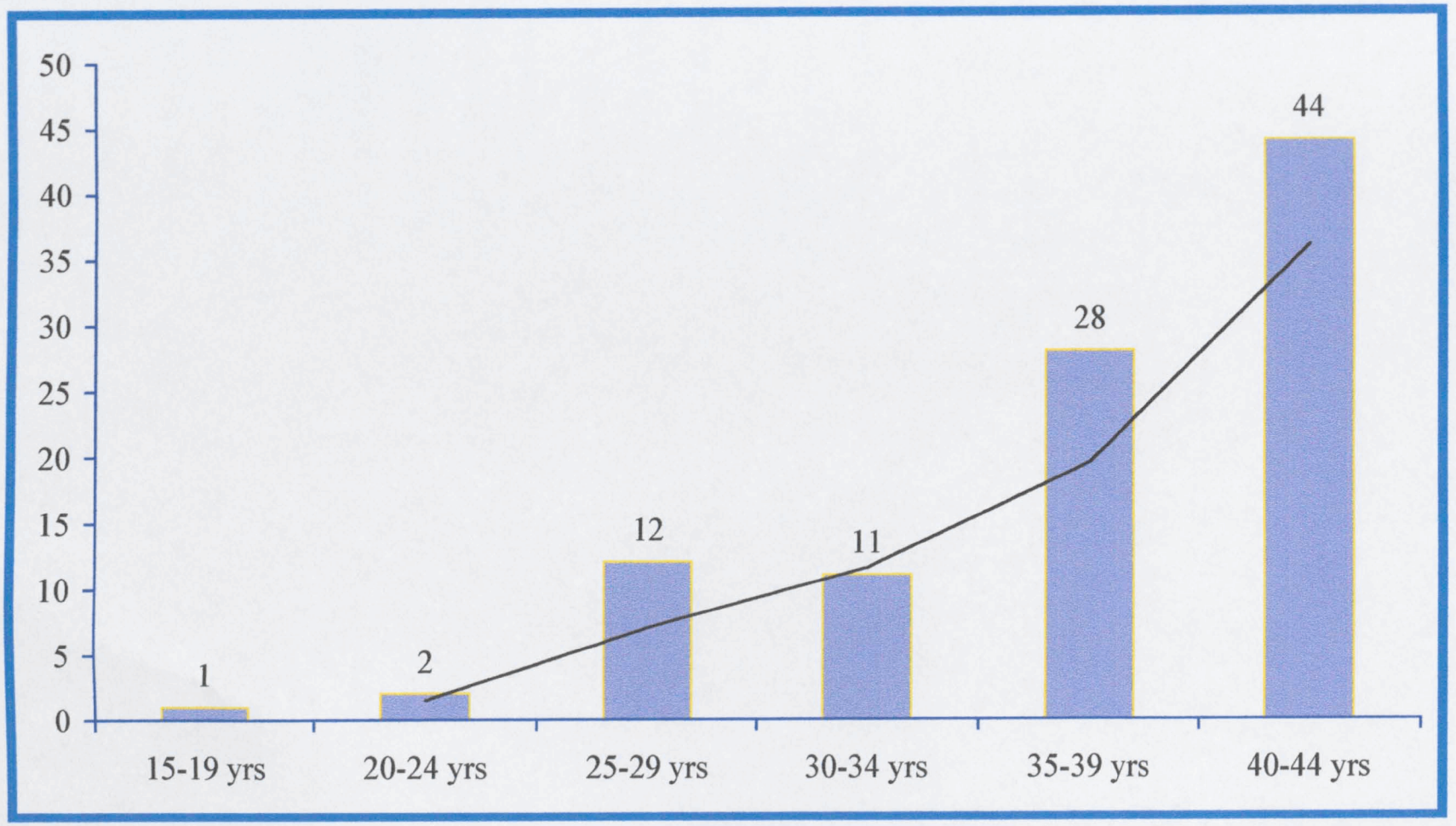


Table 2.1: Age-specific distribution of female breast cancer in the present cohort study

Cases	Total cases	15-19		20-24		25-29		30-34		35-39		40-44		>45	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Affected * (BC)	82	1	1.2	-	-	9	10.9	10	12.2	24	29.3	38	46.3	-	-
Unaffected	16	-	-	2	12.5	3	18.8	1	6.3	3	18.8	5	31.3	2	12.5
Affected * (OV)	6	-	-	-	-	-	-	-	-	1	16.7	1	16.7	4	64
Total	104	1	0.96	2	1.92	12	11.53	11	10.57	28	26.93	44	42.3	6	5.76

*The affected women histologically have been confirmed with breast or ovarian cancer.
BC= Breast cancer OV= Ovarian Cancer

Figure 2.3: Age-specific distribution of breast cancer in the Iranian population



As Table 2.1 and Figure 2.3 show, in this study the prevalence rate of breast cancer has increased by the age so that data collection was unbiased, however, premenopausal state was chosen as a main criterion.

2.1.2 Methods

2.1.2.1 Breast/Ovarian cancer gene predisposing scanning

In an ideal situation it would be preferable to have the capacity to screen all exons of both genes. However, this may only be practicable for a small cohort of patients, i.e. those at very high risk of being mutation carriers. Also local practices and funding may prevent the full screen of both genes. Thus, it is considered acceptable to carry out a limited screen, which targets areas of the genes where common mutations exist, typically exons 2, 5, 11 and 20 of BRCA1 and exons 10 and 11 of BRCA2.

For such a these reasons, in this study BRCA1 exons 11 and BRCA2 exons 10 and 11 by the Protein Truncation Test (PTT) [[12, 13, 14]] and BRCA1 exons 2, 3, 5, 13 and 20 and BRCA2 exons 9, 17, 18 and 23 by the Single Strand Conformation Polymorphism (SSCP) assay [[15]] and Heteroduplex Analysis (HA) [[16]] were analysed by amplification from genomic DNA with the polymerase chain reaction (PCR). Although mutations are scattered throughout both BRCA1 and BRCA2 some coding regions were particularly chosen for several reasons: first, many putative mutations have been reported in these regions [[17]] second, it has been shown that they have a significant role in protein function (exon 2-5 -Ring finger domain [[18]]; exon 11 and exon 20 -BRCT domain) [[19, 20]] and third, exons 10 and 11 cover a large segment of the genes.

All indices have been analysed for germline mutation in both BRCA1 and BRCA2 genes regardless to their mutation status.

2.1.2.2 Material preparation (Genomic DNA purification)

The source material for testing varies; most laboratories extract genomic DNA from blood samples due to its simplicity of preparation and stability, whereas others also collect RNA from the same patient. Generally cDNA/RNA is not used as a template for mutation screening, the possibility remains that truncation mutations decrease mRNA stability and

thus might decrease the detection of the mutated allele derived mRNA after RT-PCR/PTT [21]. Therefore, in the case of breast cancer in particular, patients are not always available to obtain fresh samples.

In addition mRNA expression level might change in different hormonal levels (Oestrogen and Progesterone) such as during menstruation period, pregnancy, lactation, menopausal state and etc.

In this study genomic DNA was used as a genetic material. Fresh whole blood was collected in K-EDTA tubes and stored at -20 °C until genomic DNA extraction became feasible (as a source of material); no more than 10 days later.

Using a Wizard Genomic DNA purification kit from Promega, Southampton, UK; catalogue no. LA1620 and in accordance with the manufacturer's protocols, genomic DNA was extracted from peripheral blood lymphocytes in Department of Biotechnology at the Pasture Institute in Tehran- Iran.

The Wizard ® Genomic DNA Purification Kit is designed for isolation of DNA from white blood cells. It is based on a four-step process [22]. The first step in the purification procedure lyses the cells and the nuclei. For isolation of DNA from white blood cells, this step involves lysis of the red blood cells in the Cell Lysis Solution, followed in second step by lysis of the white blood cells and their nuclei in the Nuclei Lysis Solution. The cellular proteins are then removed by a salt precipitation step (3rd step), which precipitates the proteins but leaves the high molecular weight genomic DNA in solution. Finally, the genomic DNA is concentrated and desalted by isopropanol precipitation (4th step).

Wizard ® Genomic DNA Purification Kit contains:

- Cell Lysis Solution
- Nuclei Lysis Solution
- Protein Precipitation Solution
- DNA Rehydration Solution

2.1.2.3 Protocols for genomic DNA isolation from whole blood

The purification of genomic DNA from fresh whole blood following collection in EDTA-, heparin- and citrate-anticoagulant tubes, produced no adverse effects upon subsequent manipulations of the DNA, including PCR [23]. Anticoagulant blood samples may be stored at 2–8°C for up to two months, but DNA yield is reduced with length of storage. The yield of genomic DNA will vary depending on the quantity of white blood cells present. The following protocol was routinely used for isolating DNA form a 3ml sample volume:

1) Add 9.0ml of cell lysis solution to a sterile 15ml centrifuge tube.

2) Gently rock the tube of blood until thoroughly mixed; then transfer blood to the tube containing the cell lysis solution. Invert the tube 5–6 times to mix.

3) Incubate the mixture for 10 minutes at room temperature (invert 2–3 times once during the incubation) to lyse the red blood cells. Centrifuge at 2000 ×g for 10 minutes at room temperature for each 3ml sample.

4) Remove and discard as much supernatant as possible without disturbing the visible white pellet. Approximately 50–100µl of residual liquid will remain in the 15ml tube. If the blood sample has been frozen, repeat Steps 1–4 until the pellet is white. There may be some loss of DNA from frozen samples.

Note: Some red blood cells or cell debris may be visible along with the white blood cells. If the pellet appears to contain only red blood cells, add an additional aliquot of cell lysis solution after removing the supernatant above the cell pellet, and then repeat steps 3-4.

5) Vortex the tube vigorously until the white blood cells are resuspended (10–15 seconds). Completely resuspend the white blood cells to obtain efficient cell lysis.

6) Add 3ml nuclei lysis solution to the tube containing the resuspended cells. Pipette the solution 5–6 times to lyse the white blood cells. The

solution should become very viscous. If clumps of cells are visible after mixing, incubate the solution at 37°C until the clumps are disrupted. If the clumps are still visible after 1 hour, add additional nuclei lysis solution (1.0ml for 3ml sample volume) and repeat the incubation.

7) Optional: Add RNase (15µl per 3ml sample volume) to the nuclear lysate and mix the sample by inverting the tube 2–5 times. Incubate the mixture at 37°C for 15 minutes, and then cool to room temperature.

8) Add 1.0ml protein precipitation solution to the nuclear lysate and vortex vigorously for 10–20 seconds. Small protein clumps may be visible after vortexing.

9) Centrifuge each 300µl sample at 13000–16000 ×g for 3 minutes at room temperature (centrifuge at 2000 × g for 10 minutes at room temperature for 3ml sample volumes). A dark brown protein pellet should be visible at this stage.

10) For the 3ml sample volume, transfer the supernatant to a 15ml centrifuge tube containing 3ml isopropanol at room temperature.

11) Gently mix the solution by inversion until the white thread-like strands of DNA form a visible mass.

12) Centrifuge at 2000 ×g for 1 minute at room temperature for 3ml sample. The DNA will be visible as a small white pellet.

13) Decant the supernatant and add one sample volume of room temperature 70% ethanol to the DNA. Gently invert the tube several times to wash the DNA pellet and the sides of the micro centrifuge tube. Centrifuge as in Step 12.

14) Carefully aspirate the ethanol using either a drawn Pasteur pipette or a sequencing pipette tip. The DNA pellet is very loose at this point and care must be used to avoid aspirating the pellet into the pipette. Invert the tube on clean absorbent paper and air-dry the pellet for 10–15 minutes.

15) Add 250µl DNA rehydration solution to the tube and incubate at 65°C for 1 hour. Periodically mix the solution by gently tapping the tube. Alternatively, rehydrate the DNA by incubating the solution overnight at room temperature or at 4°C.

16) Store the DNA at 2–8°C.

Note 1: If additional nuclei lysis solution was added in step 6, add a total of 1.3ml Protein Precipitation Solution for 3ml sample volume at step 8.

Note 2: At Step 10, some supernatant may remain in the original tube containing the protein pellet. Leave this residual liquid in the tube to avoid contaminating the DNA solution with the precipitated protein.
Following extraction, the concentration of DNA from each sample was measured using Genequant in accordance with manufacturer's recommendations.

2.1.2.4 DNA amplification by Polymerase Chain Reaction (PCR)

DNA segments can be hugely amplified using the polymerase chain reaction (PCR) [[24, 25]]. Two synthetic oligonucleotides are synthesized, each complementary to sequences on opposite strands of the target DNA at position just beyond the ends of segment to be amplified. The oligonucleotides serve as replication primers, with the 3' ends oriented towards each other and positioned to prime DNA synthesis across the desired DNA segment.

The basic principle of PCR involves three main steps: denaturation, annealing and extension. In PCR experiments a negative control was always used, to exclude the possibility of any contamination.

Consideration of the following made the experiments reproducible and PCR product more efficiently:

- *Pipetting and DNA template*

It is better to add water to the PCR tube first, followed by the other ingredients: there was no difference in PCR yield when each other component of the reaction was added in a different order.

To minimize the chance of primer binding to the DNA template and to prevent the polymerase from working (even theoretically) prior to the first denaturing step, it is useful to keep the vials on ice while pipetting the ingredients of the reaction.

- *First PCR program*

The aim of a PCR reaction is to amplify a specific locus without any non-specific by-products in high yield. Therefore, annealing should take place at a sufficiently high temperature to allow only perfect primer-template complementarity to occur in the reaction. For any given primer pair, the PCR program can be selected based on the composition (GC content) of the primers and the length of the expected PCR product. In the majority of the cases, products expected to be amplified are relatively small (from 0.2 to 2-3 kb). The activity of the Taq polymerase is about 2000 nucleotides/minute at optimal temperature (72-78°C) and the extension time in the reaction can be calculated accordingly.

As the activity of the enzyme may not be always optimal during the reaction, an easy rule applied successfully to consider an *extension time* (in minutes) equal to the number of kb of the product to be amplified (1min for a 1kb product, 2min for a 2kb product etc.). Later on, after the product(s) become "known", extension time may be further reduced. Some researchers use a 1-3 minutes *first denaturing step* before the actual cycling starts [26, 27]. This is supposed to help denaturing the target DNA better. In addition, a final *last extension time*, of 5-10 minutes, is described in many papers (supposedly to help finish the elongation of many or most PCR products initiated during the last cycle).

An *annealing time* of 45-60 seconds was sufficient for all primer pairs tested in this work. The *annealing temperature* was chosen based on the melting temperature of the primer-template duplex, which can be

calculated. It is desirable (but not absolutely necessary) for the two primers to have a close melting temperature or T_m (say, within 5°C or so). If the melting temperature (T_m) difference between the two primers is high, the lower T_m can be increased by increasing the length of that primer at the 3' end (this can also keep the size of the amplified locus constant) or the 5' end.

For most of the primers used during my work, a *denaturing time* of 60 seconds at 94°C was sufficient to achieve satisfactory PCR yields.

- *Number of cycles.*

In general, 30 cycles is sufficient for a conventional PCR reaction.

Table 2.2: Designing a first PCR program

Stage	Temperature	Time
First denaturing	94 °C	Optional
Denaturing	94 °C	30-60 sec
Annealing	54-60 °C	30-60 sec
Extension	72 °C	30-90 sec
Last extension	72 °C	Optional

2.1.2.5 Primer design in practice

Both BRCA1 and BRCA2 are large genes consisting of 24 and 27 exons respectively. Mutations in BRCA genes are distributed throughout the coding region [17]. BRCA1 exons 2, 3, 5, 11, 13 and 20 and BRCA2 exons 9,10,11, 17, 18 and 23 were analysed by amplification from genomic DNA with the PCR. A pair consists of at least 20 mer that was not self-complementary or capable of forming intra molecular secondary structures, was used in each reaction.

The software named PRIMER DESIGNER version 2.01, Scientific and Educational software 1990-1991 was used to calculate melting temperature for priming, to avoid of presence of any primer dimmer and hairpin structures in PCR products.

2.1.2.6 Oligonucleotide synthesis

Oligodeoxynucleotides used as primers for DNA amplification by PCR, were synthesised at Dr Arthur Moir's laboratory (Biomolecular Synthesis Service, The University of Sheffield) on an Applied Biosystem DNA/RNA synthesiser, model 381A.

2.1.2.7 PCR program

Basically, two sets of reaction components and thermocycling producer were used for DNA amplification for SSCP and PTT methods (See chapters 3 and 4 respectively). PCR products were used directly for SSCP analysis but for PTT agarose gel electrophoresis is necessary to determine yield and size of the products. Usually 100-200ng/μl of PCR products is sufficient for PTT detection. The presence of abnormally sized products is indicative of splicing mutations or genetic rearrangements such as deletions or duplications.

2.1.2.8 Horizontal agarose gel electrophoresis

PCR products were separated by agarose gel electrophoresis through 0.7%-2% agarose gels run in 1X TAE buffer for 25-60 minutes at 50-110 Volts. A fourth volume of loading dye solution was added to each sample prior to loading onto the gel. Samples were run on horizontal gel former (Biometra) and a comb positioned. Following electrophoresis, the gels were stained for 10-15 minutes in a solution of ethidium bromide (0.5 μg/ml). PCR products were visualized by exposure of the gel to U.V.-light.

The sizes of DNA fragments were estimated by comparison to 100bp or 1000bp DNA ladder run on the same gel.

2.1.3 Mutation detection methods

Mutation testing methods can be divided into two groups:

- *Screening method:*

Mutation detection methods test a DNA sample for the presence or absence of one specific mutation; this is widely applied in diagnostic laboratories.

- *Scanning method:*

Mutation scanning methods screen a sample for any deviation from the standard sequence.

Testing for known and unknown mutations in a gene posses different problems and requires different methods. All routine diagnostic methods such as Sequencing, PTT, SSCP, HA, DGGE, etc suffer from two limitations. They are quite laborious and expensive for use in a diagnostic services, which needs to produce answers quickly and within a modest budget. They detect differences between the patient's sequence and the published normal sequence but they do not generally distinguish between pathogenic and enhance non-pathogenic changes.

2.1.4 Combined Single Strand Conformational Polymorphism assay (SSCP) and Heteroduplex Analysis (HA)

SSCP [15] is the simplest and most used method of mutation detection. PCR is used to amplify the region of interest and the resultant DNA is separated as single-stranded molecules by electrophoresis in a non-denaturing polyacrylamide gel. A strand of single-stranded DNA folds differently from another if it differs by a single base, and it is believed that mutation induced changes of secondary structure of the DNA results in different mobilities for the two strands. These mutations are detected as the appearance of new bands by silver staining of bands. Separation of mutant fragments could be increased in low pH buffer system. Abnormal pattern of single strand DNA mobility has been detected in 88% of mutant fragments

in range of 579-797 bp [28]. SSCP and HA [16] reputedly each have sensitivities of between 60 and 80% [16]. The main advantages of both techniques are that they are rapid and easy to perform, relatively inexpensive and require little specialised equipment or kits (See chapter 3 in great detail).

2.1.4.1 Polyacrylamide gel electrophoresis (PAGE)

For SSCP polyacrylamide gels were prepared as follow:

Add 2 ml 10x TBE, 0.8-4ml glycerol (2-10% concentration), 14ml 59% acrylamide/1% bis, and 22.8ml distilled water for a final volume of 40ml to prepare 19.5cm x 22cm x 1mm gel. Mix the gel to dissolve glycerol entirely. Add 130µl 20% ammonium persulfate (AMPS) and 50µl TEMED prior to casting the gel (Table 2.3).

Table 2.3: Formulation for polyacrylamide preparation standard gels [19.5cm x 22cm x 1mm]

Components	One gel (20 wells)	Two gels (40 wells)	Final concentration
dH_2O	Up to 40 ml	Up to 80 ml	---
Acrylamide/Bis (59:1)	14 ml	28 ml	14%(w/v)
TBE x10	2 ml	4 ml	0.5M
Glycerol (in different %)	0.8 to 4 ml	2 to 8 ml	2-10 % (w/v)
AMPS	130 µl	260 µl	20% (w/v)
TEMED	50 µl	100 µl	---
Total volume	40 ml	80 ml	---

Follow the instructions provided with the 21cm x 30cm gel apparatus (from Bio-Rad, Hertfordshire, UK) for the recommended gel casting techniques for 1.0mm thick gels. The number of samples loaded on the gel determined which type of comb (1.0 mm) was used.

2.1.4.2 Running the SSCP assay

1) Denaturing the samples: Add 5µl of PCR product to 5µl loading buffer (See Appendices table 6) and denature the strands by incubating the samples at 99°C for 10 minutes, followed immediately by placing the samples on ice for 20 minute.

2) Prepare two litre of 0.5x TBE electrophoresis running buffer. Assemble the cast in apparatus (from Bio-Rad, Hertfordshire, UK) containing the gel into the lower buffer chamber base. Add the running buffer to both lower and upper buffer chamber.

3) All volume (10µl) of denatured samples load on a 21 x 30 cm non-denaturing 14% polyacrylamide gel [acrylamide/bis (59:1), containing 3–10% glycerol] and run at 245 volts constant power, 12°C -16°C for 16 to 20 hours. The gel is then stained with silver and photographed.

NOTE: It is important to keep the gel at proper temperature constantly so that secondary structures of single-stranded molecule are maintained during the run. This can be accomplished by using temperature controller, which connected to the apparatus (from Bio-Rad, Hertfordshire, UK). It can be set to the desired run temperature. The buffer re-circulator and stirring mechanism insure uniform buffer temperature.

2.1.4.3 Silver staining protocol

The silver staining technique takes 1-1.5 hours but it may start to see the results after about 30 minutes. Always stain in non-metallic containers. Multiple gels can be stained at once (commonly four to six at a time).

1) Following electrophoresis, fix gel(s) in a solution of 10% absolute ethanol and 0.5% acetic acid for 10 minutes at room temperature.

2) Stain with 0.1% silver nitrate (made from 1% stock) for 20 minutes at room temperature.

3) Rinse gels once with distilled water to remove excess silver solution.

4) Develop with a solution stock 740ml distilled water, 60ml NaOH (from 5M NaOH stock), 3ml formaldehyde 0.048% for 20 minutes at room temperature.

5) Fix stain with a solution of 0.75% $NaCO_3$ (made from 7.5% stock) for 10 minutes at room temperature (See also Appendix VI, Table 6).

2.1.5 Protein Truncation Test (PTT)

PTT is a mutation detection method based upon the in vitro transcription and translation of PCR amplified sequences. It specifically detects frameshift or nonsense mutations, which lead to the termination of mRNA translation and subsequently protein truncation [12, 13, 14]. This sort of analysis has been successfully applied to a number of clinical conditions including hereditary breast and ovarian cancer.

In the BRCA1 gene, missense mutations are seldom found, and are generally thought to be non-pathogenic. For such a gene, the PTT has several advantages. It conveniently ignores the non-pathogenic silent or missense base substitutions and reveals the approximate location of any mutation (See chapter 4 in great detail).

2.1.5.1 Principle of the Protein Truncation Test

PTT analysis detects premature stop codons in a single reaction by amplifying DNA fragments of the coding region using a special primer, which carries at the 5' end a T_7 promoter followed by eukaryotic translation initiator sequence, transcribing the fragments into mRNA, and translating the mRNA into radiolabeled polypeptides [12, 13, 14]. Products from the transcription/translation reaction can be analysed by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) [29] followed by autoradiography.

SDS-PAGE is a discontinuous system that has a non-restrictive large pore stacking gel that is layered on top of a resolving gel. Each of these gels was

made with a different buffer, the tank buffers was also have a different composition from that present in the gel.

The sample to be fractionated is denatured and coated with detergent by heating in the presence of SDS and a reducing agent. The SDS coating gives the protein a high net negative charge that is loaded on a polyacrylamide gel and high voltage (125 volts) is applied, causing the protein components to migrate toward the positive electrode (anode). Since all of the proteins have a net negative charge that is proportion to their size, the proteins are separated solely on the basis of their molecular mass- a result of the sieving effect of the gel matrix. The molecular mass of a protein can be estimated using SDS-PAGE by comparing the gel mobilities of a band with protein standards. Sharp banding of the protein component is achieved by using a discontinuous gel system, having stacking and separating gel layers differ in either salt concentration or pH or both [30]. Following gel electrophoresis, gel bands may be visualized by autoradiography [31]. If the product is full length, no truncating mutation is present in the amplified sequence. In samples where premature termination has occurred the protein product will be smaller than that generated from the non-mutant sequence. Assessment of the size difference between these two products allows the position of the putative mutation to be determined.

Note: The key feature of PTT is a specifically designed, tailed, sense forward primer used in the PCR amplification step (See section 2.1.8 Outline of PTT method). This primer contains four specific regions. A T_7 promoter sequence at the 5'-end directs the production of RNA, using the corresponding phage RNA Polymerase. The promoter sequence is separated from a eukaryotic translation initiation sequence (Kozak sequence) by a spacer sequence of 3-6bp [32, 33]. The 3'-end contains the target gene sequence (approximately 17-20bp) in-frame with the ATG codon from the Kozak sequence.

2.1.5.2 Agarose gel electrophoresis

After PCR amplification, agarose gel electrophoresis is necessary to determine yield (quantity and quality) and size of the products. Size alterations indicate the existence of small deletions/duplications or the presence of mutations affecting splicing.

2.1.5.3 In-vitro synthesis of RNA

The next step in PTT is the *in-vitro* transcription/translation reaction [34].

Promega ™ developed a system called TNT® Coupled Reticulocyte Lysate Systems offers an alternative for eukaryotic *in-vitro* translations: a single tube, coupled transcription/translation system. In most cases, the TNT® Lysate reactions produce protein in a 90 minutes reaction. In comparisons of [^{35}S] incorporation, the TNT® Lysate reactions incorporate significantly more radiolabel than do standard *in-vitro* translation reactions. This system then improved and named TNT® Quick Coupled Transcription/Translation Systems, which further simplifies the process by combining the RNA Polymerase, nucleotides, salts and recombinant RNasin® Ribonuclease Inhibitor with the reticulocyte lysate to form a single TNT® Quick Master Mix.

The latest generation of TNT® kit called TNT® T7 Quick for PCR DNA (Promega, Southampton, UK; catalogue no. L5540) designed for optimum expression of PCR templates ranging from 350-5000bp. For most PCR templates, the TNT® T7 Quick for PCR DNA reactions produce up to 5 times more protein [35]. The PCR products may be added directly to the TNT® T7 Quick for PCR DNA reaction without purification.

To use the TNT® T7 Quick for PCR DNA system, a PCR fragment containing a T_7 promoter is added to the TNT® T7 PCR Quick Master Mix and incubated for 60–90 minutes at 30°C. The synthesized proteins then can be analyzed by SDS-PAGE followed by autoradiography or

phosphorimaging [36] (See Appendix XI for Technical Manual # TM 235, Promega corporation).

2.1.5.4 Protein detection

The most commonly used procedure for the detection of newly synthesized proteins is incorporation of radiolabeled amino acids during synthesis, using [^{35}S] methionine, [^{35}S] and cysteine. TNT® T7 Quick for PCR DNA System is, at present, only available for [^{35}S] methionine. [^{35}S] Methionine prevents the background labelling of a rabbit reticulocyte lysate 42kDa protein, which can occur using other labels [37].

2.1.5.5 SDS-Polyacrylamide gel preparation

For separation of the translation products, appropriate SDS-PAGE conditions must be chosen for simultaneous detection of nearly full-length products as well as small products. Usually a 6% and 12% polyacrylamide gel is used for PTT products of up to 60-75kDa. In this study full-length artificial protein of BRCA1 and BRCA2 exon 11, 126kDa and 183kDa respectively, were detected using 6% to 12% polyacrylamide gel.

2.1.5.6 SDS-PAGE analysis and visualisation by autoradiography

After electrophoresis, the SDS-PAGE gels are typically treated with solutions to prevent cracking during drying. After drying the gels, generally 24 hours exposure to X-ray film results in a clear signal [13, 32]. The extent of [^{35}S] incorporation may be estimated by passing a radioactive Geiger counter over the surface of a dried gel before autoradiography.

In general, analysis of the translation products should lead to strong signals or bands for the desired translation products, occasionally accompanied by several smaller, weaker non-specific bands. These lower abundance products are often derived from internal translation initiation sites (AUG) and, in exceptional cases, might obscure the analysis and/or detection of truncated fragments [38].

Abnormal, short protein products indicate the presence of truncation mutations. Whether or not both truncated and wild type translation products are detected in the same sample depends upon the disease. In X-linked syndromes (e.g. Duchenne Muscular Dystrophy), the analysis of male patients is simplified by the presence of only one allele, which either contains a mutation or does not.

To assess the capacity of a new Promega transcription/translation system (TNT® T7 Quick for PCR DNA) all positive cases and few normal cases for BRCA2 exon 11, were analysed by the PTT method in duplicate. The presence of truncating mutations is typically analysed using two overlapping segments; the first segment covers 2799bp and the second overlapping covers 2620bp. The second screening, the entire BRCA2 exon 11 sequence was screened in one experiment that consists of 4959 base pairs (see chapter 4, Table 4.3, Figure 4.2) gave no detectable differences.

2.1.6 SDS-PAGE in practice

- Using 2 x Mini-Protean system (Bio-Rad, Hertfordshire, UK, catalogue no.165-3301) make thin minigels (7cm x 10cm) in 0.75 mm diameter with 10 well combs.
- The amount of reagents indicated in Table 2.4 and 2.5 are sufficient for the preparation of one 7 x 10 cm gel, 0.5-1.00 mm thick.
- Add the ammonium persulfate (AMPS) and TEMED immediately prior to pouring the gels, as these reagents promote the polymerisation of acrylamide.
- Pour the resolving gel mix into assembled gel plates, leaving sufficient space at the top for the stacking gel to be added later. Gently overlay the gel mixture with 0.1% SDS or N-butanol and allow the gel to polymerise for at least 15-30 minutes. After polymerisation, remove the SDS or N-

butanol and rinse the surface of the resolving gel with water to remove any unpolymerised acrylamide and blot with Postlip papers to dry.

- Fill the remaining space with the stacking gel solution and insert the comb immediately. After the stacking gel has polymerised, remove the comb and rinse the wells with water to remove unpolymerized acrylamide. At least 1 cm of stacking gel should be present between the bottom of the loading wells and the resolving gel.
- Assemble the plates according to manufacturer's instruction.
- Fill the apparatus with SDS-PAGE running buffer 1x (See Table 2.7).
- Load sample on the SDS-Polyacrylamide gel.

2.1.7 Assembling of the PTT reaction components

1) Sample preparation

Before begin: To reduce the chance of RNase contamination, gloves should be worn when setting up experiment, and microcentrifuge tubes and pipet tips should be RNase-free.

* Aliquot all [^{35}S] into 5µl aliquots (sufficient for 20 reactions) and keep frozen at –70°C

* Make Master Mix of distilled water (1.3 µl) and [^{35}S] (0.2 µl) per reaction in a 0.5µl microcentrifuge tubes

* Add 100-200ng/µl of PCR products to the Master Mix microcentrifuge tubes (containing of water and [^{35}S]) up to 3 µl.

*Remove the TNT ® T7 PCR Quick Master Mix from storage at -70°C. Immediately place the Master Mix on ice and rapidly thaw by hand warming and place on ice.

Note: Except for the actual translation incubation, all handling of the Lysate mix should be done at 4°C. Any unused Master Mix should be frozen as soon as possible after thawing to minimise loss of translational activity. Do not freeze/thaw the Lysate mix more than two times.

* Add 10 µl of TNT ® T7 PCR Quick Master Mix to Master Mix microcentrifuge tubes
[Great care must be taken to not introduce air bubbles into the mix.]

2) Incubate the sample at 30°C for 90 minutes.

3) Add 112.5-117µl loading sample buffer (45µl per 5µl translated mix) to the sample and boil it for 5-10 minutes. Cool it on ice.

4) Load sample (25µl-30µl) on 6% and 12 % SDS-Polyacrylamide gel

5) Apply high voltage (125 volts) for 60-75 minutes. Run until blue dye goes down to the bottom or just off.

6) Fix SDS-Polyacrylamide gel in 7% acetic acid for an hour with shaking.

7) Keep SDS-Polyacrylamide gel in a solution consist of 3% glycerol, 20% methanol for an hour (to prevent gel cracking when dried) with shaking.

8) Dry down for one hour at 80°C by auto dryer.

9) Visualize by autoradiography for 24 hours (Depend on [^{35}S] incorporation in protein product).

10) Developed the film using Kodak developer for 1-3 minutes until bands comes out, rinse with tap water and fix by Kodak fixing solution for 1-3 minutes, rinse with tap water and leave it to be air died.

All this procedure must be perform under safety condition (observing radioactive protection protocol).

2.1.8 Outline of PTT method

1) Coding sequence without introns (large exons in genomic DNA) is PCR amplified. The essential feature of PTT is a specifically designed tailed sense (**forward**) primer. This contains four different regions:

- At it's 5'-end a T7 RNA-polymerase promoter sequence, which facilitates the *in-vitro* production of RNA. Usually, additional nucleotides are present upstream of the T7 promoter. Even the addition of a single G nucleotide upstream of the promoter increases the transcriptional efficiency [39].
- A 5-7 base pairs spacer that separates T7 promoter from a consensus Kozak sequence, facilitating the initiation of protein synthesis[32].
- A eukaryotic translation initiation sequence (Kozak sequence) that includes a ATG start codon, facilitating the initiation of protein synthesis[12, 14].
- The 3' region contains a gene specific sequence designed so that the sequence amplified reads in-frame from the ATG. At the 3' end of the target, the primer can include a stop codon if the amplified sequence does not contain the native stop codon [31].

5'-GGATCC TAATACGACTCACTATAGGG AACAG CCACCATGG GAGAACAACTGTCTACAAACT-3'

Spacer	T7 promoter	Spacer	Kozak sequence Including ATG	Target sequence

*Large deletions, duplications and splicing mutations may be detected by agarose gel electrophoresis at this stage.

**In this study, no translation termination codon incorporates in the reverse primer.

2) After amplification, the PCR product is added to a coupled *in-vitro* transcription-translation system.

3) For detection a labelled amino acid is included, which is usually methionine. The label can be a radionucleotide such as [^{35}S] which is visualised by autoradiography.

4) The polypeptides produced are separated by size using SDS-PAGE. If the product is only full length, no truncating mutation is present. Truncating mutations result in shorter products, the size of which gives the approximate position of the mutation.

5) The area of the gene where the truncating mutation is occurred is then sequenced to identify the alteration of the gene precisely.

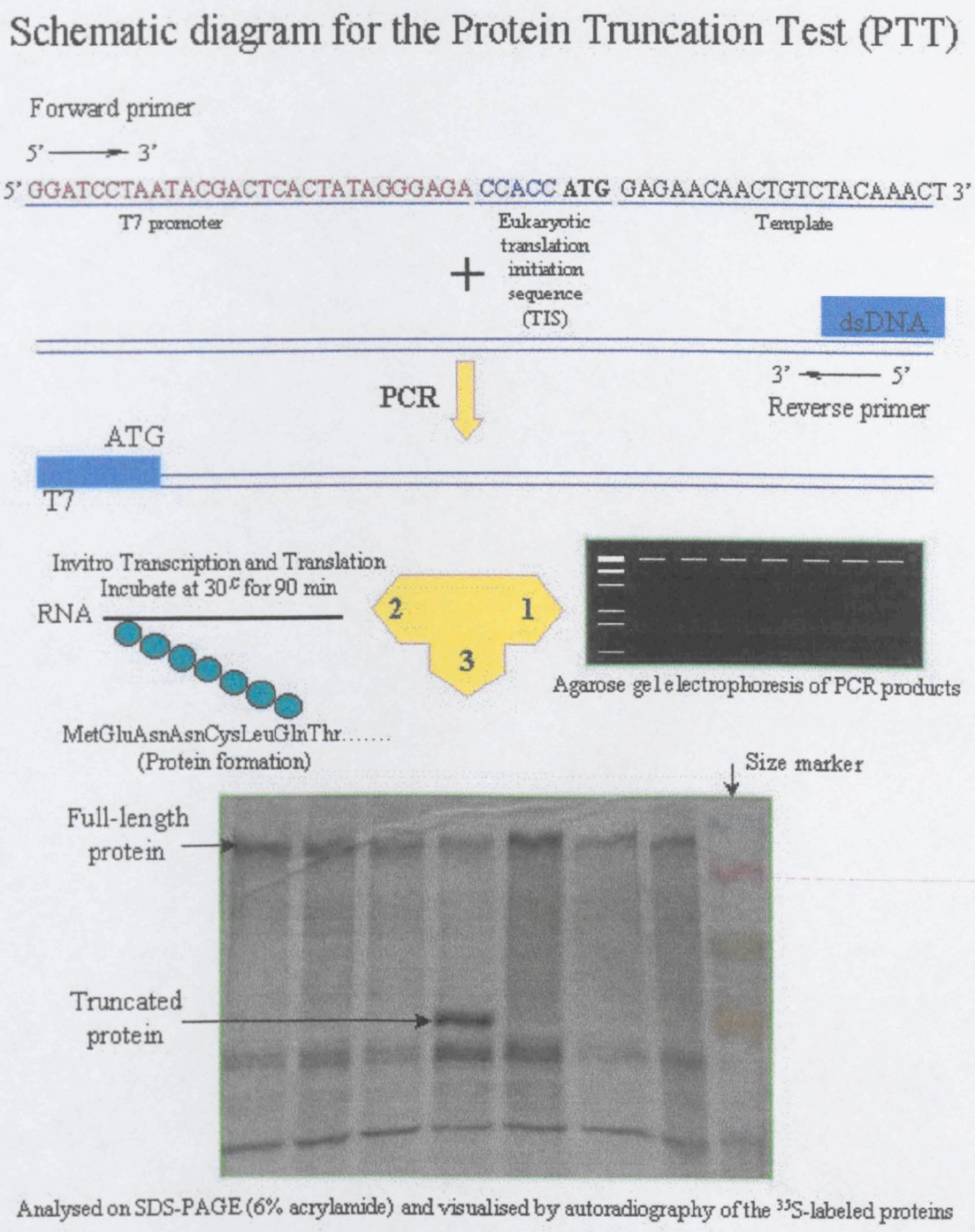


Figure 2.4: Schematic diagram for protein truncation test

2.1.9 Advantages of PTT

PTT has clear advantages over other screening methods when large portions of the mutations are due to small genetic changes, causing truncation mutations [4, 38].

1) An advantage of PTT over conventional assays is that the length of the truncated protein pinpoints the position of the mutation, thereby facilitating its confirmation by sequence analysis. Difficulties can be expected in the case of in-frame deletions comprising 4kDa or less, for example those due to exon skipping, caused by splice site mutations. In such events, the truncated protein is only slightly smaller than the wild-type product and thus difficult to detect by SDS-PAGE. Fortunately, such mutations are accompanied by smaller RT-PCR products, which can be easily detected by agarose gel analysis [12, 13, 38].

2) PTT ignores missense mutations; thus polymorphisms detected by other methods (e.g. SSCP/HA) do not have to be eliminated by sequencing [12, 15].

3) PTT allows the analysis of large stretches of coding sequence (up to 5kb genomic DNA) [12], for instance the entire BRCA1 or BRCA2 exon 11 sequence can be analysed in one experiment. This significantly reduces workload. However, if large areas are analysed the resolution of the SDS-PAGE decreases. Solution: use a couple of different percentages of polyacrylamide gel 4%-20% to cover the large range of proteins.

Alternatively the area to be analysed can be amplified in overlapping sections. To prevent mutations near the 5'-end of each section being missed (polypeptide produced is so small it falls off the gel), the overlaps must be 200-300 bp. Thus, a mutation present at the end of one section should be detected twice, once at the end of one section and again at the beginning of the following section.

4) Detects truncating mutations i.e. disease causing and not missense mutations, which often represent non-disease related sequence variation [12, 13].

2.1.10 Disadvantages of PTT

1) Insensitive to mutations immediately upstream and downstream of PCR primer sites.

The boundaries of the translated fragments represent the most critical regions for PTT analysis and are primarily determined by the resolution of SDS-PAGE analysis. Early (N-terminal) mutations-close to the 5' end- may result in products, which have too little, or no label incorporated, or their electrophoretic migration might fall outside of the resolution range. Late (C-terminal) mutations -close to the 3' end- might result in a size difference, which is not resolved near the top of the gel. Finally, mutations at translation initiation and termination sites represent a special case. Here, an internal control of the overlapping segment is not available; special attention (e.g., sequence analysis or SSCP) is required to reveal these mutations [38].

2) PTT may be limited to amplification of the mutated allele, which may lead to failure to detect very small in-frame deletions/insertions or missense mutations. Amplification failure can have several explanations including a mutation of the primer binding site (an altered or deleted sequence), large insertions, translocations and inversions which go beyond amplifiable lengths, or duplications when one or both primer binding sites lie within the duplicated segment. Mutations that affect the amount of RNA produced or which render the mutated mRNA unstable may also not be detected [38].

3) Not applicable to all genes: dependent on the proportion of mutations that are truncating: E.g. APC, BRCA1, BRCA2 and Dystrophin all have

approximately 90-95% truncating mutations. In addition, restricted to truncating mutations.

4) Radioactive detection method. [^{35}S] Methionine commonly used to make radiolabel newly synthesized proteins, which can then visualised by autoradiography.

5) Cannot detect missense mutations. Basically PTT may detect either frameshift or nonsense mutations (truncating mutation) that lead to generate stop codon. However, some missense mutations may cause a disease but does not generate stop codon lead to detect by PTT (See chapter 4 Figure 4.6).

Table 2.4: Resolving gels for SDS-PAGE

Components	Gel 4%	Gel 6%	Gel 10%	Gel 12%	Gel 20%
40% bis:acryl (29:1)	0.5 ml	0.75 ml	1.25 ml	1.5 ml	2.5 ml
1.5 M Tris (pH=8.8)	1.25ml	1.25 ml	1.25 ml	1.25 ml	1.25 ml
10% (w/v) SDS	50µl	50 µl	50 µl	50 µl	50 µl
20% (w/v) AMPS (fresh)	25µl	25 µl	25 µl	25 µl	25 µl
TEMED	4µl	4 µl	4 µl	4 µl	4 µl
Distilled water	3.17ml	2.92 ml	2.42 ml	2.17 ml	1.17 ml

Total volume = 5ml for one gel

Table 2.5: 5% Stacking gels for SDS-PAGE

Components	Two gel	Four gels
40 % bis:acryl (29:1)	0.638 ml	1.276 ml
1 M Tris (pH=6.8)	0.625 ml	1.250 ml
10% (w/v) SDS	50 µl	100 µl
20% (w/v) AMPS (fresh)	25 µl	50 µl
TEMED	4 µl	8 µl
Water	3.66 ml	7.32 ml

* Total volume= 5ml for two gel

Table 2.6: Formulation for 2x SDS-PAGE sample buffer

Components	Concentration	vol/ml
Tris-base (pH=6.8)	1 M	0.5
Glycerol	---	0.8
SDS	10% (w/v)	1.6
2-Mercaptoethanol	---	0.4
Bromophenol blue	Saturated	0.1
Water	---	4.5

* Total volume 8ml
* Store working solution at 4°C.

Table 2.7: Formulation for 1x SDS-PAGE running buffer

Components	Concentration	g/litre
Tris-base (pH=8.6)	2.5mM	6
Glycine	190mM	28.8
SDS	0.1% (w/v)	2

* Dissolve above ingredients in 800 ml distilled water.
*Adjust pH to 8.3
* Adjust volume to 1 litre with distilled water.
* Store at room temperature.

2.1.11 Meta-PCR technique

Meta-PCR is a method for creating chimaeric DNA molecules and increasing the productivity of mutation scanning techniques [27]. Many mutation-scanning techniques are capable of locating mutations in DNA fragments much larger than the average exon. Meta-PCR that can maximize the length of sequence scanned by these techniques, improving their productivity and realizing their full potential. Meta-PCR is a simple, versatile, and powerful method for generating chimaeric DNA molecules. Currently, up to five PCR amplifiable fragments can be combined to form a single linear amplimer [27]. The Meta-PCR reaction is self-assembling and takes place in two coupled stages carried out in a single reaction vessel. The order of fragments is reproducible and determined by primer design (See chapter 5 in great detail).

2.1.12 Direct nucleotide sequencing

Direct nucleotide sequencing is the most reliable technique in detecting variant DNA sequence. Sanger and colleagues invented a technique in 1977, which is an enzymatic method and involves DNA synthesis by a primer dependent DNA polymerase from a ssDNA template [40]. This method consist of three stages:

1. Annealing a short oligonucleotide (primer) to a DNA template; complementary to a region adjacent to the sequence of interest.

2. The Primer/template duplex is as a substrate for chain extension where dNTPs and radioactively labelled dATPs are incorporated into the extended DNA strand.

3. The reaction is terminated by adding dideoxynucleotides to the extended DNA strands in the four separate reactions

These reactions produce a series of DNA fragments, after separation of the four sets of reactions in adjacent slots by electrophoresis on a long denaturing polyacrylamide gels, that can then be visualised by

autoradiography. This method characterises the location and nature of the sequence change and is therefore the final step of any detection method.

Recently, a new sequencing technique has been developed and applied widely named cycle sequencing. However, a new protocol based on cycle sequencing and fluorescence detection technology has been developed [41]. Fluorescent-labelled dideoxy terminators are sets of different fluorescent dyes that are covalent coupled to each of the four dideoxynucleotide triphosphate (ddNTPs). Typically this method is using as automated ABI DNA sequencer; used relatively this technique is the best method for mutation detection although it is expensive.

2.1.12.1 Disadvantages of direct nucleotide sequencing (DS)

The sensitivity of DS reaches to 98% [42] however failure to detect the entire exon deletions, or error in RNA transcripts processing outside to the coding regions (exonic part of the genes) has been reported. The proportion of BRCA1 and BRCA2 genes mutations wrongly attributable to such inefficiencies is unknown, but it has been estimated at to 5%-15% [43].

In this study each sequence variant was re-amplified from the original genomic DNA to minimise the possibility of errors. Amplified DNA was sequenced using BIG DYE dideoxy-terminator chemistry (Pre-Sequencing Kit, Amersham Life Science) as described in the manufacturer's protocol on an automated ABI 373A DNA sequencer in Dr A. Moir's laboratory. Each fragment was sequenced in both directions with the original PCR primers.

2.1.12.2 BIG DYE sequencing protocol

1. Add 1.5µl exonuclease and 7.5µl PCR product to a 0.5ml eppendorf. Spin, and then incubate at 30°C for 15 minutes follow at 80°C for 15 minutes)

2. Add 1.5µl alkaline phosphatase. Spin and repeat incubation as step one.

3. Make up the following master mix:

4µl	Ready React
4µl	half term
3.2µl	Primer (**EITHER** forward or reverse at conc. of 1pmol/µl)
4.8µl	distilled water
4µl	Prepared template

i.e. a separate master mix is required for forward and reverse of same PCR product.

4.Spin, and then amplify at 94°C for 30 sec, at 50°C for 15 sec and extending at 60°C for 4 min for 25 cycles on an Applied Biosystems DNA thermal cycler, Applera Europe BV, Cheshire, UK.

PCR product then precipitates and washes as described below;

2.1.12.3 Sodium acetate precipitation

1. Add 50µl 95% ethanol and 2µl 3M Sodium Acetate (pH4.6) to a 0.5µl eppendorf.
(NB. 3M sodium acetate = 4.92g in 20ml distilled water) (Rmm = 82.03)

2. Add all sequencing products, Vortex and stand at room temperature for at least 15 minutes (preferably 30 minutes or more). (NB.30mins works fine.)

3.Centrifuge at 13,000rpm, 16°C, for 20 minutes.
IMPORTANT: Proceed to the next step immediately. If not possible, then spin the tubes for 2 minutes more immediately before performing the next step.

4. Carefully aspirate the supernatant.
IMPORTANT: The supernatant must be removed completely, as unincorporated dye terminators are dissolved in them. The more residual supernatant left in the tubes, the more unincorporated dye terminators will remain in the samples.

5. Add 250µl 75% Ethanol.

6. Centrifuge for 5 minutes, 13,000rpm, 16°C.

7. Remove supernatant carefully as in step 4.

8. Repeat steps 4-7

9. Open lids and air dry.

2.1.13 Features of the 'ideal' mutation screening method

Breast cancer is now considered as a health problem worldwide. Most countries are now offering a diagnostic and predictive genetic test to the high-risk families as a public or private health services. Due to complexities of BRCA genes analysis, new techniques are rapidly emerging to overcome these difficulties. The ideal method should have features, which can then be readily reproduced by molecular genetics laboratories. Some of these features are mentioned below:

- Able to scan kilo base lengths
- 100% detection rate
- No false positives or negatives
- Single step
- No complex equipment or facilities
- No harmful reagents
- No electrophoresis
- High throughput
- Low elapsed and bench time
- Mutation identification
- Low cost

It is important to note that no currently available technique can guarantee the identification of all cancer-predisposing mutations in for example the BRCA1 or BRCA2 genes. [6]

2.2 Materials and general equipments

A detailed list of materials, equipments and suppliers used in this study can be found in Appendix IX, X unless otherwise indicated all other chemicals were from BDH. The preparation of stock reagents and buffers is described in Appendix VIII. The distilled water referred to as dH_2O in the text was prepared commercially.

2.3 References

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3 Combined Single Strand Conformation Polymorphism assay (SSCP) and Heteroduplex Analysis (HA)

3.1 Introduction

SSCP is widely regarded as the simplest and most commonly used method of mutation detection. The PCR is used to amplify the region of interest from genomic DNA and the resultant widely regarded on products are separated as single-stranded molecules by electrophoresis in a non-denaturing polyacrylamide gel [1]. Different single-stranded DNA sequences fold differently from each other even when two sequences differ by a single base; it is believed that mutation induced changes of secondary structure of the DNA confer different electrophoretic properties upon the two strands. These differences that arise owing to mutation can be detected by the appearance of new species following electrophoresis and detection by silver staining of bands (Figure 3.1).

The secondary structure of single-stranded DNA changes under different physical conditions e.g. temperature and ionic environment. Hence the sensitivity of SSCP depends on these (and possible other) conditions.

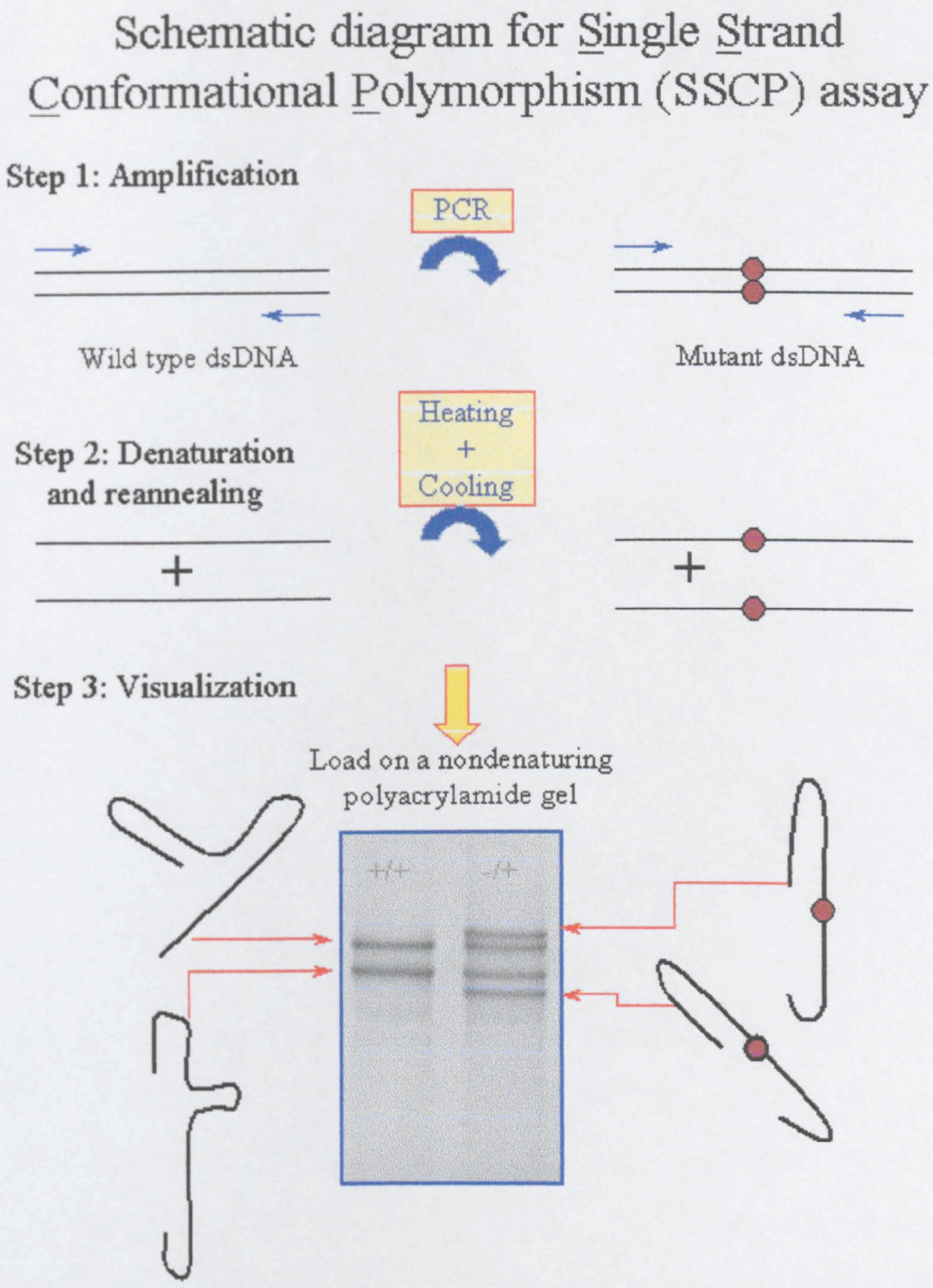


Figure 3.1: Schematic diagram for SSCP assay

Whilst some empirical rules have emerged for the choice of separation conditions for sequence variants in particular sequence contexts, it is impossible to predict whether a certain mutation can be detected under given conditions, especially when the mutation falls within a new sequence context. The success of mutation detection in PCR-SSCP is generally very high; more than 80% of mutation can be detected in a single run when fragments are shorter than 300bp[2]. As sensitivity is not 100%, the absence of a new band does not prove that there is no mutation in the analysed molecule.

The sensitivity of PCR-SSCP decreases with increasing fragment length, less than 300bp being the optimum. For mutation detection in longer fragments (exons >300bp and whole cDNAs) overlapping short primer sets can be used, or long PCR products digested with appropriate restriction enzymes prior to SSCP.

3.2 The principle of Single Strand Conformational Polymorphism assay

For SSCP, wild type and test samples are typically amplified by PCR from genomic DNA, denaturation at 99°C and rapid cooling is followed by electrophoresis on a non-denaturing polyacrylamide gel that allows the single strand molecules to form secondary structure, which affects their rate of migration, products can be detected by silver staining. Some of each product may reanneal to give double-stranded DNA, which typically runs faster through the gel than single-stranded DNA. Since each strand takes up a different structure, two bands are seen in wild-type DNA. A homozygous mutation produces two bands in a different position than the wild type amplicon and a heterozygote will produce four bands (two wild type, two mutant). The sample is then sequenced to determine the specific location and nature of the mutation.

If fragments of less than 300 bp (size dependent) are tested, insertions, deletions and most single base substitutions are likely to be detectable. In SSCP, the precise pattern of bands seen is very sensitive to the experimental conditions.

The ability to detect single base changes rests on several factors, which when optimised can have a significant influence upon band resolution.

1) Fragment size: the estimated efficiency for detecting single base changes is 89-99% for 100-300 bp fragments, but drops to 67% for 300-450 bp fragments [2].

2) Gel temperature: some fragments may be separated from wild type at room temperature, others at 12°C.

3) Bis/acrylamide ratio: the bis/acrylamide ratio determines the per cent of gel cross-linking. One to two per cent cross-linking is optimal (i.e. 5% acrylamide with 0.05% bis = 1% cross-linking).

Box 3.1

SSCP assay is based on several observations:

1. Under nondenaturing PAGE, denatured DNA runs as single strands.
2. Secondary structures form that are sequence dependent
3. A single base change can alter the secondary structure in a way that affects electrophoretic mobility of the single strands.

Additionally, glycerol (5-10%) can be added to the gels, which are then run at 12°C-16°C to improve resolution. Detection is accomplished by silver staining. Optimal conditions must be determined for each fragment (sequences dependent).

To test whether the chosen SSCP conditions and analysis are suitable for a given amplicon it is a common to start with a set of three DNA samples that have been previously characterized and are known to represent three

different genotypes within the defined DNA region (AA [homozygote (Ho) A], Ht [heterozygote (Ht) AB] and BB [Wild type (Wt) B]. (Figure 3.2)

Figure 3.2: Three genotypes with different patterns of ssDNA and dsDNA mobility

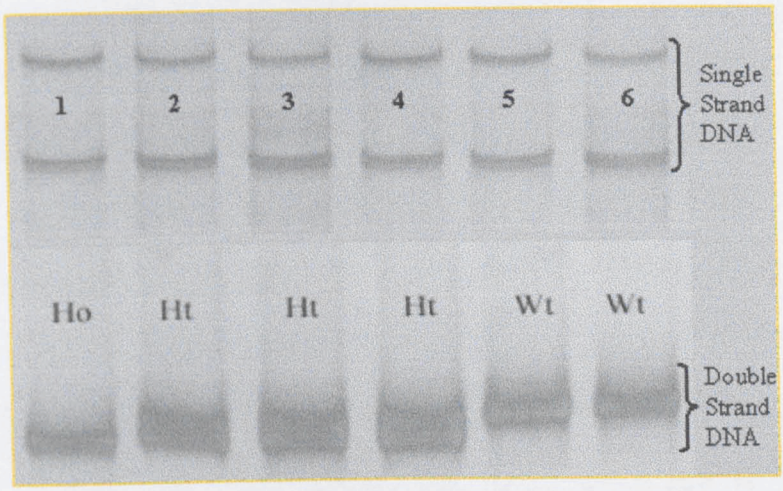


Figure 3.2: Lanes 1-6 (top row) show normal pattern of single-stranded DNA electrophoretic mobility and bottom row depict different pattern of double-stranded DNA mobility from three different genotypes within the defined DNA region seen on polyacrylamide gel using SSCP/HA.

3.2.1 Advantages of SSCP assay

1) Simple, minimal number of steps.
2) Adequately sensitive (60% - 80%)
3) Low cost – requiring no special equipment.
4) Non - radioactive detection method can be used.

3.2.2 Disadvantages of SSCP assay

1) Sensitivity decreases with increasing fragment length. Maybe overcome by:

(a)Multiplexing differently sized fragments and loading onto single gel lane
(b)Restriction enzyme digestion prior to electrophoresis

2) Inefficient for fragments longer than 250-300 bp
3) Dose not reveal the nature or position of mutation
4) Control sample must be used
5) **Uncertain interpretation**

3.2.3 Heteroduplex Analysis (HA)

A Heteroduplex is a double-stranded DNA molecule in which the two DNA strands show imperfect base complementarity. When dsDNA is denatured, the two strands separate; upon renaturation, complementary DNA strands reanneal and form a homoduplex. However, when there is a mutation in one of the strands then a heteroduplex is formed. The electrophoretic mobility of heteroduplexes in polyacrylamide gels is usually less than that of the corresponding homoduplex, and these two species can often be readily distinguished [3, 4] (Figure 3.2). This concept of heteroduplex formation also forms the basis of mutation screening when the normal (probe) and patient's DNA is mixed, denatured and annealed. Most, but not all of these methods, rely on detecting mismatched bases formed when complementary strands of a mutation and the wild type allele are allowed to hybridise to form a heteroduplex. This is likely to occur naturally when DNA sample from an individual who is heterozygous for a particular genotype is denatured then cooled to allow mutant strands to base pair with complementary strands from the wild type allele. In fragments of less than 200 bp insertions, deletions, and most single base substitutions can be detected using this approach. Heteroduplex analysis is mostly used combined with single strand conformational polymorphism assay (SSCP) on a single gel to improve sensitivity [1] (Figure 3.2).

Several mutations in a gene can also be combined to form a multiplex heteroduplex analysis. Such a method has been successfully reported in early-onset breast cancer [5].

- **Advantages of HA**

Advantages and disadvantages of HA similar to those for SSCP:

1) The SSCP and Heteroduplex Analysis techniques are each estimated to have sensitivities between 60 and 80% in detecting single-base-pair substitutions and the combined mutation detection rate should be higher [6].

2) The size limit for the DNA fragment is higher than SSCP (up to 800bps) [7].

In view of the nature of this experiment described in this chapter, I have included details of the methodology as they pertain SSCP, in order to clarify the results and their interpretation.

3.3 SSCP in practice

Both BRCA1 and BRCA2 are large genes containing 24 and 27 exons respectively (See Appendix II and table 1). In both genes the first exon is non-coding and exon 4 consists of a repetitive sequence of the Alu family that is omitted from most transcripts.

In this study 104 samples for germline mutations in the BRCA1 gene exons 2, 3, 5, 13, 20 and BRCA2 gene exons 9,17, 18, 23 were analysed by SSCP/HA assay based on PCR-amplified genomic DNA.

In order to improve the SSCP analysis, BRCA2 exon 18 was amplified in two segments.

To avoid any missed frameshift mutations that might be present close to the N-terminal (early) and C-terminal (late) coding region of BRCA1 and BRCA2 gene exon 11, these regions were screened by SSCP/HA. The entire coding sequences of exon 11 in both BRCA1 and BECA2 gene was also analysed by Protein Truncation Test (See chapter four for more detail).

3.3.1 Primer design

Table 3.1 and 3.2 show the sequences of primers used in this work for the amplification of the above BRCA1 and BRCA2 genes. In all cases splice junctions were included in the analysis.

Table 3.1: Details of primers used for amplification and screening of the BRCA1 gene by SSCP/HA

Exon	Forward primer	Reverse primer	Anl. Temp Used (°C)	Product length (bp)
2	5'- aaa cct tcc aaa tct taa a -3'	5'- gtc ttt tct tcc cta gta tgt -3'	54	302
3	5'- aac gaa ctt gag gcc tta tg -3'	5'- ttg gat ttt tcg ttc tca ctt -3'	54	308
5	5'- ctc tta agg gca gtt gtg ag -3'	5'- atg gtt tta tag gaa cgc tat g -3'	56	278
11- 5'end	5'-ttc agt ttt tga gta cct tgt tat t -3'	5'-cta tca tta cat gtt tcc tta ctt c-3'	55	362
11- 3'end *	5'-cat tga aga ata gct taa atg act g- 3'	5'- act ggg gca aac aca aaa acc t- 3'	57	343
11- 3'end *	5'- aag aaa tta gag tcc tca gaa -3'	5'- aca caa aaa cct ggt tcc aat - 3'	53	501
13	5'- aat gga aag ctt ctc aaa gta -3'	5'- atg ttg gag cta ggt cct tac -3'	55	320
20	5'- ata tga cgt gtc tgc tcc ac -3'	5'- agt ctt aca aaa tga agc gg -3'	56	259

*C-terminal (3'end) of BRCA1 exon 11 was analysed using conventional PCR-SSCP assay and digested PCR-SSCP with BamHI with different sizes 343bp and 501bp respectively.
(Annealing temperature=Anl. Temp)

Table 3.2: Details of primers used for amplification and screening of the BRCA2 gene by SSCP/HA

Exon	Forward primer	Reverse primer	Anl. Temp Used (°C)	Product length (bp)
9	5'- cta gtg att tta aac tat aat ttt tg -3'	5'- ctg tag ttc aac taa aca gag g -3'	55	168
11- 5' end	5'-ggt act tta att ttg tca ctt tgt gt-3'	5'- gta ttc cac ttt tga atg ttg tac -3'	56	373
11- 3'end	5'- gta tta gga acc aaa gtc tca c -3'	5'-tag tga ttg gca aca cga aag g -3'	57	385
17	5'- cag aga ata gtt gta gtt gtt gaa -3'	5'-aga aac ctt aac cat act gc -3'	55	304
18 part1	5'- cag tgg aat tct aga gtc aca c -3'	5'- gcc act ttt tgg gta tct gc -3'	57	229
18 part2	5'- gca gat acc caa aaa gtg gc -3'	5'- gac tga ttt tta cca aga gtg c -3'	57	234
23	5'- cac ttc ttc cat tgc atc ttt ctc -3'	5'- gag att cca taa act aac aag cac -3'	57	265

(Annealing temperature=Anl. Temp)

3.3.2 PCR amplification protocol

The DNA region to be screened was amplified by PCR, using DNA from the three samples described above as templates. After optimising conditions (dilutions of DNA template, primer, Mg^{2+} concentrations and PCR thermocycling settings), the PCR components prepared (Table 3.3).

Table 3.3: Modification of standard PCR components for optimum SSCP templates

Components	Volume	Final concentration
1) distilled water (pH 7.0)	Up to 20μL	
2) 10x PCR Buffer*	2.0μL	
3) $MgCl^{2+}$ (25mM)	1.4-1.6μl	1.7-2mM
4) dNTPs (2.5 mM each nucleotide)	1.5μL	200μM
5) primer mix (5pM/μL each primer)	1μL	0.25pM/μL
6) genomic DNA template (50-100ng)	1.0μL	
7) Red Hot Taq DNA polymerase (2-5 Unit)	1.0μL	

* 2μl of 10x PCR Reaction Buffer consist of: 750mM Tris-HCl (pH 8.8 at 25°C), 200mM$(NH4)_2$ SO4 and 0.1%(v/v) Tween 20

3.3.3 Thermocycling procedure

The DNA template was denatured with one cycle at 94°C for 2 minutes. Denaturing at 94°C for 30 sec, annealing at 55°C -60°C for 1 minutes and extending 72°C for 1 minute for 30 cycles. A final extension at 72°C for 5 minutes was performed. Annealing temperatures were varied according to predicted primer-template melting temperature.

3.3.4 Interpretation of results

The interpretation of the results of an SSCP/HA gel requires experience, which therefore require that controls of known mutations be used in each analysis. Some double-stranded DNA often reforms after the denaturation step, which will produce bands closer to the bottom of the gel. It is helpful to include an undenatured sample to help in identifying these bands. Those heteroduplexes molecules formed when a mutant and wild type strand anneal, can also predict a sequence alteration. These heteroduplexes migrate just above the double-stranded DNA. While heteroduplexes are most prominent in the case of insertion or deletion, they can sometimes be observed with point mutations [8].

Since the exact migration properties of single strand conformers can vary from gel to gel, it is useful to include all available control samples at least once on each gel. While it is expected that the two strands will migrate differently, this is not always the case. Similarly, in a heterozygote, not all four strands are always resolved. It is important to note that a mutant sample should give a clearly different pattern from a wild type sample. In addition, a heterozygote should display approximately equal intensity in all four bands. The final proof of the alteration ultimately comes from nucleotide sequencing (Figures 3.3-3.7).

3.3.5 Modifications of SSCP/HA detection

The condition of the SSCP gel can be varied to produce gels in which the samples form alternative conformations. The number of possible conformations is so large that there is no theoretical basis for choosing conditions. Clearly, the more conditions run, the greater and the potential sensitivity can be obtained.

The variables that have been employed for each amplicon for analysing of both BRCA1 and BRCA2 genes are listed in Table 3.4 and 3.5 respectively.

Table 3.4: SSCP gel-running conditions for each BRCA1 amplicon*

Exon/ Amplicon	Polyacrylamide/ cross linker (59:1)	Temperature (°C)	Glycerol concentration	Running time/hours
2	14%	12	3%	15
3	14%	12	3%	16
5	14%	12	3%	14
11- 5'end	14%	15	1%	17
11- 3'end	14%	14	5%	20
13	14%	12	2%	16
20	14%	12	3%	16

*See Table 3.1 for the amplicons sizes

Table 3.5: SSCP gel-running conditions for each BRCA2 amplicon*

Exon/ Amplicon	Polyacrylamide/ cross linker (59:1)	Temperature (°C)	Glycerol concentration	Running time/hours
9	14%	12	3%	16
11- 5' end	14%	15	2%	17
11- 3'end	14%	12	3%	20
17	14%	12	5%	18
18 part1	14%	12	3%	16
18 part2	14%	12	5%	15
23	14%	25	5%	16

*See Table 3.2 for the amplicons sizes

Note: The same constant power (245 volts) was applied for all conditions above.

The type and the position of the alteration within a sequence context of amplicon have a significant influence on the conformation of single-

stranded molecule. In addition, each of above variables can alter the conformation of single-stranded molecule. Gels with higher percentage of acrylamide and lower cross-linking can detect more mutations [5]. It is not possible to predict how a given change in conditions will affect the mobility of a specific fragment (Figure 3.12).

3.4 Results

- **Mutant, sequence variant, and polymorphism results**

Six distinct sequence variants were detected by analysing 104 samples for germline mutations in the BRCA1 gene exons 2, 3, 5, 13 and 20 and BRCA2 gene exons 9, 17, 18 and 23 by amplification from genomic DNA with PCR. Two of these were frameshift (putative mutations), three missense (unknown significant changes) and one sequence variant included a 12bp duplication [BRCA1, IVS20+48] (Table 3.5 and Figures 3.3 -3.13).

A common polymorphism in BRCA2 [IVS16-14T>C] was detected in affected and unaffected individuals (Figures 3.9 -3.11). Seventy-eight samples of British affected women were analysed within the BRCA2 intron-exon 17 boundary, fifty-nine individuals carried this polymorphism (Figure 3.11).

All these alterations were characterised by direct sequencing. DNA sequences obtained from affected patients' carriers and normal individuals in conjunction with the sequence data in public databases for BRCA1 and BRCA2 genes [GenBank Accession numbers: U14680, L78833, U43746 and Z74739] were compared and identified the nucleotides variation.

- **Ambiguous results**

For SSCP, in some cases different mobilities were detected in two strands but direct sequencing revealed the presence of a normal sequence. This can be a result of low sensitivity of SSCP method and/or inefficient generation of PCR products (Figure 3.12 – 3.15).

Table 3.6: Germline mutations detected by SSCP in the BRCA1 and BRCA2 genes

Gene/Exon	Mutation and nucleotide change	Tumour type	Stop codon occurred at	Coding effect	Scanning method	Family history	Age at diagnosis
BRCA1-Exon 2	185-186delAG	IDC	39 TGA	Frameshift	SSCP	2BC-1PS	**37**
BRCA1-Exon 2	181-182insT	IDC	40 TGA	Frameshift	SSCP	1-OV	**41**
BRCA2-Exon 17†	IVS16-14T>C IVS16- 6T>G	n.a.	----	Close to splice site	SSCP	n.a.	n.a.
BRCA2-Exon 18	8345A>G (N2706S)	IDC	----	Missense	SSCP	Negative	**38**
BRCA1-Exon 20	IVS20+48 dup gtattccactcc	IDC	----	Polymorphism	SSCP	2BC<42	**27††**
BRCA2-Exon 23	9266C>T T3013I	IDC	----	Missense	SSCP	Negative	**31**

††This patient also harbour a frameshift mutation in BRCA2 exon 11.

†This is close to splice site variant were detected in 81 out of 104 individuals (61 heterozygous and 20 homozygous).

BC= Breast Cancer OV= Ovarian Cancer PS= Prostate Cancer n.a.= not applicable

IDC= Invasive Ductal Carcinoma

Figure 3.3: Schematic diagram of the BRCA1(a) and BRCA2(b) cDNAs given the characterisation of the mutations detected by SSCP/HA and direct sequencing

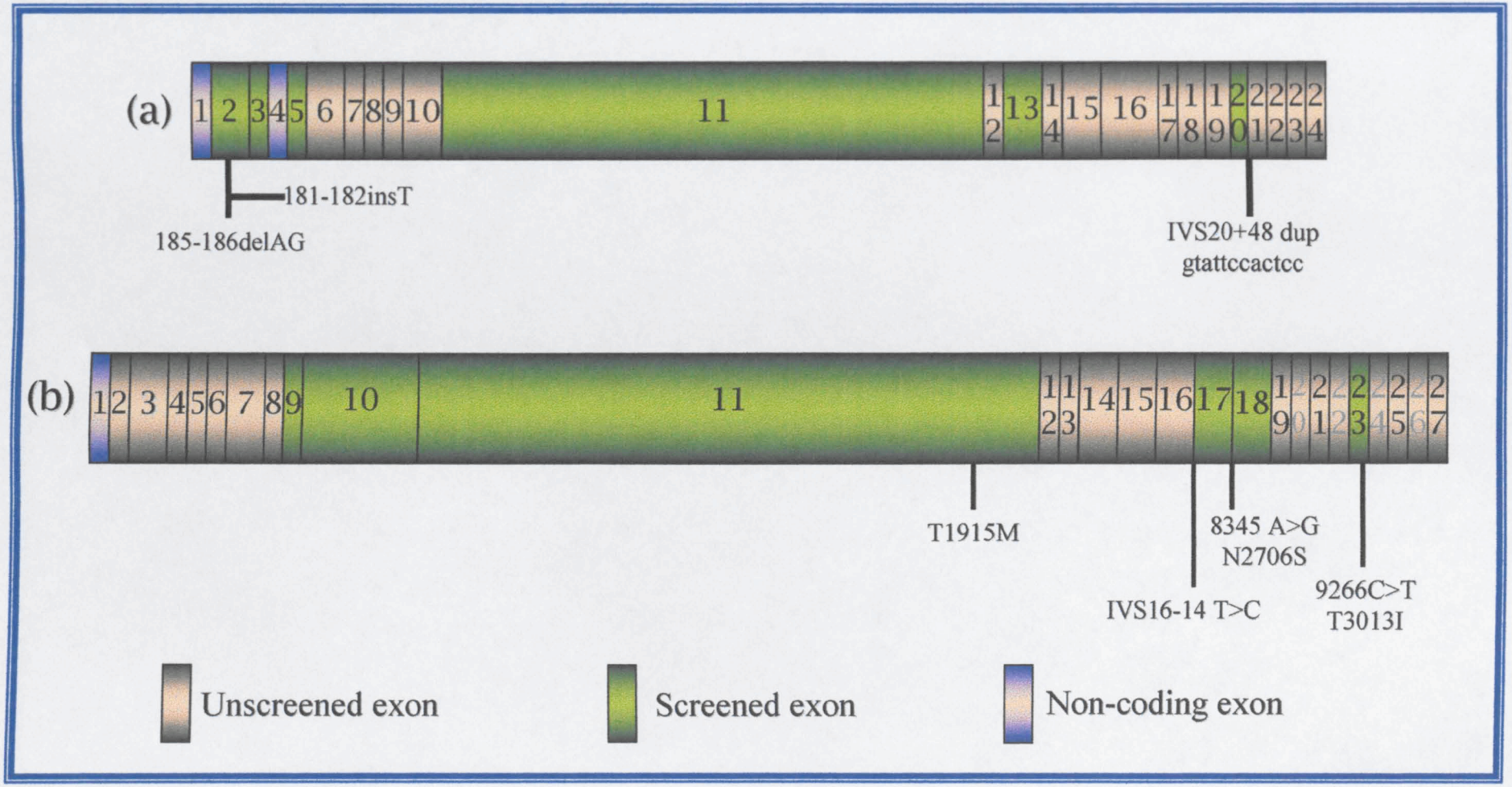
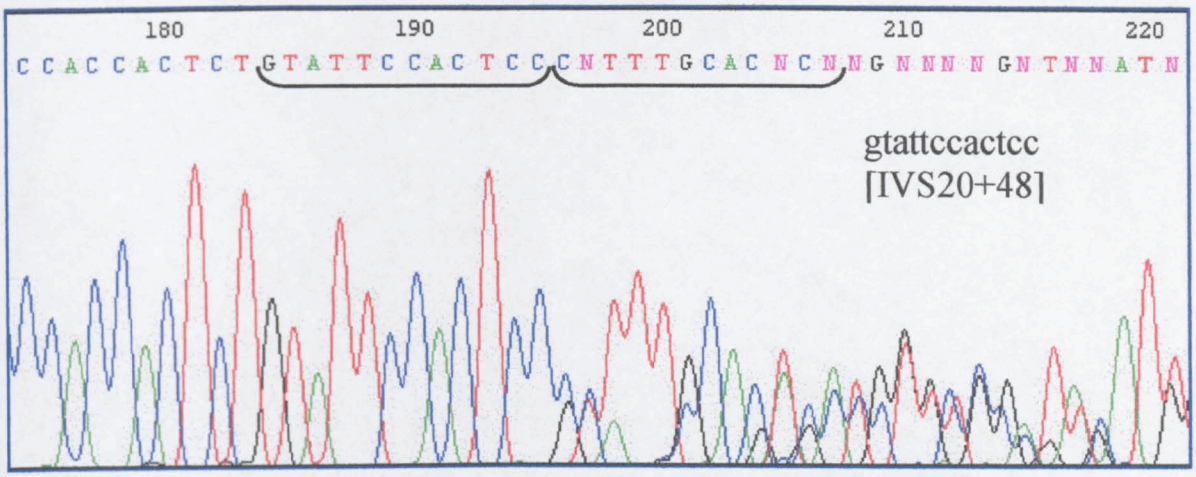
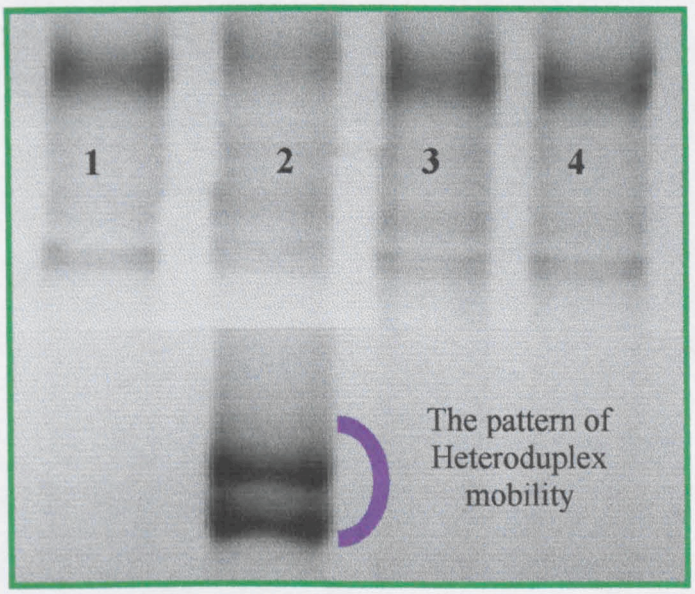


Figure 3.4: Electropherograms (a, c) and PAGE image (b) of sequence variant of BRCA1 [IVS20+48] 12bp duplication gtattccactcc

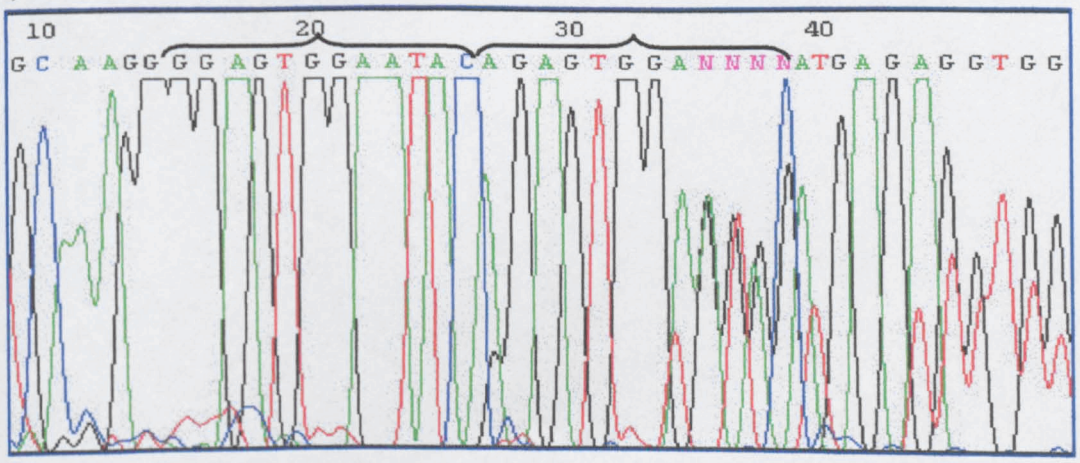
a) Forward sequence



b) PAGE image



c) Reverse sequence



Wild type sequence of BRCA1 intron19- exon20-intron20 boundary

1 ttt cag cat gat ttt gaa gtc aga gga gat gtg gtc

37 aat gga aga aac cac caa ggt cca aag cga gca aga

73 gaa tcc cag gac aga aag gta aag ctc cct ccc tca

109 agt tga caa aaa tct cac ccc acc act ctg **tat tcc**

145 **act ccc** ctt tgc aga gat ggg ccg ctt cat ttt gta

Sequence variant of BRCA1 intron19-exon20-intron20 boundary
IVS20+48 dup gtattccactcc

1 ttt cag cat gat ttt gaa gtc aga gga gat gtg gtc

37 aat gga aga aac cac caa ggt cca aag cga gca aga

73 gaa tcc cag gac aga aag gta aag ctc cct ccc tca

109 agt tga caa aaa tct cac ccc acc act ctg **tat t cc**

145 **act ccg tat tcc act ccc** ctt tgc aga gat ggg ccg

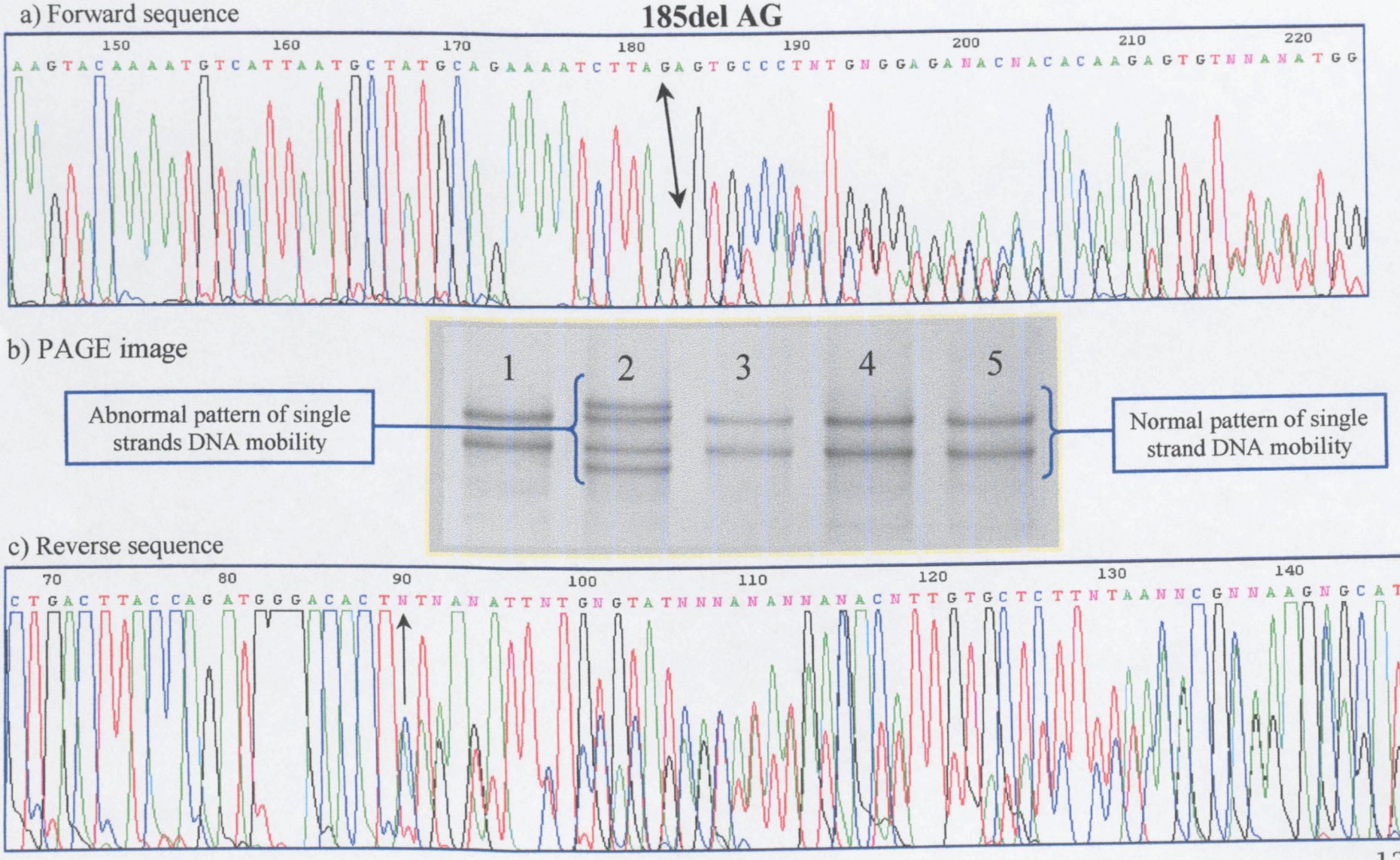
181 ctt cat ttt gta aga ctt att aca tac ata

Blue colour = Exonic sequences
Red colour = Intronic sequences
Bold lower case underlined blue colour = Intronic 12bp duplication gtattccactcc

Figure 3.4 (b) lane 2, depicts the abnormal pattern of heteroduplex mobility seen on polyacrylamide gel. This sample (lane 2) were characterised by direct sequencing and revealed 12bp duplication gtattccactcc [IVS20+48], which shown on figure 3.4 (a and c).

Note: This variant [IVS20+48 dup gtattccactcc] has been reported elsewhere and seems to be a polymorphism.

Figure 3.5: Electropherograms (a, c) and PAGE image (b) of mutant sequences of BRCA1 gene-Exon 2



Wild type sequence of BRCA1 exon 2 and exon 3 boundary

```
1   atg gat tta tct gct ctt cgc gtt gaa gaa gta caa
      M   D   L   S   A   L   R   V   E   E   V   Q

37  aat gtc att aat gct atg cag aaa atc tta gag tgt
      N   V   I   N   A   M   Q   K   I   L   E   C

73  ccc atc tgt ctg gag ttg atc aag gaa cct gtc tcc
      P   I   C   L   E   L   I   K   E   P   V   S

109 aca aag tgt gac cac ata ttt tgc aaa
      T   K   C   D   H   I   F   C   K
```

Mutant sequence of BRCA1 exon2 [185-186 del AG]

```
1   atg gat tta tct gct ctt cgc gtt gaa gaa gta caa
      M   D   L   S   A   L   R   V   E   E   V   Q

37  aat gtc att aat gct atg cag aaa atc tta gtg tcc
      N   V   I   N   A   M   Q   K   I   L   V   S
                          Site for AG deletion

73  cat ctg tct gga gtt gat caa gga acc tgt ctc cac
      H   L   S   G   V   D   Q   G   T   C   L   H

109 aaa gtg tga cca cat att ttg caa a
      K   V   -   P   H   I   L   Q
```

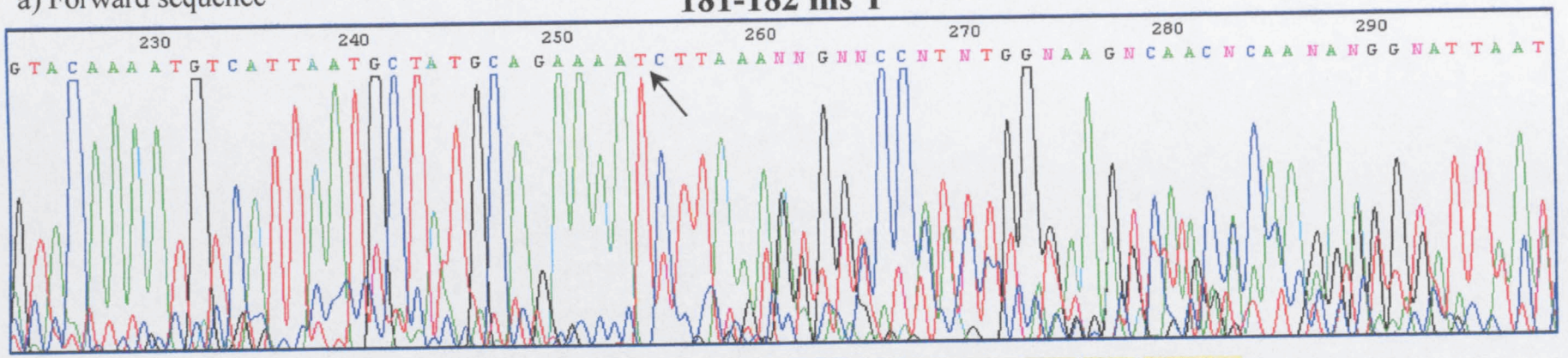
Lower cases (blue colour) =Coding sequence of exon 2
Underlined Blue colour = two base pair that is deleted in a mutant case
Red colour = Coding sequence of exon 3
Upper case letter (red and blue colour) = Amino acid sequence

Note: Figure 3.5 (b) lanes 1 and 3-5 show normal pattern and lane 2 depict the abnormal patterns of single strand DNA mobility seen on polyacrylamide gel.

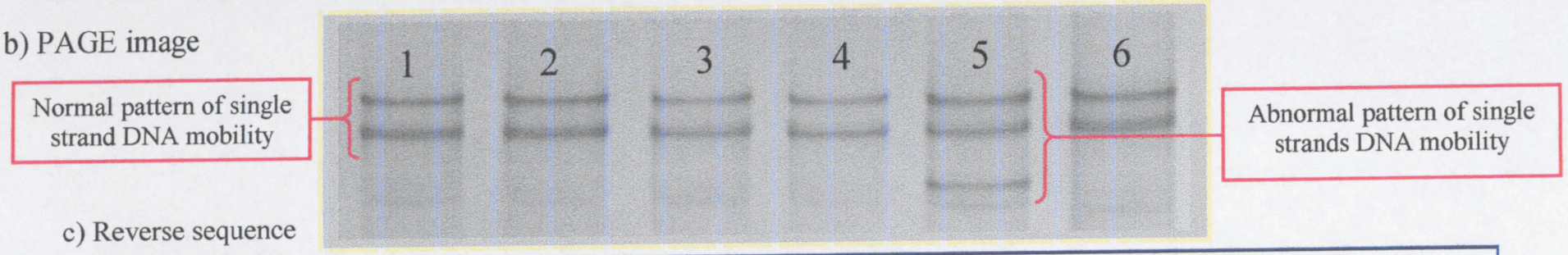
This sample (lane 2) were characterised by direct sequencing and revealed two base pair (AG) deletion in BRCA1 exon 2 at nucleotides 185-186 that leading to a TGA at codon 39 (in cDNA sequences). Figures 3.5 (a and c) show this alteration.

Figure 3.6: Electropherograms (a, c) and PAGE image (b) of mutant sequences of BRCA1 gene-Exon 2

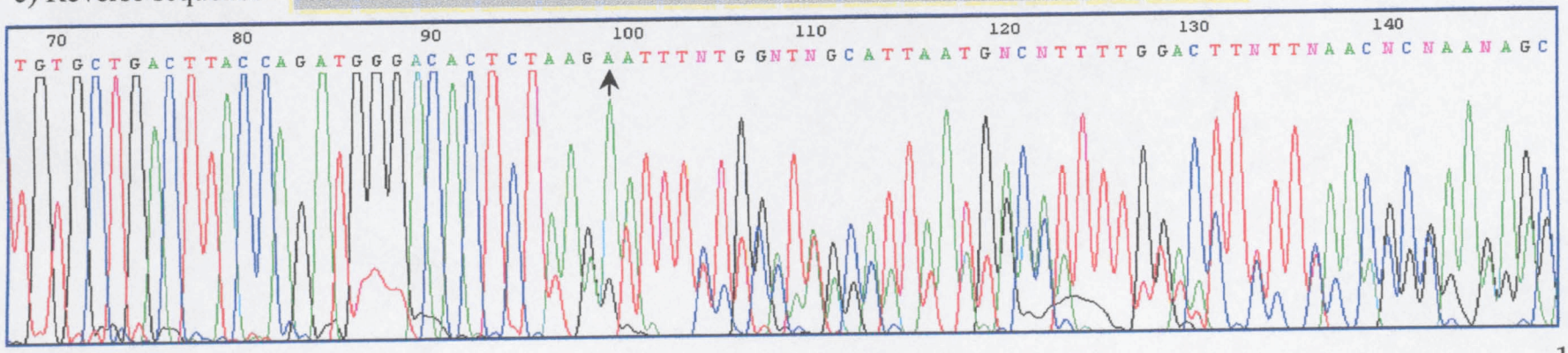
a) Forward sequence



b) PAGE image



c) Reverse sequence



Wild type sequence of BRCA1 exon 2 and exon 3 boundary

```
  1  atg gat tta tct gct ctt cgc gtt gaa gaa gta caa
       M   D   L   S   A   L   R   V   E   E   V   Q

 37  aat gtc att aat gct atg cag aaa atc tta gag tgt
       N   V   I   N   A   M   Q   K   I   L   E   C

 73  ccc atc tgt ctg gag ttg atc aag gaa cct gtc tcc
       P   I   C   L   E   L   I   K   E   P   V   S

109  aca aag tgt gac cac ata ttt tgc aaa
       T   K   C   D   H   I   F   C   K
```

Mutant sequence of BRCA1 exon2 [181-182 ins T]

```
  1  atg gat tta tct gct ctt cgc gtt gaa gaa gta caa
       M   D   L   S   A   L   R   V   E   E   V   Q

 37  aat gtc att aat gct atg cag aaa att ctt aga gtg
       N   V   I   N   A   M   Q   K   I   L   R   V
                   Site for T insertion
 73  tcc cat ctg tct gga gtt gat caa gga acc tgt ctc
       S   H   L   S   G   V   D   Q   G   T   C   L

109  cac aaa gtg tga cca cat att ttg caa a
       H   K   V   -   P   H   I   L   Q
```

Lower case letter (blue colour) = Coding sequence of exon 2
Underlined Blue colour= one base pair that is deleted in a mutant case
Red colour = Coding sequence of exon 3
Upper case letter (red and blue colour) = Amino acid sequence

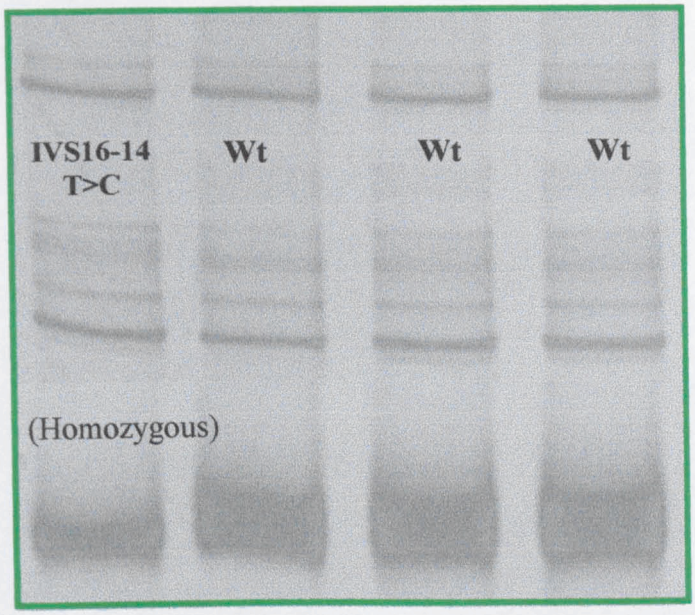
Note: Figure 3.6 (b) lanes 1-4 and 6 show normal pattern and lane 5 depict the abnormal patterns of single strand DNA mobility seen on polyacrylamide gel.

This sample (lane 5) were characterised by direct sequencing and revealed a one base pair (T) insertion in BRCA1 exon 2 at nucleotide between 181 and 182, which leads to a TGA at codon 40 (in cDNA sequences). The frameshift and nonsense mutations are likely disrupting the function of the BRCA1 protein. Figures 3.6 (a and c) show this alteration.

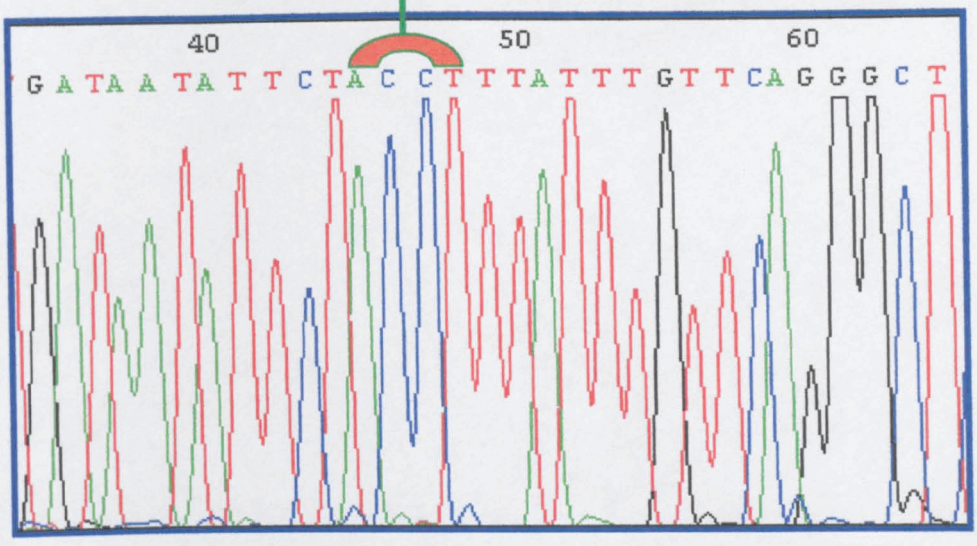
Figure 3.7: Electropherograms (a, b, c) and PAGE images (d, e) for two sequence variants of BRCA2 intron-exon 17 boundary (1) and exon 18 (2) in an individual

1) BRCA2 intron-exon 17 boundary [IVS-14 T>C]

d) PAGE image

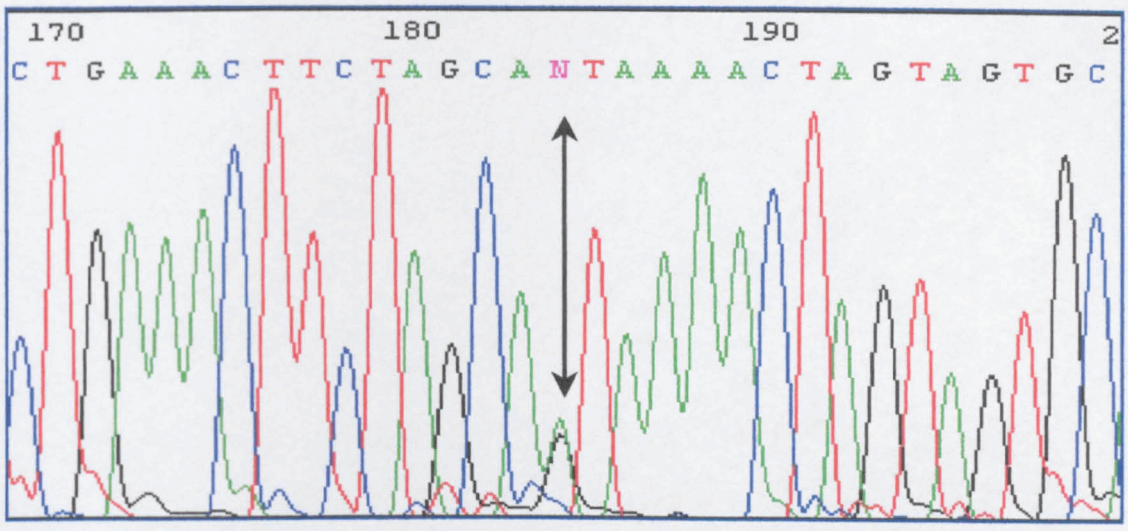


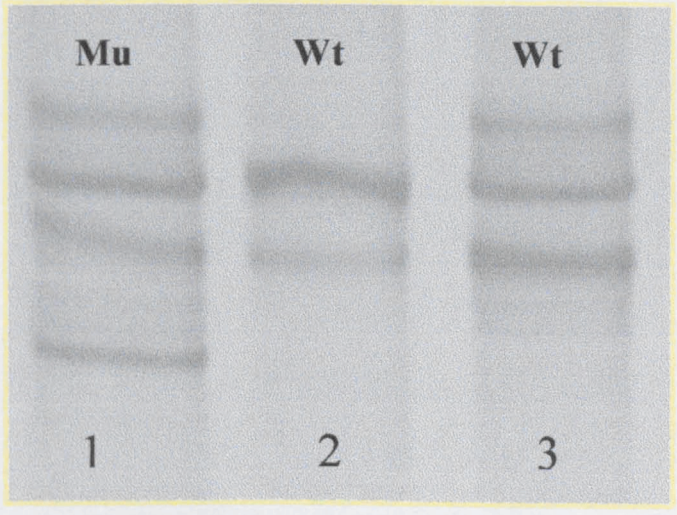
a) Forward sequence
IVS16-14 T>C (Homozygous)



2) BRCA2 exon 18 [8345 A>G (N2706)]

b) Forward sequence





e) PAGE image

c) Reverse sequence

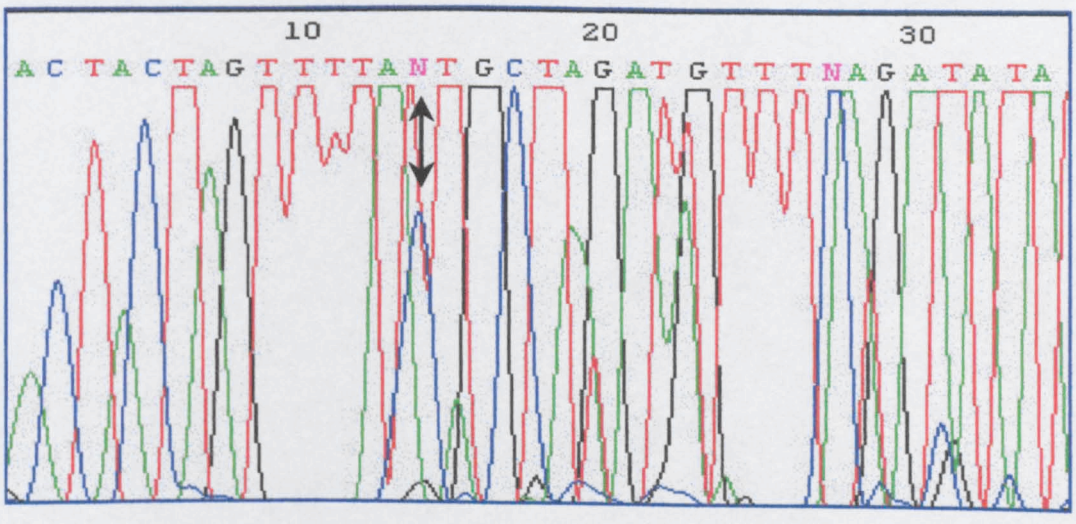


Figure 3.7: Depicted two sequence variants in an individual.

1) A one variant was observed in BRCA2 intron-exon 17 boundary [IVS16-14 T>C] (Homozygous).

Figure 3.7 (d) lane 2-4 show normal pattern and lane 1 depict the normal patterns of single strand DNA mobility and homoduplex pattern of same sample seen on polyacrylamide gel.

Direct sequencing identified a single base pair substitution T>C at IVS16-14 in this sample (Figure 3.7 a).

2) A single base pair substitution was detected in BRCA2 exon 18.

Figure 3.7 (e) lanes 2, 3 reveal normal pattern and lane 1 depict the abnormal patterns of single strand DNA mobility seen on polyacrylamide gel. Direct sequencing characterised A>G substitution at 8345 nucleotide. (Figure 3.7 b, c). Substitution of Asparagine to Serine (both are uncharged polar amino acid) on BRCA2 gene expression is more unlikely to be effective.

Wild type sequence of BRCA2 intron-exon 17 boundary

```
1   cag aga ata gtt gta gtt gtt gaa ttc agt atc atc
        R   E   -   L   -   L   L   N   S   V   S

37  cta tgt ggt ttt tat gat aat att cta ctt tta ttt
    S   Y   V   V   F   M   I   I   F   Y   F   Y

73  gtt cag ggc tct gtg tga cac tcc agg tgt gga tcc
    L   F   R   A   L   C   D   T   P   G   V   D

109 aaa gct tat ttc tag aat ttg ggt tta taa tca cta
    P   K   L   I   S   R   I   W   V   Y   N   H

145 tag atg gat cat atg gaa act ggc agc tat gga atg
    Y   R   W   I   I   W   K   L   A   A   M   E

181 tgc ctt tcc taa gga att tgc taa tag atg cct aag
    C   A   F   P   K   E   F   A   N   R   C   L

217 ccc aga aag ggt gct tct tca act aaa ata cag gca
    S   P   E   R   V   L   L   Q   L   K   Y   R

253 agt tta aag cat tac att acg taa tca tat acg gca
    Q   V   -   S   I   T   L   R   N   H   I   R
```

Lower case letter (blue colour) = Exonic segment
Red colour = Intronic segment
Upper case letter (red colour) = Amino acid sequence

Polymorphic sequence of BRCA2 intron-exon 17 boundary [IVS16-14T>C]

```
1   cag aga ata gtt gta gtt gtt gaa ttc agt atc atc
        R   E   -   L   -   L   L   N   S   V   S

37  cta tgt ggt ttt tat gat aat att cta cct tta ttt
    S   Y   V   V   F   M   I   I   F   Y   L   Y

73  gtt cag ggc tct gtg tga cac tcc agg tgt gga tcc
    L   F   R   A   L   C   D   T   P   G   V   D

109 aaa gct tat ttc tag aat ttg ggt tta taa tca cta
    P   K   L   I   S   R   I   W   V   Y   N   H
```

```
145  tag atg gat cat atg gaa act ggc agc tat gga atg
      Y   R   W   I   I   W   K   L   A   A   M   E

181  tgc ctt tcc taa gga att tgc taa tag atg cct aag
      C   A   F   P   K   E   F   A   N   R   C   L

217  ccc aga aag ggt gct tct tca act aaa ata cag gca
      S   P   E   R   V   L   L   Q   L   K   Y   R

253  agt tta aag cat tac att acg taa tca tat acg gca
      Q   V   -   S   I   T   L   R   N   H   I   R
```

Lower case letter (Blue colour) = Exonic segment
Underlined Bold Italic Red colour= A base pair substitution in polymorphic sequence
Red colour = Intronic segment
Upper case letter (red colour) = Amino acid sequence

Note: This common polymorphism in BRCA2 [IVS16-14T>C] was identified in both Iranian and British population. It may be useful as a marker in population study (See also figures 3.9, a-c and 3.11, a-e).

Wild type sequence of BRCA2 exon18

```
  1  ata tga tac gga aat tga tag aag cag aag atc ggc
        Y   D   T   E   I   D   R   S   R   R   S

 37  tat aaa aaa gat aat gga aag gga tga cac agc tgc
      A   I   K   K   I   M   E   R   D   D   T   A

 73  aaa aac act tgt tct ctg tgt ttc tga cat aat ttc
      A   K   T   L   V   L   C   V   S   D   I   I

109  att gag cgc aaa tat atc tga aac ttc tag caa taa
      S   L   S   A   N   I   S   E   T   S   S   N

145  aac tag tag tgc aga tac cca aaa agt ggc cat tat
      K   T   S   S   A   D   T   Q   K   V   A   I

181  tga act tac aga tgg gtg gta tgc tgt taa ggc cca
      I   E   L   T   D   G   W   Y   A   V   K   A
```

```
217  gtt aga tcc tcc cct ctt agc tgt ctt aaa gaa tgg
       Q   L   D   P   P   L   L   A   V   L   K   N

253  cag act gac agt tgg tca gaa gat tat tct tca tgg
       G   R   L   T   V   G   Q   K   I   I   L   H

289  agc aga act ggt ggg ctc tcc tga tgc ctg tac acc
       G   A   E   L   V   G   S   P   D   A   C   T

325  tct tga agc ccc aga atc tct tat gtt aaa g
       P   L   E   A   P   E   S   L   M   L   K
```

Lower case letter (Blue colour) = Exonic segment
Upper case letter (red colour) = Amino acid sequence

Sequence variant of BRCA2 exon 18

```
  1  ata tga tac gga aat tga tag aag cag aag atc ggc
       Y   D   T   E   I   D   R   S   R   R   S

 37  tat aaa aaa gat aat gga aag gga tga cac agc tgc
       A   I   K   K   I   M   E   R   D   D   T   A

 73  aaa aac act tgt tct ctg tgt ttc tga cat aat ttc
       A   K   T   L   V   L   C   V   S   D   I   I

109  att gag cgc aaa tat atc tga aac ttc tag cag taa
       S   L   S   A   N   I   S   E   T   S   S   S

145  aac tag tag tgc aga tac cca aaa agt ggc cat tat
       K   T   S   S   A   D   T   Q   K   V   A   I

181  tga act tac aga tgg gtg gta tgc tgt taa ggc cca
       I   E   L   T   D   G   W   Y   A   V   K   A

217  gtt aga tcc tcc cct ctt agc tgt ctt aaa gaa tgg
       Q   L   D   P   P   L   L   A   V   L   K   N

253  cag act gac agt tgg tca gaa gat tat tct tca tgg
       G   R   L   T   V   G   Q   K   I   I   L   H

289  agc aga act ggt ggg ctc tcc tga tgc ctg tac acc
       G   A   E   L   V   G   S   P   D   A   C   T
```

```
325  tct tga agc ccc aga atc tct tat gtt aaa g
     P   L   E   A   P   E   S   L   M   L   K
```

Lower case letter (Blue colour) = Exonic segment
Underlined Bold Italic red colour= A base pair substitution in sequence variant sample.
Upper case letter (red colour) = Amino acid sequence

Figure 3.8: PAGE image of sequence variants of Homozygous and heterozygous of BRCA2 exon 17 detected by SSCP/HA

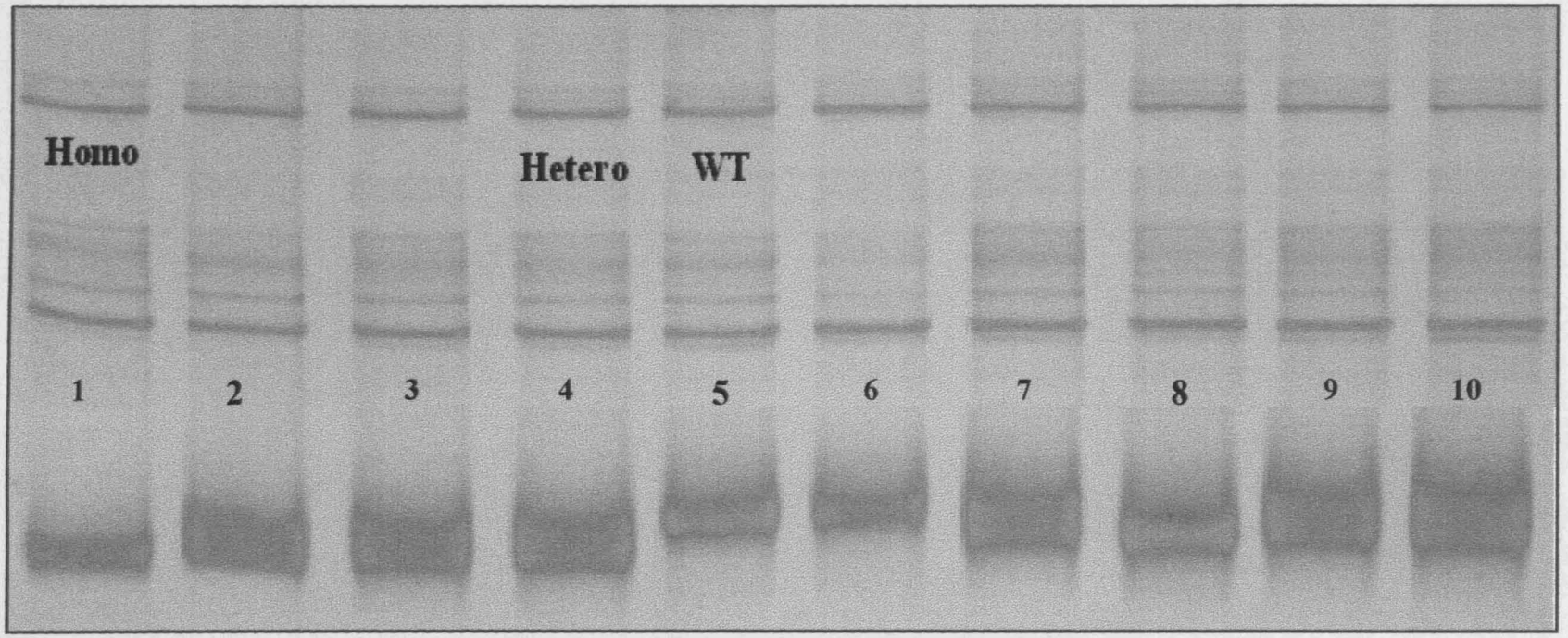
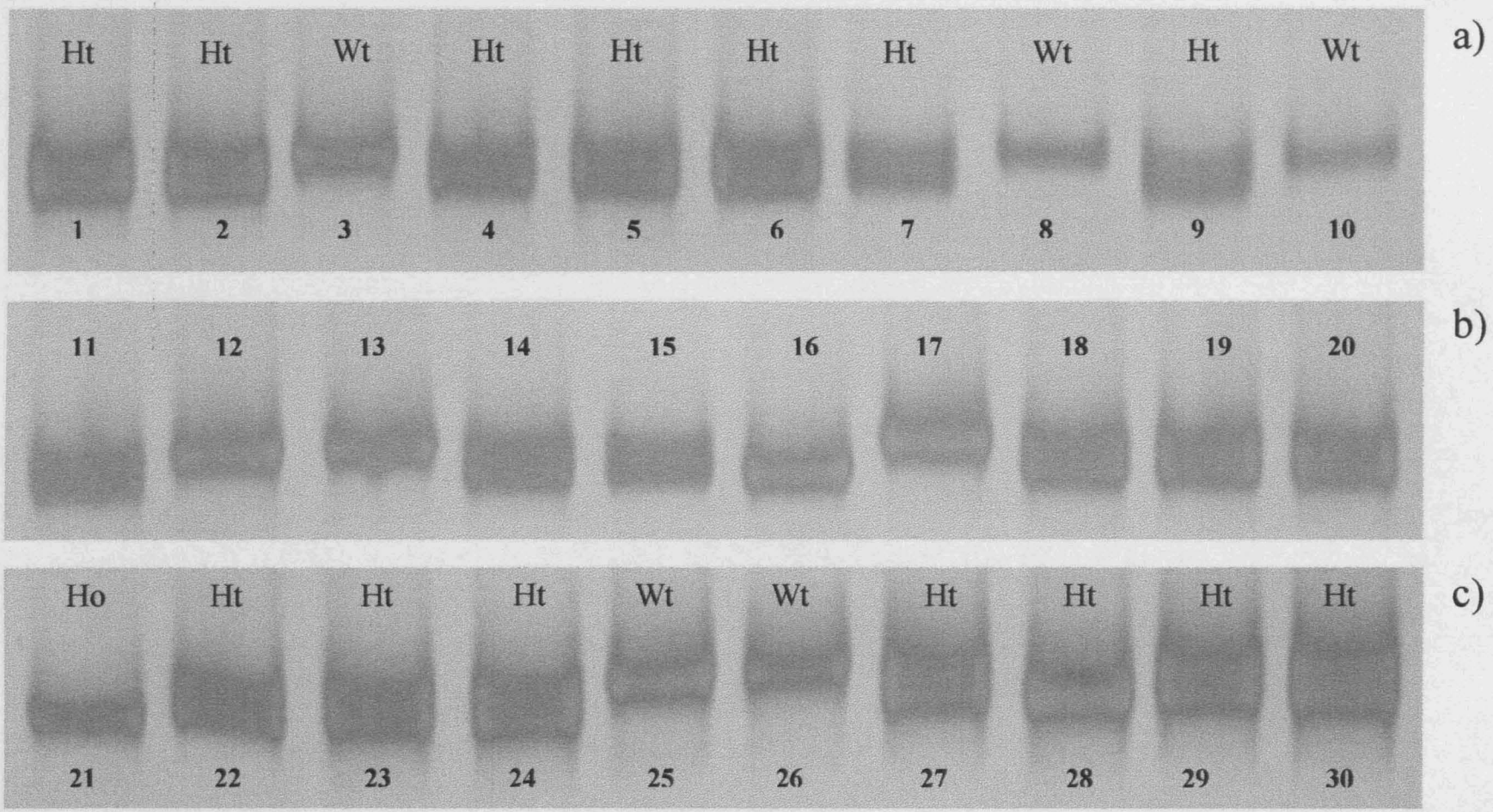


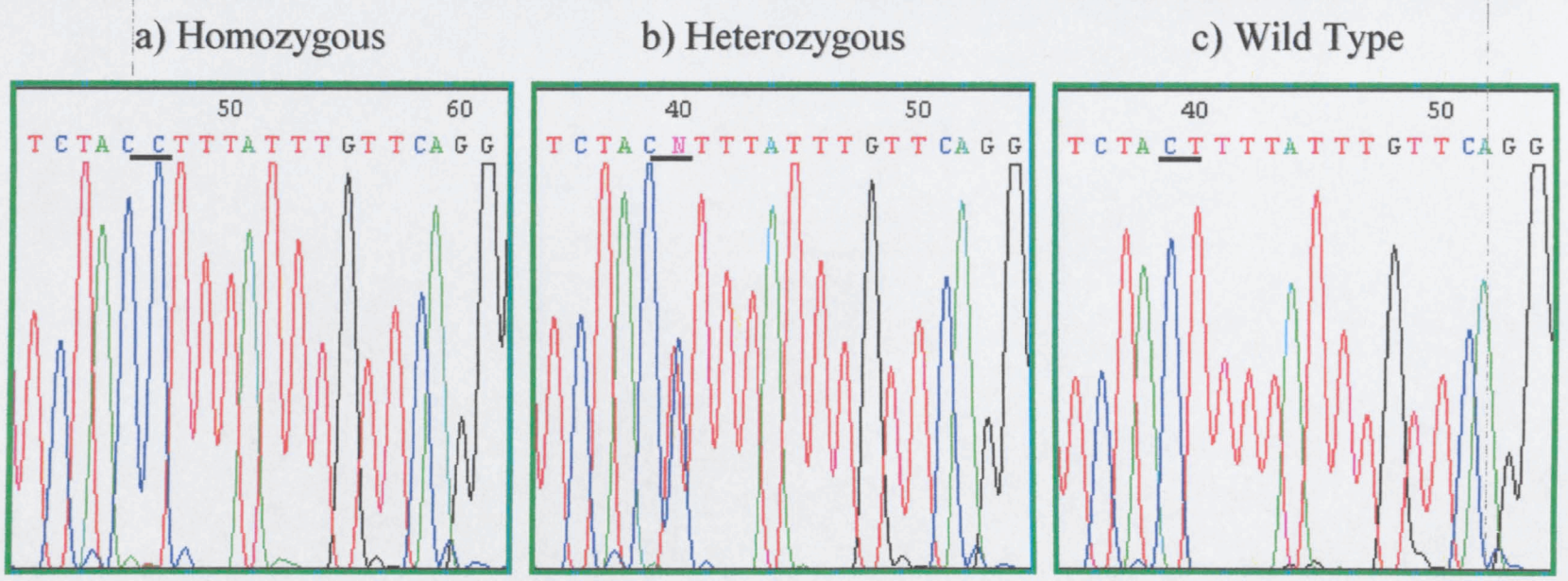
Figure 3.8: Lanes 1-10 (top row) show normal pattern of single strand DNA electrophoretic mobility and bottom row depict different pattern of double strand DNA mobility from three different genotypes (lanes 1, 4, 5) within the defined DNA region seen on polyacrylamide gel.

Figure 3.9: PAGE images (a, b, c) of Heteroduplex Analysis (HA) of sequence variants in BRCA2 [IVS 16-14 T>C] in Iranian affected women with breast cancer



Single base pair changes between the different genotypes are evident.
The designations are as follows: Lanes Wt, TT (homozygote T); Lanes Ht, TC (heterozygote C); Lane Ho,CC (homozygote C).

Figure 3.10: Electropherograms from different genotypes of BRCA2 intron-exon 17 boundary



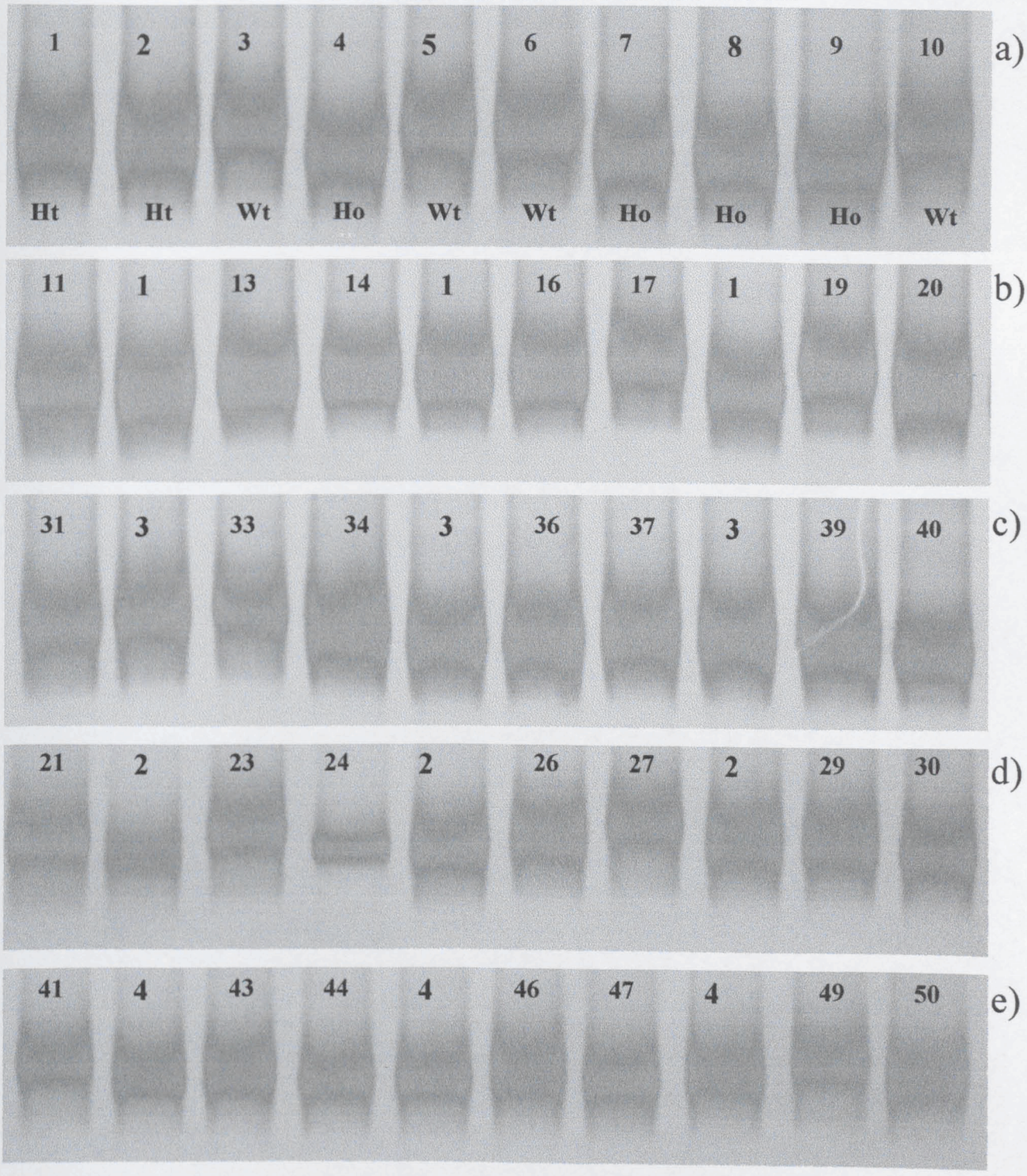
Eighty-three similar splice site variants were detected among affected and unaffected individuals

Wt=Wild type (23 cases) Ho=Homozygous (20 cases) Ht=Heterozygous (61 cases)

Figure 3.9 and 3.11 show different patterns of dsDNA mobility within the defined DNA region seen on polyacrylamide gel. Figure 3.10 shows electropherograms from three different genotypes close to splice site of BRCA2 exon17.

Figure 3.11: PAGE images of Heteroduplex Analysis (HA) of sequence variants in BRCA2 [IVS 16-14 T>C]

in British affected women with breast cancer



Single base pair changes between the different genotypes are evident. The designations are as follows: Lanes Wt, TT (homozygote T); Lanes Ht, TC (heterozygote C); Lane Ho, CC (homozygote C).

Figure 3.12: Electropherograms (a, c) and PAGE image (b) of sequence variant in BRCA2 exon23 detected by SSCP/HA

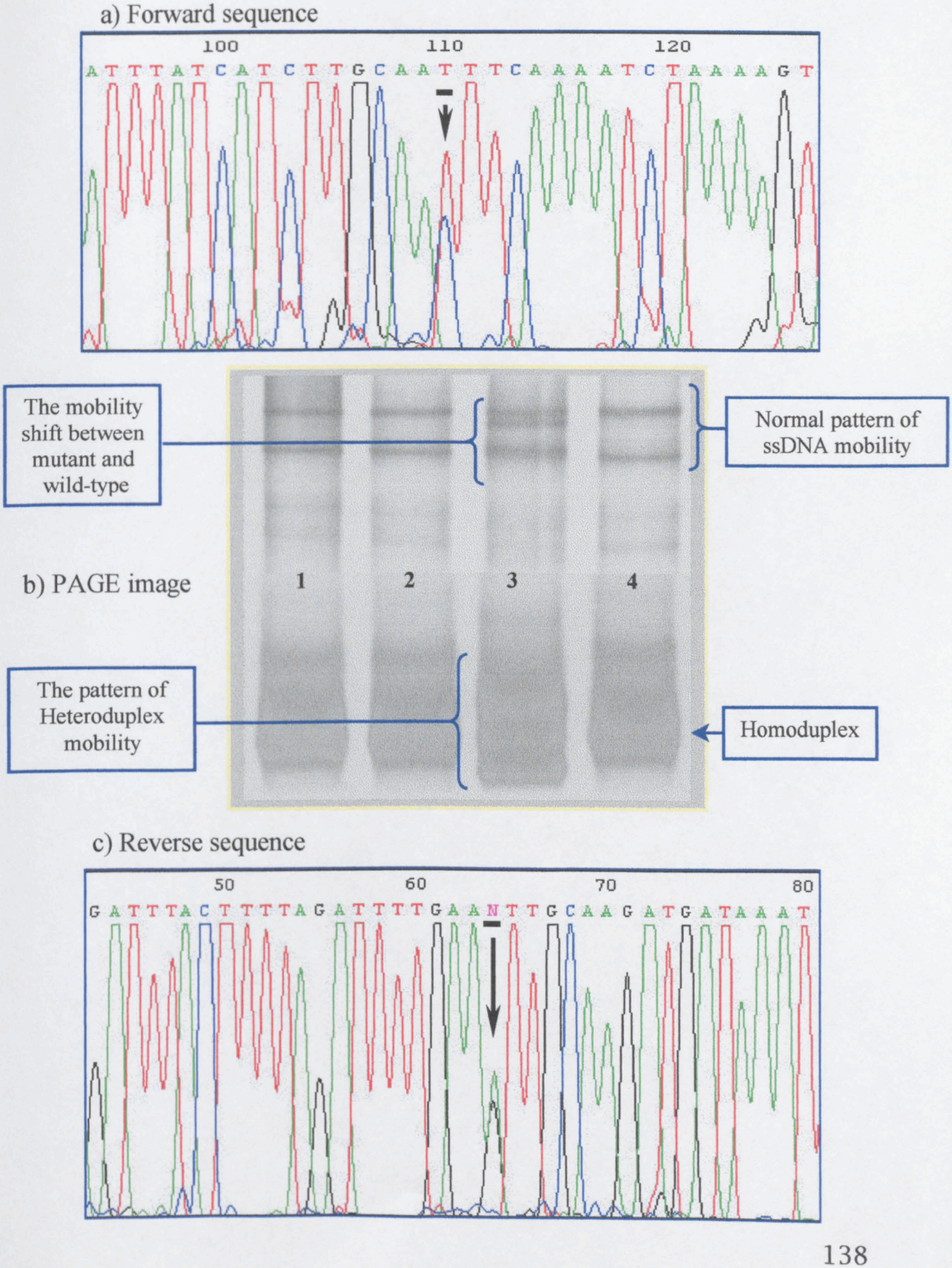


Figure 3.13: Heteroduplex Analysis of BRCA2 gene exon 23

Different pattern of heteroduplex mobility on PAGE (a, b, c) from a same sample

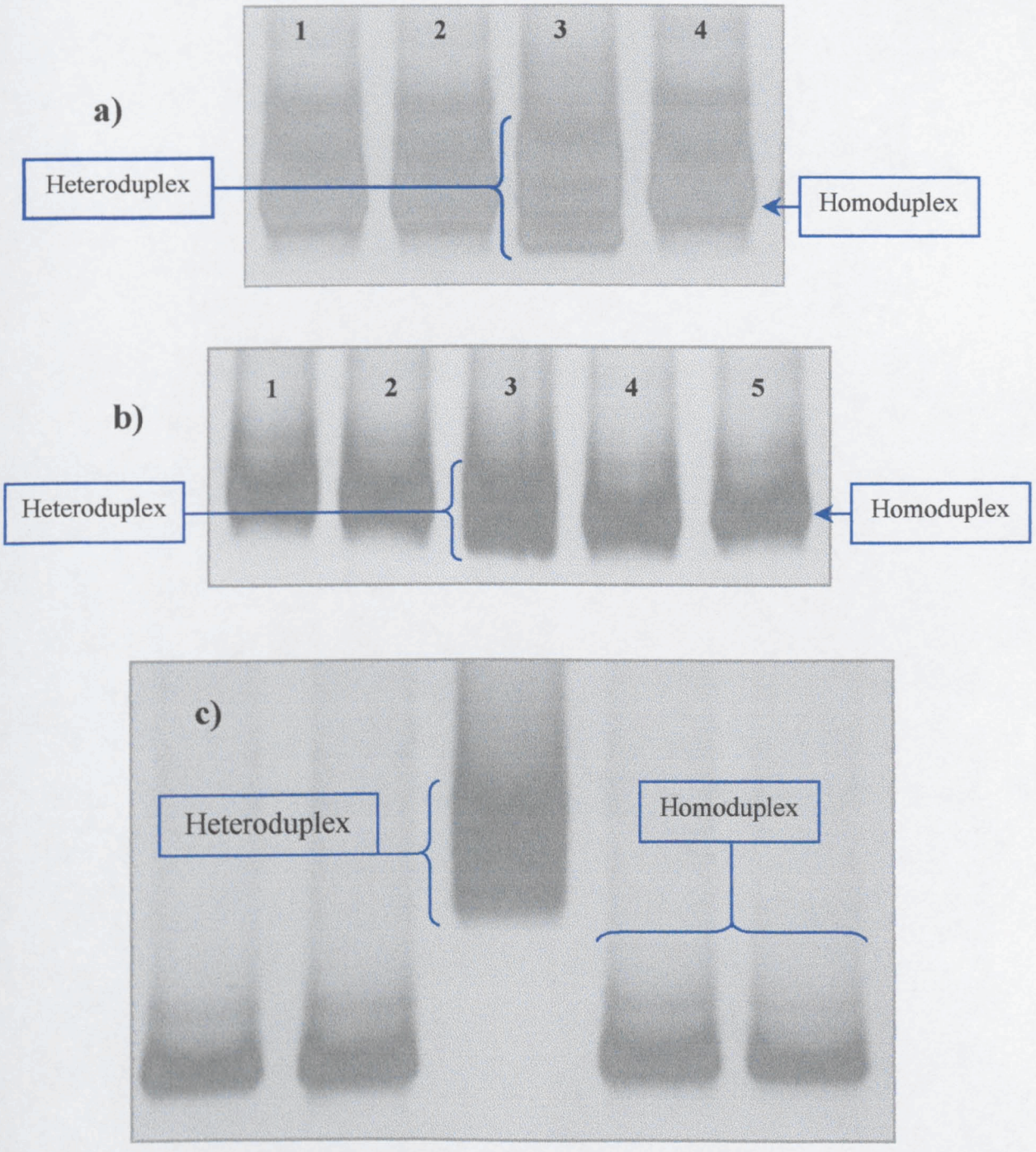


Figure 3.12 shows the sequence variant and wild type of PCR products of BRCA2 exon 23 run on 14% Polyacrylamide gel at 16°C, 3% glycerol for 20 hours. Under these conditions, it is possible to resolve three distinct bands representing (lane 3) the two single strands (top two bands) and the lower band corresponding to the reannealed double-stranded DNA.
Note that the single-stranded sequences migrated to different positions in the wild type and mutant samples, demonstrating the desired sequence variant (Figure 3.12 a, c).

Figure 3.13 (a, b, c) shows the heteroduplex analysis of mutant and wild type of PCR products of BRCA2 exon 23 with different patterns of heteroduplex mobility. Optimising the running temperature and the glycerol concentration made a significant effect on the formation of heteroduplexes.
PAGE images 3.13 a, b and c depict the PCR products of BRCA2 exon 23 that has been run on 25°C with glycerol concentration 5%; 16°C with glycerol concentration 10%; 16°C with glycerol concentration 5% respectively. These patterns might be resulted in inefficient PCR products or reflect of alteration of conditions.

The wild type sequence of BRCA2 exon 23

```
1   cac ttc ttc cat tgc atc ttt ctc atc ttt ctc caa
      H   F   F   H   C   I   F   L   I   F   L   Q

37  aca gtt ata ctg agt att tgg cgt cca tca tca gat
      T   V   I   L   S   I   W   R   P   S   S   D

73  tta tat tct ctg tta aca gaa gga aag aga tac aga
      L   Y   S   L   L   T   E   G   K   R   Y   R

109 att tat cat ctt gca act tca aaa tct aaa agt aaa
      I   Y   H   L   A   T   S   K   S   K   S   K
```

```
145  tct gaa aga gct aac ata cag tta gca gcg aca aaa
       S   E   R   A   N   I   Q   L   A   A   T   K

181  aaa act cag tat caa caa cta ccg gta caa acc ttt
       K   T   Q   Y   Q   Q   L   P   V   Q   T   F
```

Lower blue colour = Exonic segment
Red colour = Intronic segment
Upper case letter = Amino acid sequence

The sequence variant of BRCA2 exon 23

```
  1  cac ttc ttc cat tgc atc ttt ctc atc ttt ctc caa
       H   F   F   H   C   I   F   L   I   F   L   Q

 37  aca gtt ata ctg agt att tgg cgt cca tca tca gat
       T   V   I   L   S   I   W   R   P   S   S   D

 73  tta tat tct ctg tta aca gaa gga aag aga tac aga
       L   Y   S   L   L   T   E   G   K   R   Y   R

109  att tat cat ctt gca att tca aaa tct aaa agt aaa
       I   Y   H   L   A   I   S   K   S   K   S   K

145  tct gaa aga gct aac ata cag tta gca gcg aca aaa
       S   E   R   A   N   I   Q   L   A   A   T   K

181  aaa act cag tat caa caa cta ccg gta caa acc ttt
       K   T   Q   Y   Q   Q   L   P   V   Q   T   F
```

Lower blue colour = Exonic segment
Underlined Bold Italic red colour= A base pair substitution in polymorphic sequence
Upper case letter = Amino acid sequence

Note: In BRCA2 gene at exon23 one base pair change (C>T) substitute Threonine (uncharged polar) to Isoleucine (non polar) amino acid. Effect of this sequence variant [9266 C>T (T3013I)] on BRCA2 gene expression is unclear.

Figure 3.14: SSCP and Heteroduplex analysis of BRCA2 exon 18 (second segment)

Abnormal pattern of single strand mobility with a normal DNA sequence pattern on PAGE

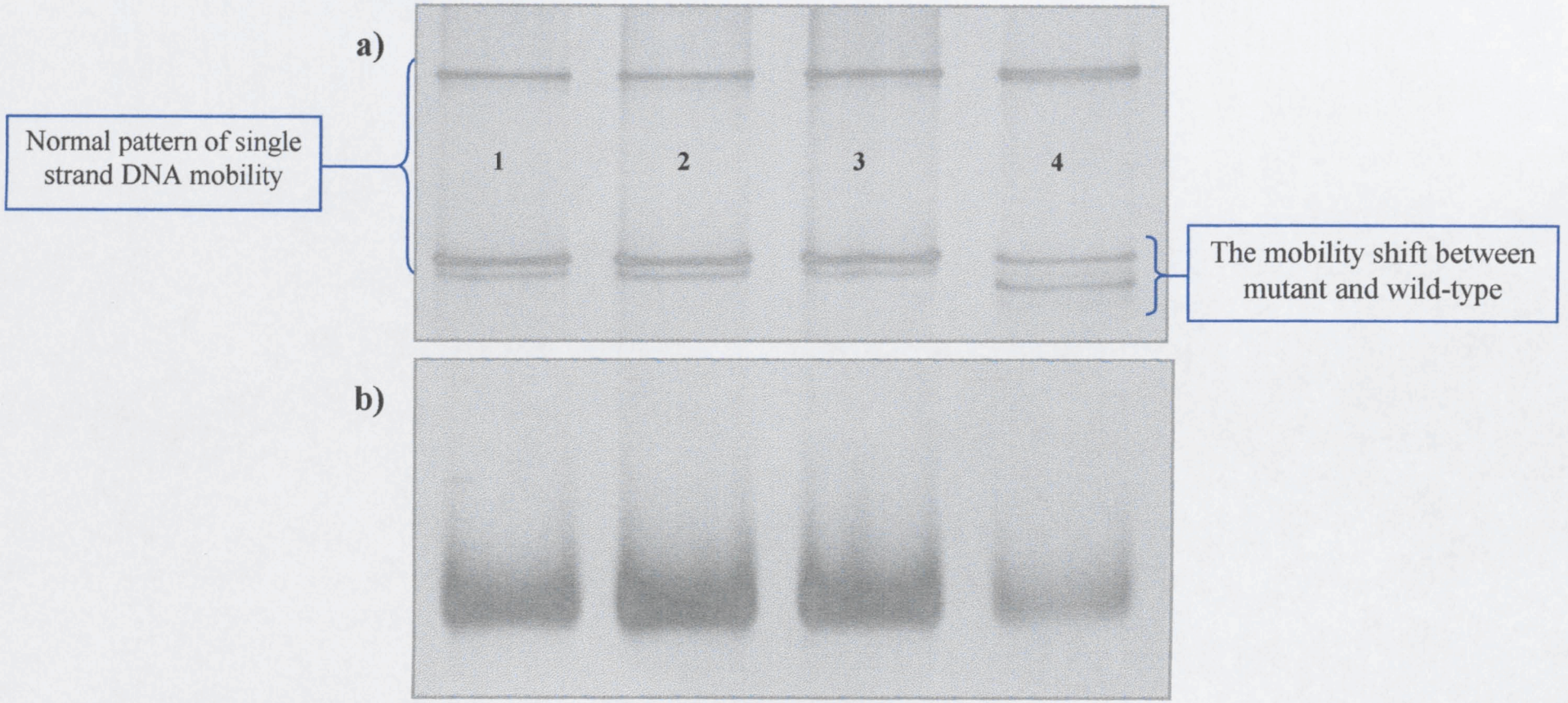


Figure 3.15: Electropherograms of a normal genotype of BRCA2 exon 18 (Segment two =235bp)

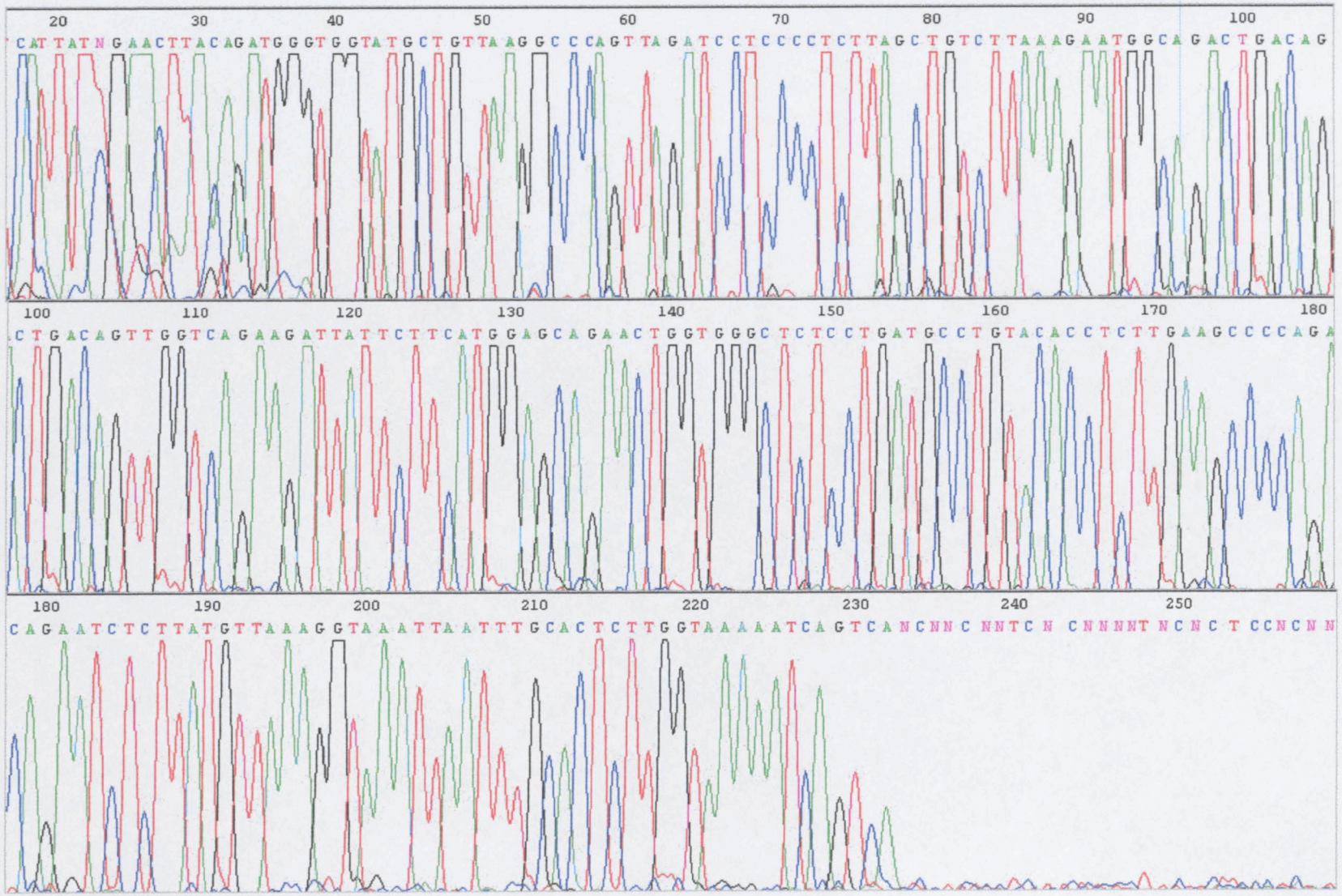


Figure 3.14 (a) lanes 1-3 show normal pattern and lane 4 revealed the abnormal pattern of single strand mobility seen on polyacrylamide gel. Direct sequencing verified a normal sequence pattern for sample (lane) four.

Figure 3.14 (b) all four samples show normal pattern of the heteroduplex mobility. Sample four was analysed three times by SSCP and direct sequencing and every time gave the same results in both methods.

Figure 3.15: The electropherograms verified the entire normal sequence of the BRCA2 exon 18 (second part =235bp) for ample four in figure 3.14 (a). Both directions (forward and reverse sequences) have shown the normal sequences pattern the specific DNA fragment.

Wild type sequence of BRCA2 exon 18 (second part =235 bp)

```
  1  gcagataccc aaaaagtggc cattattgaa cttacagatg
 41  ggtggtatgc tgttaaggcc cagttagatc ctcccctctt
 81  agctgtctta aagaatggca gactgacagt tggtcagaag
121  attattcttc atggagcaga actggtgggc tctcctgatg
161  cctgtacacc tcttgaagcc ccagaatctc ttatgttaaa
201  ggtaaattaa tttgcactct tggtaaaaat cagtc
```

3.5 The combined SSCP/HA and restriction digestion method

To evaluate the sensitivity of the PTT, restriction digestion with BamHI (recognition site = GGATCC) was applied to test one hundred samples for germline mutation in the BRCA1 exon 11-3' end (501bp).

Genomic DNA was amplified using a primer pairs and convention PCR program (Table 3.1).

PCR products were digested in total volume of 30μl (distilled water=16μl, PCR products= 10μl, Buffer 10x= 3μl, Restriction enzyme= 5U) and incubated at 37°C for 4-6 hours.

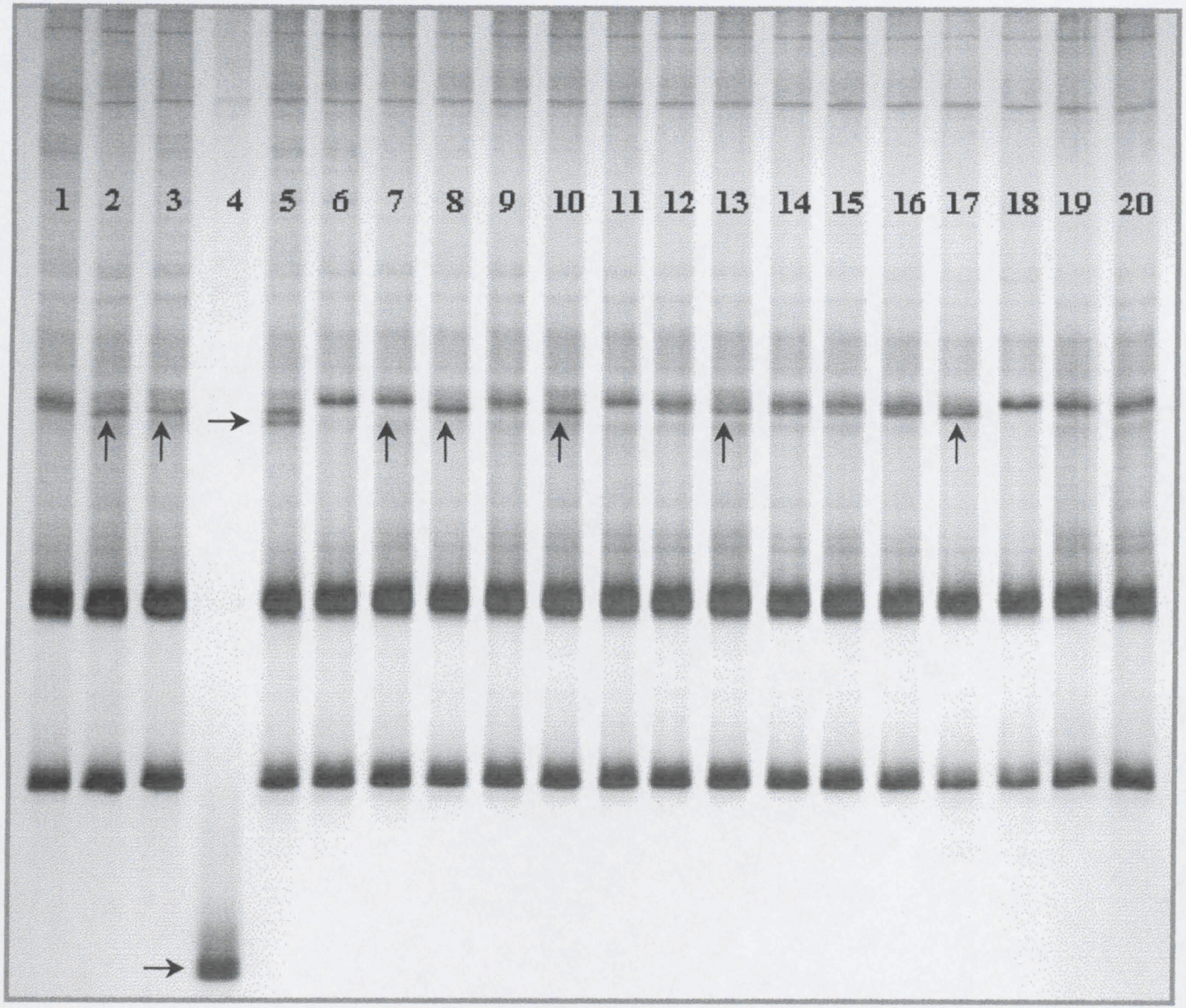
5μl of the digested products and 5μl of SSCP loading buffer were incubated at 99°C for 10 minutes, followed immediately by placing the samples on ice for 10 minute. All 10μl was then loaded on a PAGE 14% with glycerol 10% (Figure 3.16 a and b). The rest of assay was performed as a conventional SSCP method (See also chapter 2).

Wild type sequence of BRCA1 exon 11- 3'end (501 bp)

```
  1  aagaaattag agtcctcaga agagaactta tctagtgagg atgaagagct tccctgcttc
     ttctttaatc tcaggagtct tctcttgaat agatcactcc tacttctcga agggacgaag

 61  caacacttgt tatttggtaa agtaaacaat ataccttctc agtctactag gcatagcacc
     gttgtgaaca ataaaccatt tcatttgtta tatggaagag tcagatgatc cgtatcgtgg

121  gttgctaccg agtgtctgtc taagaacaca gaggagaatt tattatcatt gaagaatagc
     caacgatggc tcacagacag attcttgtgt ctcctcttaa ataatagtaa cttcttatcg

181  ttaaatgact gcagtaacca ggtaatattg gcaaaggcat ctcaggaaca tcaccttagt
     aatttactga cgtcattggt ccattataac cgtttccgta gagtccttgt agtggaatca

241  gaggaaacaa aatgttctgc tagcttgttt tcttcacagt gcagtgaatt ggaagacttg
     ctcctttgtt ttacaagacg atcgaacaaa agaagtgtca cgtcacttaa ccttctgaac

301  actgcaaata caaacaccca ggatcctttc ttgattggtt cttccaaaca aatgaggcat
     tgacgtttat gtttgtgggt cctaggaaag aactaaccaa gaaggtttgt ttactccgta

361  cagtctgaaa gccagggagt tggtctgagt gacaaggaat tggtttcaga tgatgaagaa
     gtcagacttt cggtccctca accagactca ctgttcctta accaaagtct actacttctt

421  agaggaacgg gcttggaaga aaataatcaa gaagagcaaa gcatggattc aaacttaggt
     tctccttgcc cgaaccttct tttattagtt cttctcgttt cgtacctaag tttgaatcca

481  attggaacca ggtttttgtg t
     taaccttggt ccaaaaacac a
```

Figure 3.16: Digested PCR-SSCP and Heteroduplex analysis of BRCA1 exon 11-3'end

a)



b)

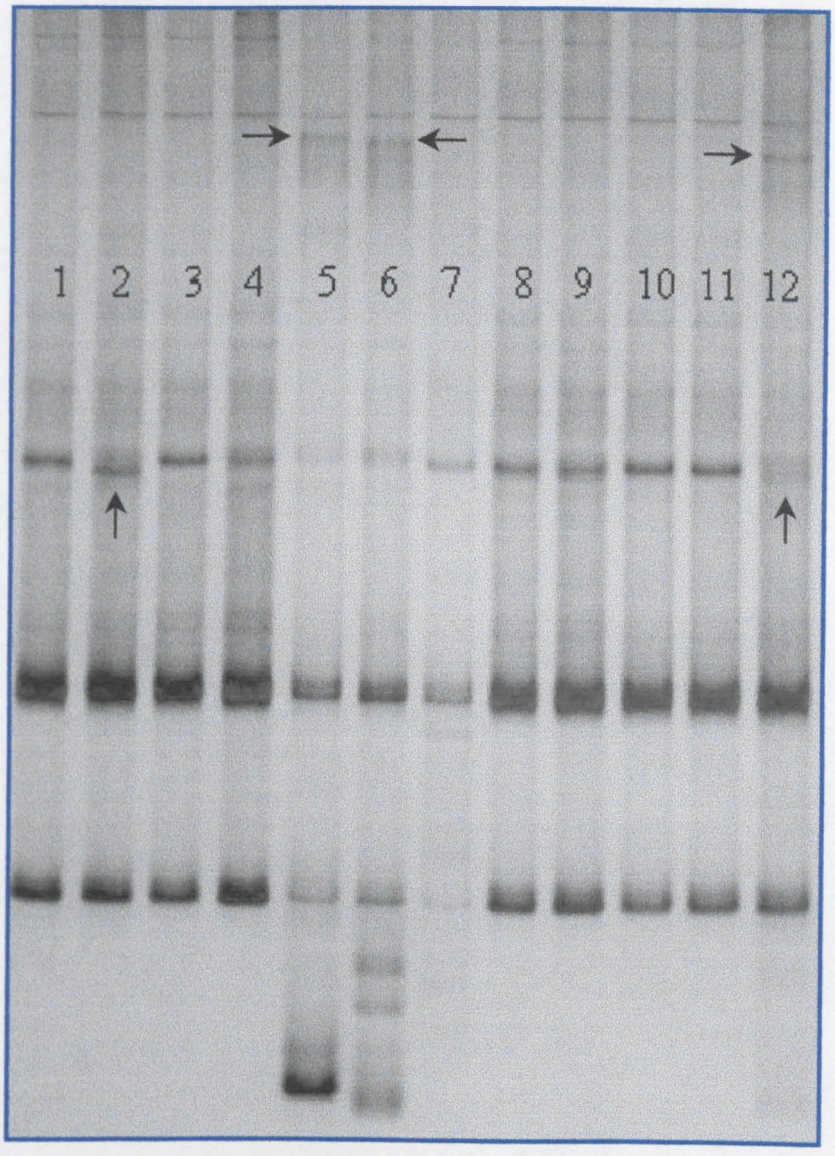
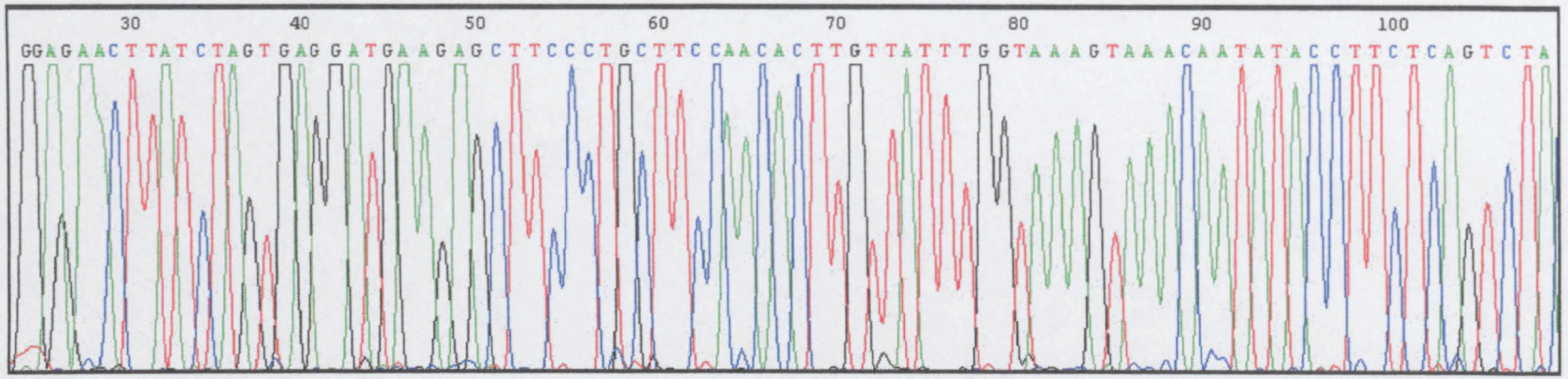
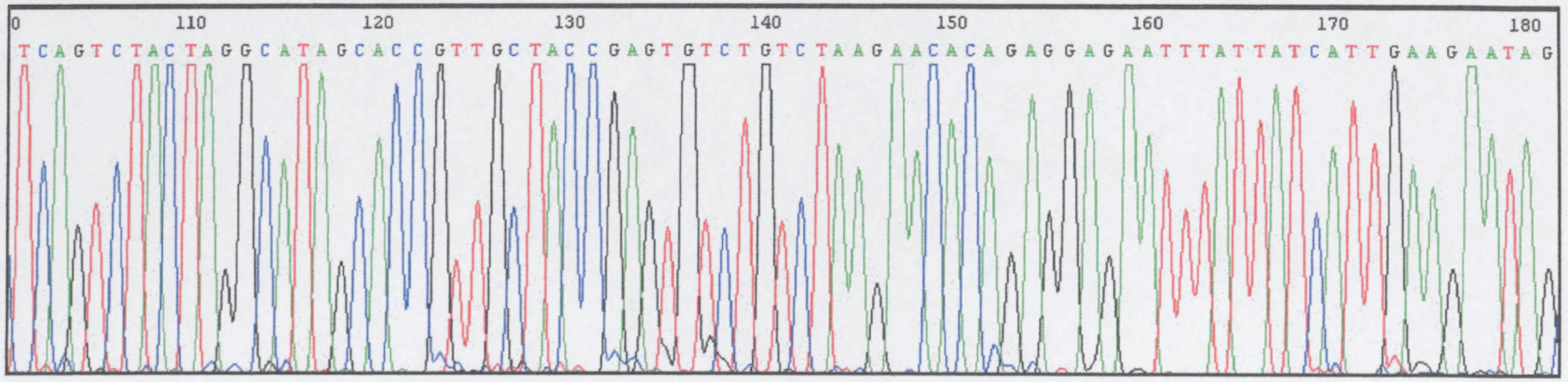


Figure 3.17: Electropherograms of a normal genotype of BRCA1 exon11 3' end (501bp)

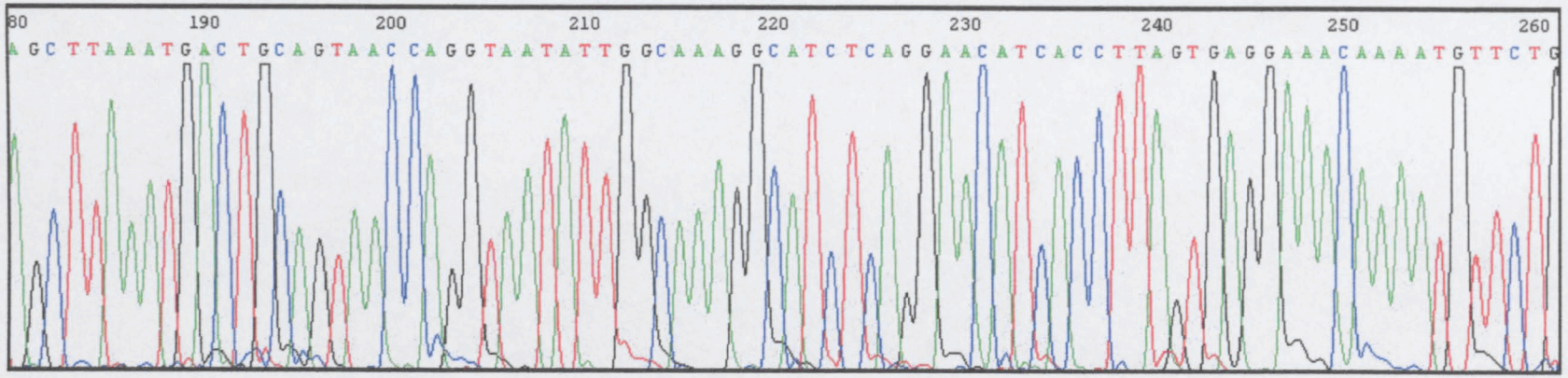
1)



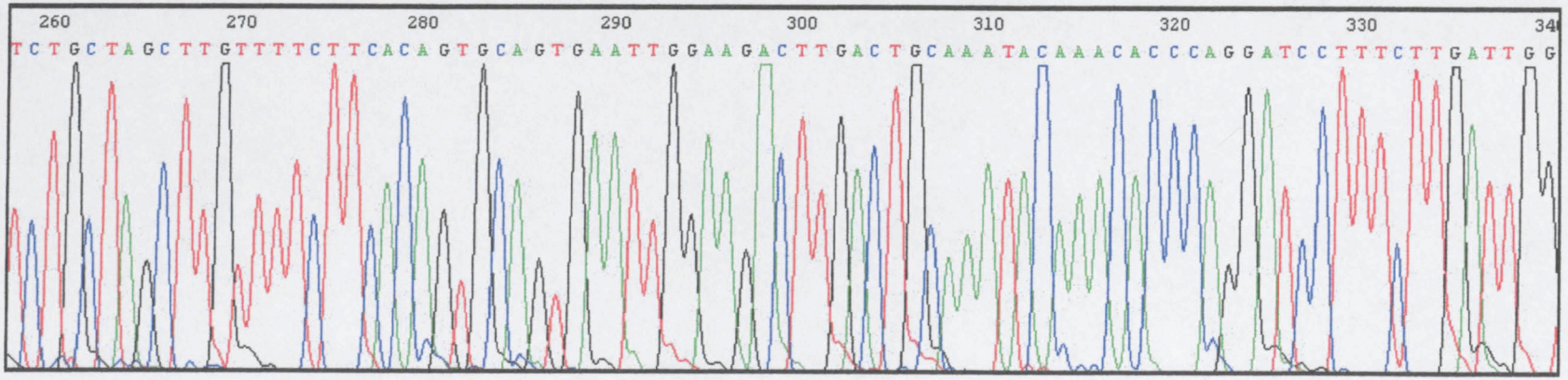
2)



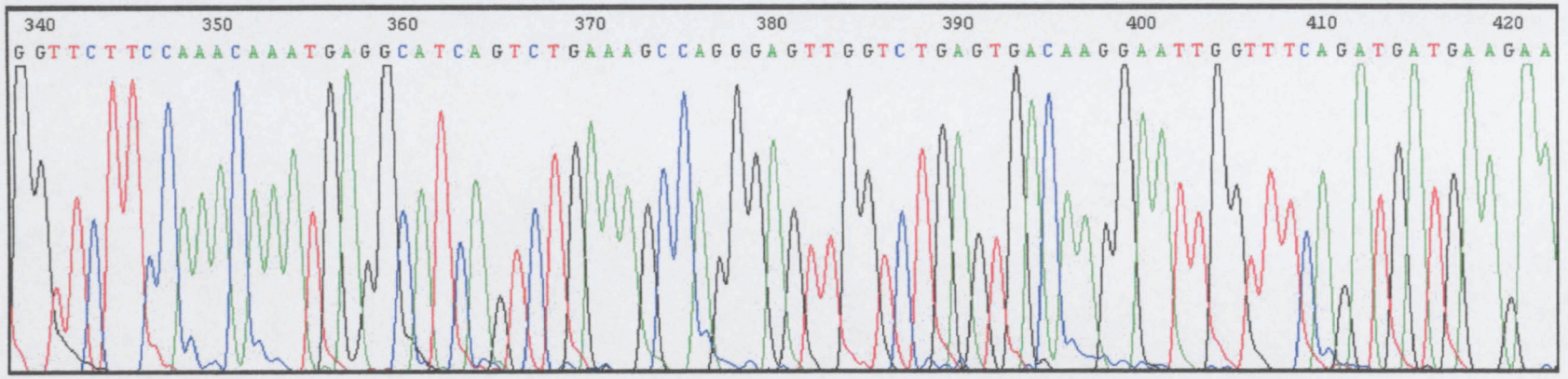
3)


180
190
200
210
220
230
240
250
260
AGCTTAAATGACTGCAGTAACCAGGTAATATTGGCAAAGGCATCTCAGGAACATCACCTTAGTGAGGAAACAAAATGTTCTG

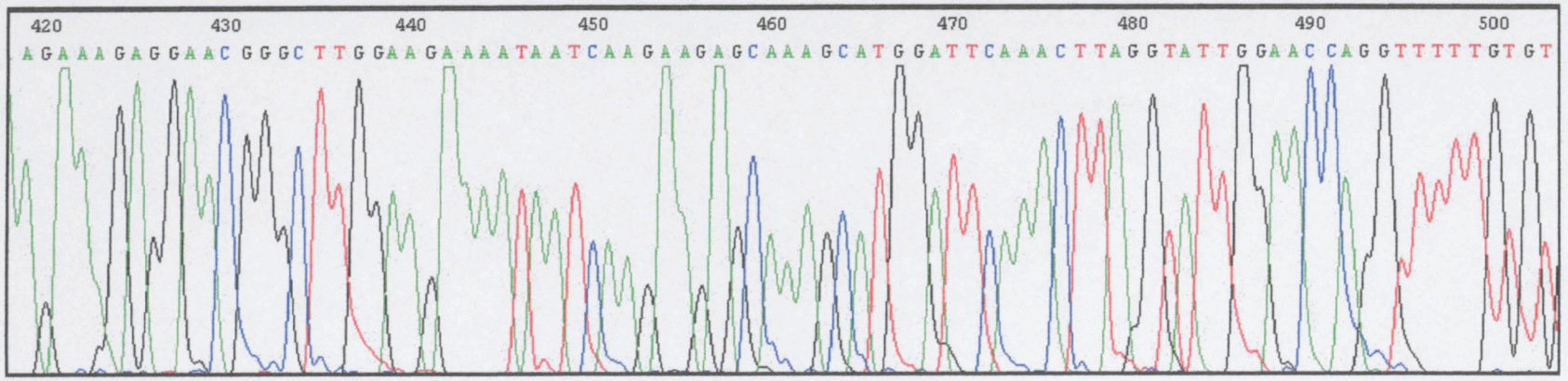
4)


260
270
280
290
300
310
320
330
340
TCTGCTAGCTTGTTTTCTTCACAGTGCAGTGAATTGGAAGACTTGACTGCAAATACAAACACCCAGGATCCTTTCTTGATTGG

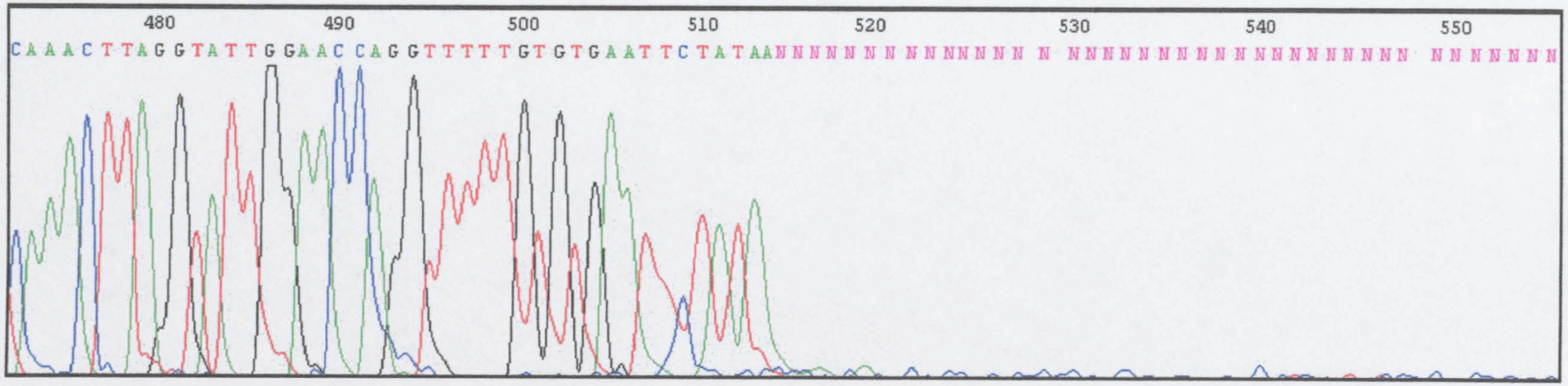
5)


340 350 360 370 380 390 400 410 420
G GTTCTTCCAAACAAATGAGGCATCAGTCTGAAAGCCAGGGAGTTGGTCTGAGTGACAAGGAATTGGTTTCAGATGATGAAGAA

6)


420 430 440 450 460 470 480 490 500
AGAAAGAGGAACGGGCTTGGAAGAAAATAATCAAGAAGAGCAAAGCATGGATTCAAACTTAGGTATTGGAACCAGGTTTTTGTGT

7)


480 490 500 510 520 530 540 550
CAAACTTAGGTATTGGAACCAGGTTTTTGTGTGAATTCTATAANNNNNNNNNNNNNN N NNNNNNNNNNNNNNNNNNNN NNNNNN

Note 1: Figure 3.16 (a) shows the normal pattern of ssDNA mobility in lane 7 and other similar lanes that differ with abnormal pattern of ssDNA mobility in lanes 2, 3, 8, 10, 13 and 17 on polyacrylamide gel.
Lane 4 seems to be the result of an inefficient PCR products and lane 5 reveals a highly abnormal pattern of ssDNA mobility.
PCR products of samples in lanes 5, 7, 8 were characterised by direct sequencing, which revealed a wild type DNA sequence.

Note 2: Figure 3.16 (b) lane 2 shows an abnormal pattern of single strand DNA mobility compared with lanes 1, 3, 4, 8, 9, 10 and 11.
Lanes 5 and 6 seem to be inefficient PCR products and lane 12 reveal abnormal pattern of single strand DNA mobility.
PCR products of samples 2, 5, 6 and 12 were characterised by direct sequencing, which revealed a wild type DNA sequence.

Figure 3.17(1-7): The electropherograms verified the entire normal sequences of the BRCA1 exon 11-3'end. Restriction digestion also did not reveal any changes in the specific DNA fragment in one hundred samples that have already been analysed by PTT.

3.6 Discussion

A total of 104 samples were investigated for germline mutations in BRCA1 gene exons 2, 3, 5, 13 and 20 and BRCA2 gene exons 9,17, 18 and 23 by PCR-amplification from genomic DNA used SSCP/HA methods.

Due to a lack of published data on BRCA gene mutations from Iranian population at the beginning of the study, there were no known positive control samples (mutant sample) to accompany the SSCP analysis. Therefore, according to the present mutation databases at the BIC, and owing to the high frequency of alterations and their significant protein functions in some of those regions (exon 2 and 20 of the BRCA1), I assumed that above regions of BRCA genes might be good starting point for screening. However, further study is required to screen the entire sequences of both BRCA1 and BRCA2 genes that lead to investigate mutational spectrum in this selected population.

Six distinct sequence variants were identified, two frameshift (putative mutations-one novel), two missense changes† of unknown significance and two polymorphisms (one intronic 12bp duplication [BRCA1, IVS20+48]* and another close to splice site [BRCA2, IVS16-14T>C]**).

† Two missense mutations in BRCA2 gene were detected once in this set of 104 samples tested. One in the exon 18 (N2706S) resulting from A>G substitution at 8345 nucleotide that lead to exchange Asparagine to Serine (both are uncharged polar amino acid) and another within the exon 23 (T1301I) changing one base pair C>T at 9266 nucleotide which substitute Threonine (uncharged polar) to Isoleucine (non polar) amino acid. It is more unlikely these variants to be deleterious mutation. Further investigation (family study) among members of Iranian breast cancer families tested and control group is required to determine whether these particular mutations segregate with cancer in family members.

* Effect of the intronic variant [IVS20+48] 12bp duplication gtattccactcc on BRCA1 expression is controversial [9, 10, 11, 12, 13, 14] however; it seems to be a polymorphism. This variant has also reported in a study at the university of Nottingham [15].

**This common polymorphism [BRCA2, IVS16-14T>C] was detected in affected and unaffected individuals, 81 out of 104 (61 heterozygous and 20 homozygous). Interestingly no abnormal patterns were observed in single strand DNA mobility and all these variants were identified by heteroduplex patterns alone. Apart from this polymorphism, no other sequence variants were detected in unaffected individuals.

To address if this polymorphism [BRCA2, IVS16-14T>C] is a marker in Iranian women, 78 samples of British affected women with breast cancer were also analysed by SSCP/HA. Fifty-nine patients had this polymorphism.

Although only six sequence variants have been detected by SSCP/HA methods in 104 probands tested. Some circumstances may have contributed to this outcome: first, I have only analysed common regions for mutations in both BRCA1 and BRCA2 genes, not the entire genes; second, only coding regions have been screened for variations, mutations in the promoter, and changes deeper within the introns would have been missed; third, SSCP is not sufficiently sensitive to detect all sequence variants.

No sequence variant was detected at the 5' and 3' ends of exon 11 in BRCA1 or BRCA2 by SSCP/HA analysis, suggesting that there was no loss of sensitivity in analysing such large fragments by PTT.

3.7 Comment

My experience to date suggests that the SSCA/HA strategy, which I have used, is efficient and relatively sensitive. Any fragment should be subjected to analysis by both SSCP/HA simultaneously because some variants might not be detected by either SSCP or HA individually. Initially, the use of SSCA/HA demonstrated the variants to be detected as double-stranded heteroduplex mobility shifts, often in the absence of variation in the single-strand conformation pattern. (See figures 3.8-3.11, which depicted only heteroduplex pattern mobility of BRCA2 gene at IVS16-14). Indeed the high frequency of frameshift alterations in the BRCA genes leads them to rapid detection via Heteroduplex Analysis. Although the SSCP assay was sensitive at detecting point mutations, the high frequency of apparent polymorphisms in the BRCA1 is proving problematic in trying to identify disease related alterations. On a number of occasions SSCP variants have been sequenced to reveal putative missense mutations, which have been either previously or subsequently established as rare polymorphisms. There are also several common polymorphisms that, although distinguishable as such, may in fact mask the presence of other sequence alterations of potentially greater significance.

3.8 References

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Chapter 4

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4 Protein Truncation Test (PTT)

4.1 Introduction

An increasing number of disease genes have been identified in which the majority of mutations result in premature termination of translation [1]. Standard techniques to detect minor changes in DNA [e.g., SSCP analysis, heteroduplex analysis and DGGE (Denaturing Gradient Gel Electrophoresis) analysis] reveal all sequence changes, including those that are not pathogenic (polymorphisms) or are of questionable pathogenicity (missense mutations). A rapid and efficient test is the Protein Truncation Test (PTT) [2, 3] which was originally designed as a tool to detect mutations that lead to premature translation termination in the Dystrophin gene [4]. The coding region of a gene is screened for the presence of translation terminating mutations using *de novo* protein synthesis from an amplified copy. It is also known as the *in-vitro* protein synthesis (IVPS) assay [5].

PTT is a mutation detection method based upon the *in-vitro* transcription and translation of typically PCR amplified sequences. It specifically detects frameshift, splice or nonsense mutations, which lead to the termination of mRNA translation and subsequent protein truncation [6].

4.2 Method

The Protein Truncation Test procedure is outlined in chapter two section 2.1.8 and shown schematically in Figure 2.4.

In summary, the coding region of a gene is screened for the presence of translation terminating mutations using *de novo* protein synthesis from an amplified DNA template. The procedure includes three important steps. The first step involves the isolation of genomic DNA and amplification of the target gene coding sequences using PCR or, alternatively, isolation of RNA and amplification of the target sequence using Reverse Transcription PCR (RT-PCR). The resulting PCR products are then used as a template for the *in-vitro* synthesis of RNA, which is subsequently translated into protein. The final step is the SDS-PAGE analysis of the synthesized protein. The shorter protein products of mutated alleles are easily distinguished from the full-length protein products of normal alleles.

4.2.1 DNA isolation

Generally cDNA/RNA is not used as a template for mutation scanning, mainly due to technical difficulties and its susceptibility to degradation. However, in hereditary breast cancer cases in particular, patients are not always available to obtain fresh sample. To date, most laboratories use genomic DNA as a source of material for genetic testing. In this study genomic DNA was extracted from fresh whole blood and used as a template for further analysis (See chapter two for more detail).

4.2.2 Primer pairs design and amplification of the target gene coding sequences

Several factors are important to design primer pairs for the PTT.

- The ATG-initiation codon in the tailed sense forward primer must be in-frame with the coding sequence. If the AUG is not in-frame, translation will start at the first internal translation initiation site and an unexpected, shorter translation product will be produced.
- If a disease gene contains several kilo bases (kb) of coding sequence, it is necessary to amplify several 2-4 kb fragments. The choice of primers ultimately determines the sensitivity obtained; they should be designed with care. If possible, the tailed forward primer of the first segment should be located upstream of the natural translation initiation site; otherwise, mutations affecting this site will not be detected [3]. A reverse primer should **not** be selected near the end of a region where a large open reading frame is present in one or both of the alternative reading frames; in such cases a frameshift mutation to an alternative reading frame will not cause a truncation and it would thus be missed [3].
- To ensure that truncation mutations in the 5'- or 3'-end of a fragment are not missed, flanking segments should have an overlap of 350-500bp if possible [6].
- It is important to place the forward primer in a region where the sequence contains a codon for the labelled amino acid to be incorporated if possible (e.g., cysteine and methionine are infrequently encoded amino acids while leucine and lysine are more common).

4.2.3 Sensitivity of the PTT

In comparison with other mutation detection techniques, the sensitivity of PTT is good with detection of mutated alleles when present at 5-10% cases. This technique may be use to identify a disease causing gene. At a point where only segments of the sequence of a candidate gene is known, tailed

primers can already be designed to scan patient samples for truncating mutations.

4.2.4 False negative of results

Failure to amplify the mutated allele or failure to detect very small deletion/insertions or missense mutations can lead to a false negative result of PTT [3]; these might be due to:

1.Mutations in the primer-binding site

2.Very small in-frame deletions/insertions because the mobility shifts are too small to detect. These problems can be overcome by the use of different percentage gels and overlapping primer sets.

3.Germline/somatic mosaism

4.Large insertions, translocations and inversions, which enlarge the region under analysis beyond amplifiable length.

4.2.5 False positive of results

Upon translation, a range of undesired background translation products may produce, usually derived from secondary sites of translation initiation (See Figure 4.3, b). Although these products could disguise truncated products, in general the translation pattern is fairly constant and changes are easy to detect.

4.2.6 Applications of PTT

Initially, PTT was developed for detecting mutations in the large dystrophin gene [4] responsible for Duchenne Muscular Dystrophy. Subsequently, additional genes have been identified which contain nonsense and frameshift mutations corresponding to a major fraction of patients exhibiting either the disease or carrier state of the disease. PTT is now widely used to identify and characterize these mutations (Table 4.1). Clinically important tumour suppressor genes such as those involved in breast cancer BRCA1 [6], BRCA2 [7] and colon cancer APC gene [3, 5] are ideally studied through the use of PTT. These genes contain one or two large exons, within which a majority of the known truncation mutations lie, thus a DNA-based diagnostic protocol can be used. Other disease characteristics make PTT the method of choice for mutation analysis. In diseases where the majority of cases are caused by a single mutation or a small number of specific mutations, PTT may present an attractive method with which to reveal the remaining mutations. For example, in Cystic Fibrosis one mutant type ($\triangle$F508) comprises 70% of disease mutations; of the remaining 30% of mutations, half cause premature termination of translation.

PTT has been very successfully used for mutation detection in the APC gene [3, 5] where 95% of the mutations in APC are translation terminating (Exon 15 is 8.5 Kb long and can be analysed in 3 - 4 overlapping fragments), BRCA1 gene [6] and Dystrophin gene [2, 3] where the frequency of missense mutations is low.

Table 4.1: Applications of PTT in human molecular genetics

Disease	% Truncating Mutations**	Gene	References
Familial Adenomatous Polyposis	95%	APC	3, 5
Hereditary desmoid disease	100%	APC	8
Ataxia telangiectasia	90%	ATM	9
Hereditary breast and ovarian cancer	90%	BRCA1	6
	90%	BRCA2	7
Cystic Fibrosis	15%	CFTR	10
Duchenne Muscular Dystrophy	95%	DMD	2, 4
Fanconi anaemia	80%	FAA	11
Hunter Syndrome	~50%	IDS	12
Hereditary non-polyposis colorectal cancer	~80%	hMSH2	13
	~70%	hMLH1	14
Neurofibromatosis type 1	50%	NF1	15
Neurofibromatosis type 2	65%	NF2	16
Polycystic Kidney Disease	95%	PKD1	17

***The percentage of truncating mutations reported which should be detectable using PTT.*

In view of the nature of this experiment described in this chapter, I have included details of the methodology as they pertain PTT, in order to clarify the results and their interpretation.

4.3 PTT in practice

PTT is applied most frequently for mutation detection in hereditary breast cancer. Most, in some cases all, mutations reported in BRCA1 and BRCA2 gene particularly in exon11cause premature translation termination [1, 18, 19], are readily detectable by PTT, and make it a very sensitive and efficient tool for mutation detection.

BRCA1 and BRCA2 are relatively large genes; consist of 22 and 26 coding regions respectively. Both have a large exon (exon 11) that comprises 61% and 45% the entire BRCA1 and BRCA2 coding region respectively (See Appendix II).

Using Wizard Genomic DNA purification kit (Promega, Southampton, UK; catalogue no. LA1620) in accordance with the manufacturer's protocols, genomic DNA was extracted from peripheral blood lymphocytes. Modified primers, containing a T_7 promoter and a perfect Kozak consensus sequence were used to generate PCR products from exon 11 of BRCA1 and BRCA2 suitable for PTT. Each pair of primers amplified 3446bp and 4959bp for BRCA1 exon 11 and BRCA2 exon 11 respectively (Table 4.3, 4.4 and Figure 4.1 a, b). This approach considerably reduces the workload involved in scanning large multi-exon genes.

To avoid any missed frameshift mutations that might be present close to the 5' and 3' end of exon 11 in both BRCA1 and BRCA2 genes, four sets of primer pairs were also used to screen these regions using SSCP method (Table 4.3 and 4.4 and also see chapter three section 3.3 for more detail).

PCR was performed using genomic DNA containing 50-100ng genomic DNA, 1µl 5pM of each primer (forward and reverse), 2µl of 2.5mM mixture dNTPs, 2.5µl of each 5x PCR Buffer A [300mM Tris-SO_4, (pH 9.1 at 25°C), 90mM$(NH4)_2$ SO_4 and 5mM $MgSO_4$] and Buffer B [300mM Tris-

SO_4,(pH 9.1 at 25°C), 90mM$(NH4)_2$ SO_4 and 10mM $MgSO_4$], 1µl ELONGASE® Enzyme Mix (Invitrogen, Paisley, UK) in accordance with manufacturer's recommendations, and distilled water was added to a final volume of 25µl. For amplification, each sample was denatured at 94°C for 1 min and followed by 31 cycles of PCR (at 94°C for 25 sec, at 56°C for 1min and at 68°C for 4 min on an Applied Biosystems DNA thermal cycler, Applera Europe BV, Cheshire, UK) were followed by an incubation at 68°C for 10 min. The PCR products were subsequently analysed on a 0.7% agarose gel and used for PTT. Usually 100-200ng/µl of PCR products is sufficient for PTT detection.

Note: In this study full-length artificial protein of BRCA1 and BRCA2 exon 11, ~126kDa and ~183kDa respectively, were analysed. The boundaries of the translated fragments represent the most critical regions for PTT analysis: 5'-end mutations result in products which might be too small to allow detection (no or little label incorporated, electrophoretic migration outside the resolution range), 3'-end mutations might result in a size difference which is not resolved near the top of the gel. These regions (5' and 3' end) of exon 11 in both genes were screened separately by SSCP assay to identify any potential reduction in PTT sensitivity of using such large fragments.

Table 4.2: Modifications of standard PCR components for optimum PTT templates

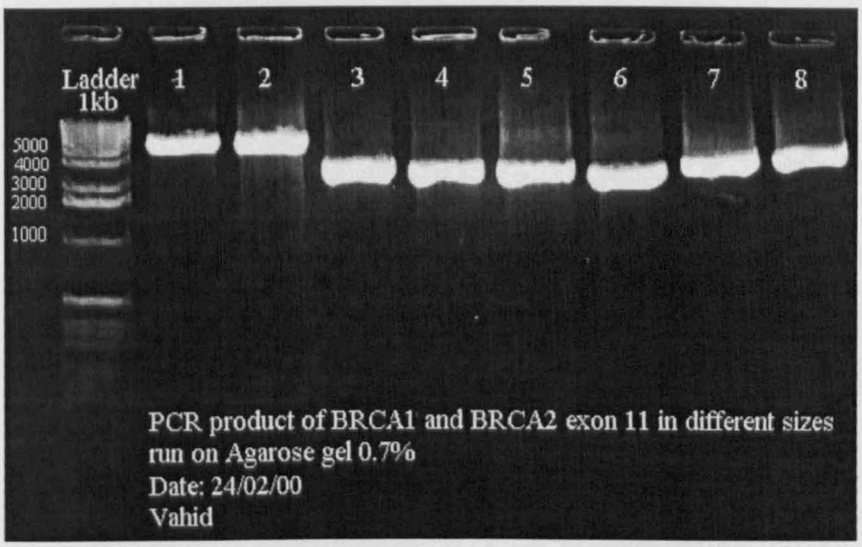
Components	Volume	Final concentration
1) Distilled Water (pH 7.0)	Up to 25μL	
2) 5x PCR Buffer A, B (each) 60mM Tris-SO_4(pH 9.1), 18mM $(NH4)_2SO_4$, with $MgSO_4$ between 1-2 mM	2.5μL	
4) dNTPs mix (2.5 mM each nucleotide)	2μL	200μM
5) Primer mix (5μM/L each primer)	2μL	0.4pM/μl
6) Genomic DNA template (100ng)	1.0μL	
7) ELONGASE® Enzyme Mix (5 Unit)	1.0μL	

The PCR products of the whole of exon 11of BRCA1 and BRCA2 genes were screened in a single reaction using a coupled transcription-translation system, TNT® T7 Quick for PCR DNA kit (from Promega, Southampton, UK; catalogue no.L5540) in accordance with manufacturer's protocols.

The 100-200ng/μl of above PCR products were used directly (without purification) as templates in coupled transcription-translation reactions with 2μCi of [^{35}S] labelled methionine (ICN PHARMACEUTICALS, HANTS, UK catalogue no.0151001H) and incubated for 90 minutes at 30°C. The samples were diluted in sample buffer (45μl per 5μl translated mix), boiled for ten minutes and analysed by SDS-PAGE (6% to 15% acrylamide) [discontinuous and denaturing sodium dodecyl sulfate - polyacrylamide gel 7cm x 10cm] on a 2 x Mini-Protean (Bio-Rad, Hertfordshire, UK, catalogue no.165-3301). The *de novo* proteins were separated and visualised by autoradiography after the gel had been dried (See chapter two section 2.1.7 for more detail).

Figure 4.1: Amplification of the BRCA1 and BRCA2 exon 11

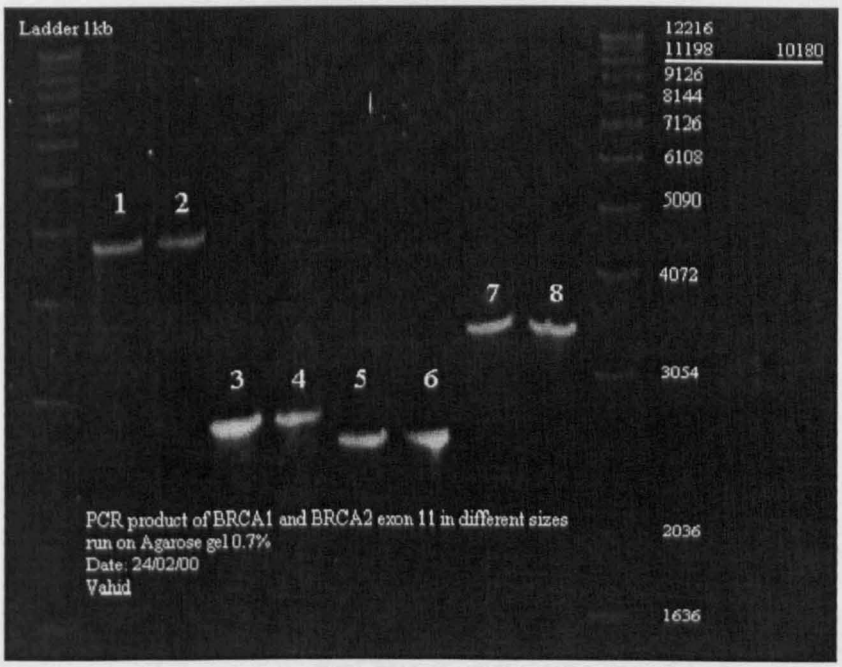
a) PCR products monitored on 0.7% agarose, confirming the integrity of the primer syntheses, intensity of PCR products and the compatibility of the primer pairs.



In both figures a and b:
Lanes 1 and 2 are PCR products of BRCA2 exon11 =4994 bp
Lanes 3 and 4 are PCR products of BRCA2 exon11 part 1=2834 bp
Lanes 5 and 6 are PCR products of BRCA2 exon11 part 2=2657 bp
Lanes 7 and 8 are PCR products of BRCA1 exon11 =3482 bp

* PCR products that used as a template for PTT includes T_7 promoter sequences, which flanking upstream of DNA specific segment, this added 35bp to the normal amplified DNA segment.

b) Again PCR products monitored on 0.7% agarose, for 16 hours at 45 volts to reveal size, quantity and quality of PCR products. Ladder 1 kb run along side to confirm the fragments sizes.



As Figures 4.1 a, b show, that under different running conditions, no dimmers were formed; the quality and the quantity of each of the PCR products were suitable for use in PTT procedures.

This approach may reduce cost and time for screening of these significant regions of BRCA genes in genetics laboratories. Therefore using similar annealing temperature (Table 4.3 and 4.4) to amplify both exon 11 of BRCA1 and BRCA2 allows amplify more samples at the same time.

Table 4.3: Primer pairs for amplification of BRCA1 exon 11 used by PTT and SSCP methods

Exon	Forward primer	Reverse primer	Anl. Temp Used (°C)	PCR products length (bp)
11	5'-(T7promotor)gct aat acg act cac tat agg aac aga cca cca tg- gct tgt gaa ttt tct gag acg g -3'	5'- aca caa aaa cct ggt tcc aat ac -3'	56	3447+35=3482*
11-5'end	5'-ttc agt ttt tga gta cct tgt tat t -3'	5'-cta tca tta cat gtt tcc tta ctt c -3'	55	362
11-3'end	5'-cat tga aga ata gct taa atg act g- 3'	5'- act ggg gca aac aca aaa acc t- 3'	57	343

*PCR products that used as a template for PTT includes T_7 promoter sequences (blue colour lower case letters), which flanking upstream of DNA specific segment, this added 35bp to the normal amplified DNA segment.

(Anl. Temp=Annealing temperature)

Table 4.4: Primer pairs for amplification of BRCA2 exon 11 used by PTT and SSCP methods

Exon	Forward primer	Reverse primer	Anl. Temp Used (°C)	PCR products length (bp)
10	5'-(T7promotor)gct aat acg act cac tat agg aac aga cca cca tg- gtg ctt ctg ttt tat act tta aca gg -3'	5'-aaa gac aga ggt acc tga atc agc-3'	58	1155+35=1190 *
11	5'-(T7promotor)gct aat acg act cac tat agg aac aga cca cca tg- tgt cac ttt gtg ttt tta tgt tta gg -3'	5'- tac cca cta aga taa ggg gct -3'	56-57	4959+35=4994*
11 segment 1	5'-gct aat acg act cac tat agg aac aga cca cca tg- tgt cac ttt gtg ttt tta tgt tta gg -3'	5'- gat ggc taa aac tgg tga ttt c -3'	56	2799+35=2834*
11 segment 2	5'-gct aat acg act cac tat agg aac aga cca cca tg- cag tta act gct act aaa acg ga -3'	5'- tac cca cta aga taa ggg gct -3'	56-57	2622+35=2657*
11-5'end	5'-ggt act tta att ttg tca ctt tgt gt -3'	5'- gta ttc cac ttt tga atg ttg tac -3'	56	373
11-3'end	5'- gta tta gga acc aaa gtc tca c -3'	5'-tag tga ttg gca aca cga aag g-3'	57	385

*PCR products that used as a template for PTT includes T_7 promoter sequences (blue colour lower case letters), which flanking upstream of DNA specific segment, this added 35bp to the normal amplified DNA segment.
(Anl. Temp=Annealing temperature)

4.4 Results

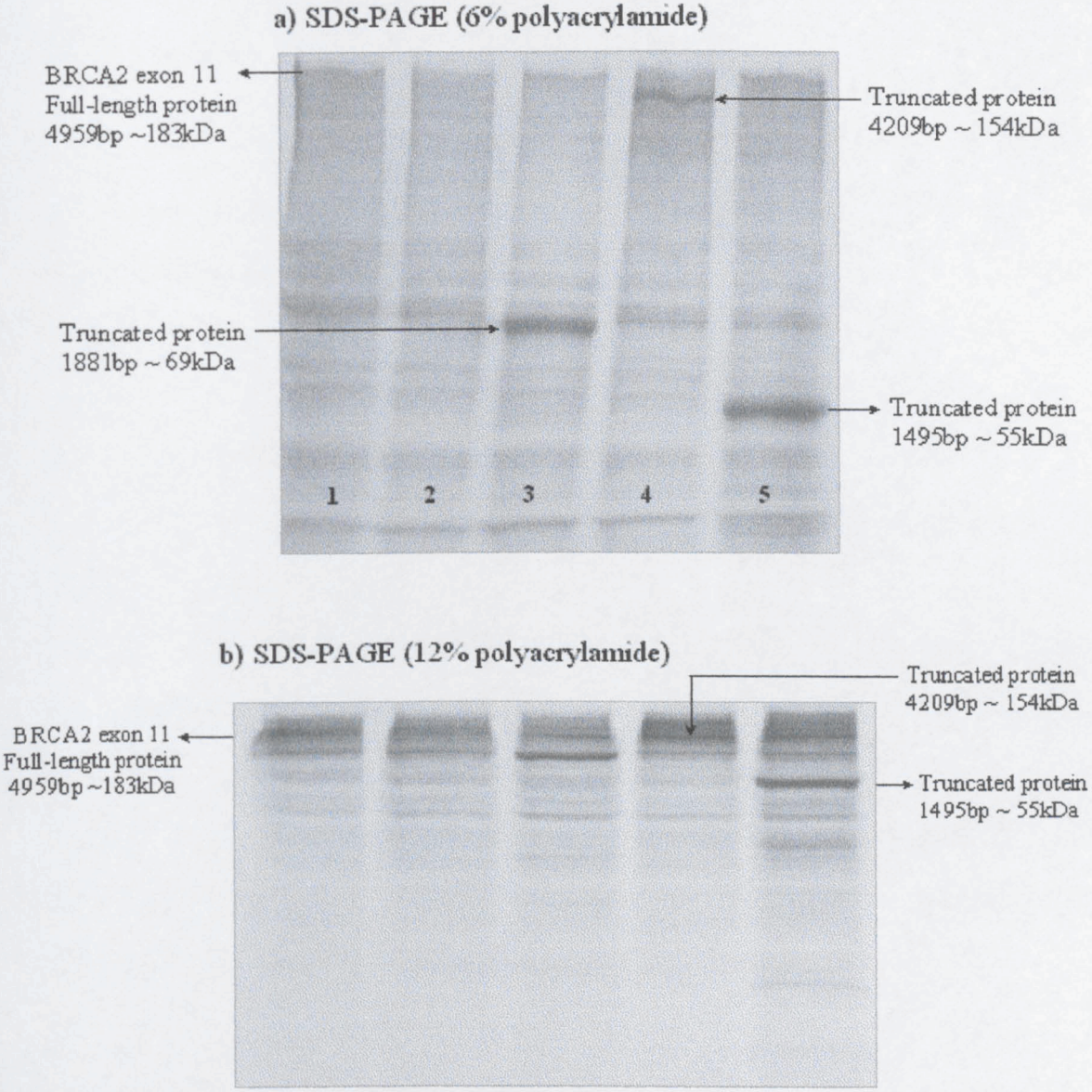
Three novel truncated mutations were detected by analysing of 104 samples for germline mutations in the BRCA1 gene exon 11 and BRCA2 gene exons 10 and 11 by amplification from genomic DNA with PCR. These alterations characterised by direct sequencing. DNA sequences obtained from affected patients' carriers and normal individuals in conjunction with the sequence data in public database for BRCA1 and BRCA2 genes [GenBank Accession numbers: U14680, L78833, U43746 and Z74739] were compared and identified the nucleotides variation. Figures 4.2-4.6 depicted these results.

Table 4.5: Germline mutations detected by PTT and D.S. in BRCA1 and BRCA2 gene in exon11

Gene	Mutation and nucleotide change	Tumour type	Stop codon occurred at	Coding effect	Screening method	Family history	Age at diagnosis
BRCA1	2335-2336delAA	IDC, MC	741 TAA	Frameshift	PTT	(2BC<40)	42
BRCA2	6261-6262 insGT	IDC	2040 TAA	Frameshift	PTT	(2BC<40)	27
BRCA2	3979-3980 insA	IDC	1264 TAA	Frameshift	PTT	Negative	40
BRCA2	5972 C>T T1915M	n.a.	----	Missense	Sequencing	Negative	41

BC= Breast Cancer n.a.= not available
IDC= Invasive Ductal Carcinoma MC= Medulary Carcinoma

Figure 4.2: SDS-PAGE images of the entire of BRCA2 exon11



Visualised by autoradiography of the ^{35}S-labeled proteins

SDS-PAGE analysis of the entire exon 11 of BRCA2 gene revealed the capability of the PTT to detect mutations within ~5kb coding sequences in a single reaction using a coupled transcription – translation system, TNT® T7 Quick for PCR DNA kit from Promega, Southamton, UK.

Figure 4.2, a and b (lane 1,2) shows the normal pattern of BRCA2 exon 11-full length protein (183kDa) and lanes 3-5 depict three different sizes of truncated proteins, which were identified on both 6% and 12% SDS-PAGE. Arrows show the size and position of the normal and those truncated proteins.

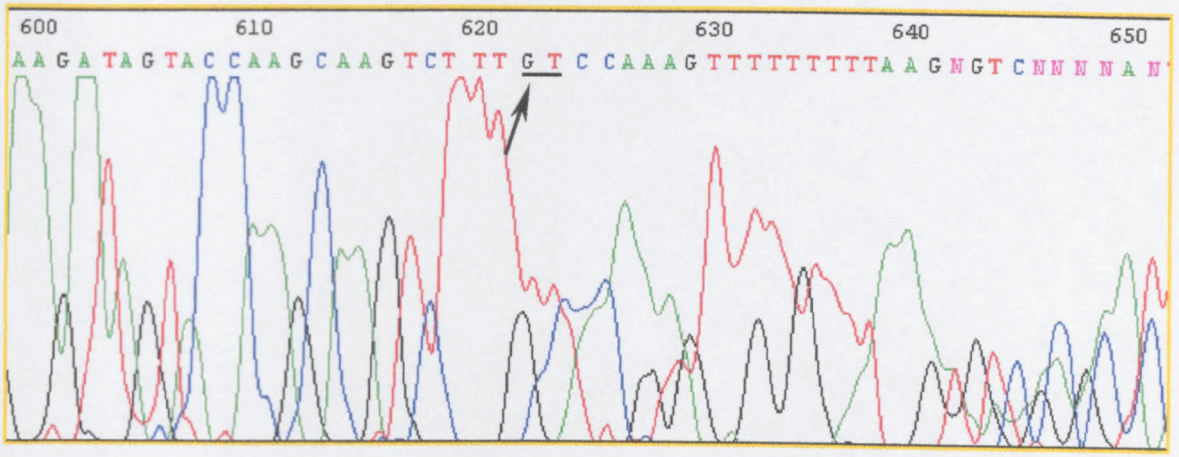
Lane 4 shows the translation products of a mutation that occurs close to the 3' end of exon 11 and produces a large truncated protein, which migrates close to the top of the gel.

Lane 5 shows a small truncated protein that resulting from a mutation that occurs close to the 5' end of exon 11 and migrates close to the bottom of the gel.

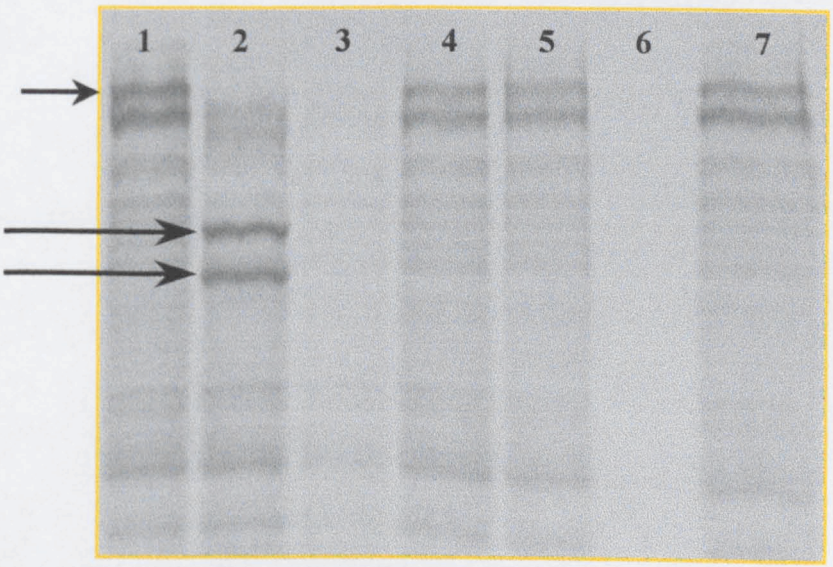
In each lane, several additional protein bands can be seen. These signals are probably generated by secondary translation initiation at internal ATG codons in the amplified segment.

Figure 4.3: Electropherograms and SDS-PAGE image of a mutant sequence of BRCA2 exon11 detected by PTT

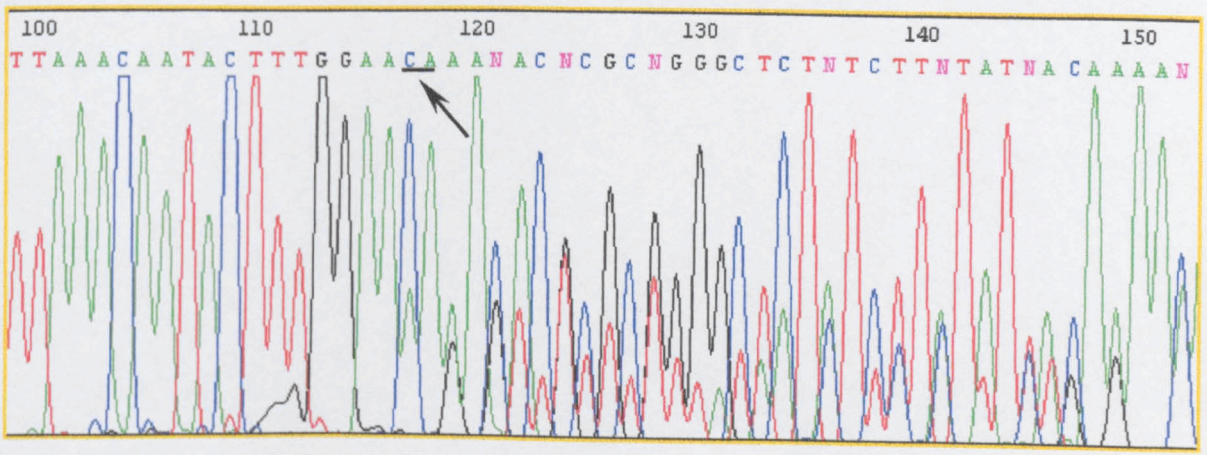
a) Forward sequence



b) SDS-PAGE image of BRCA2 exon 11 (segment two)



c) Reverse sequence



A part of wild type sequence of BRCA2 exon 11

```
1   gaa gat agt acc aag caa gtc ttt tcc aaa gta ttg
      E   D   S   T   K   Q   V   F   S   K   V   L

37  ttt aaa agt aac gaa cat tca gac cag ctc aca aga
      F   K   S   N   E   H   S   D   Q   L   T   R

73  gaa gaa aat act gct ata cgt act cca gaa cat tta
      E   E   N   T   A   I   R   T   P   E   H   L

109 ata tcc caa aaa ggc ttt tca tat aat gtg gta aat
      I   S   Q   K   G   F   S   Y   N   V   V   N

145 tca tct gct ttc tct gga ttt agt aca gca agt gga
      S   S   A   F   S   G   F   S   T   A   S   G

181 aag caa gtt tcc att tta gaa agt tcc tta cac aaa
      K   Q   V   S   I   L   E   S   S   L   H   K

217 gtt aag gga gtg tta gag gaa ttt gat tta atc aga
      V   K   G   V   L   E   E   F   D   L   I   R
```

Mutant sequence of a part of BRCA2 exon 11 [6261-6262 ins GT]

```
1   gaa gat agt acc aag caa gtc ttt gtt cca aag tat
      E   D   S   T   K   Q   V   F   V   P   K   Y
                Site for insertion GT ↑

37  tgt tta aaa gta acg aac att cag acc agc tca caa
      C   L   K   V   T   N   I   Q   T   S   S   Q

73  gag aag aaa ata ctg cta tac gta ctc cag aac att
      E   K   K   I   L   L   Y   V   L   Q   N   I

109 taa tat ccc aaa aag gct ttt cat ata atg tgg taa
      -   Y   P   K   K   A   F   H   I   M   W   -

145 att cat ctg ctt tct ctg gat tta gta cag caa gtg
      I   H   L   L   S   L   D   L   V   Q   Q   V

181 gaa agc aag ttt cca ttt tag aaa gtt cct tac aca
      E   S   K   F   P   F   -   K   V   P   Y   T

217 aag tta agg gag tgt tag agg aat ttg att taa tca
      K   L   R   E   C   -   R   N   L   I   -   S
```

Blue colour =coding sequence
Underlined Blue colour =two base pair (gt) that is inserted in a mutant case
Upper case letter (red colour) = Amino acid sequence
Upper case letter (black colour) = Not translated in mutant sequence

Note: Figure 4.3,b shows an image of SDS-PAGE (6% acrylamide) that reveals the pattern of a normal and the truncated protein of BRCA2 exon 11 segment two.

Lanes 1, 4, 5 and 7show normal pattern of *de novo* protein (2620bp ~ 96kDa); lane 3 shows a poor protein product and lane 2 depicts the truncated protein (1894bp ~ 70kDa) that run faster than normal protein on the SDS-PAGE.

In lane 2, arrows show two different sizes of truncated proteins resulted in the position of two or more consequent stop codons, which lead to generate different sizes of proteins (See the above sequences). One explanation can be, jumping the ribosome to the position of next or next after of a stop codon may produce two or more truncated proteins in the same reaction. So that different positions of bands (truncated protein) can be seen in one lane.

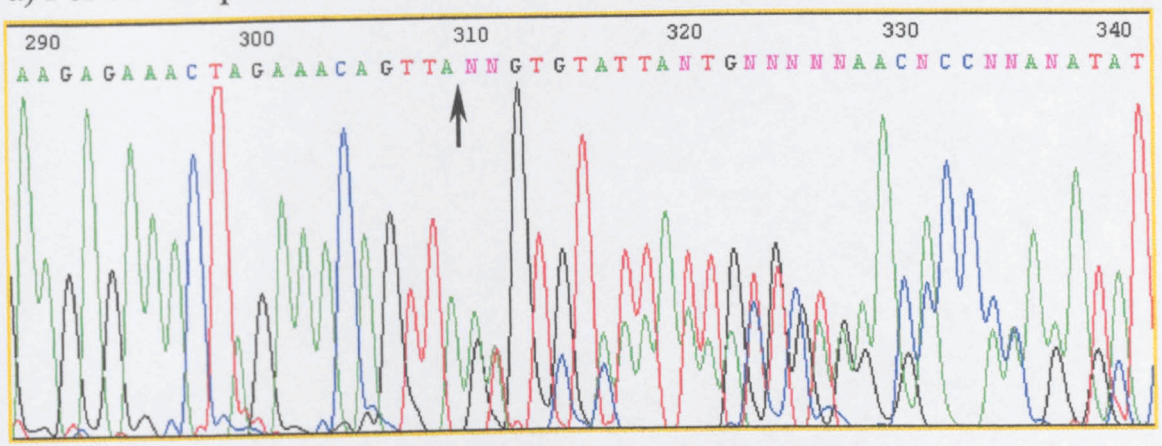
A negative control reaction containing no added template was used to allow measurement of any background incorporation (lane 6).

Direct sequencing revealed (in both directions) a mutation in sample lane 2 and characterised two base pair (GT) insertion in BRCA2 gene exon 11 between nucleotides 6261 and 6262 that leads to a TAA (stop codon) at codon 2040 on complete BRCA2 cDNA. Figures 4.4 (a and c) show this alteration.

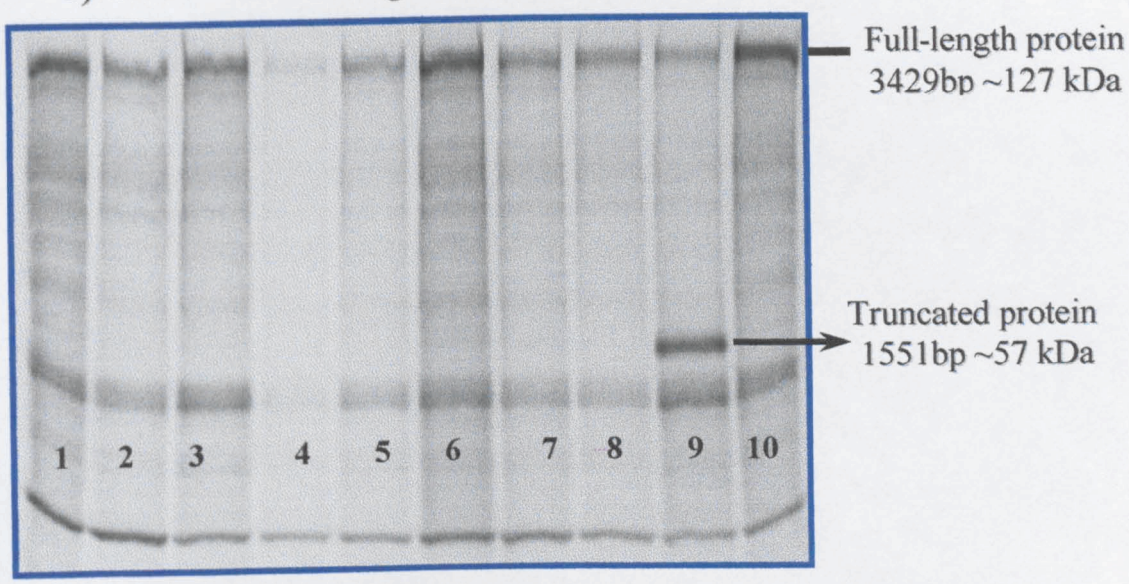
This patient harbour another sequence variant (12bp duplication gtattccactcc) that was identified in BRCA1 [IVS20+48], see chapter 3, Figure 3.4 for more detail.

Figure 4.4: Electropherograms and SDS-PAGE image of a mutant sequence of BRCA1 exon11 detected by PTT

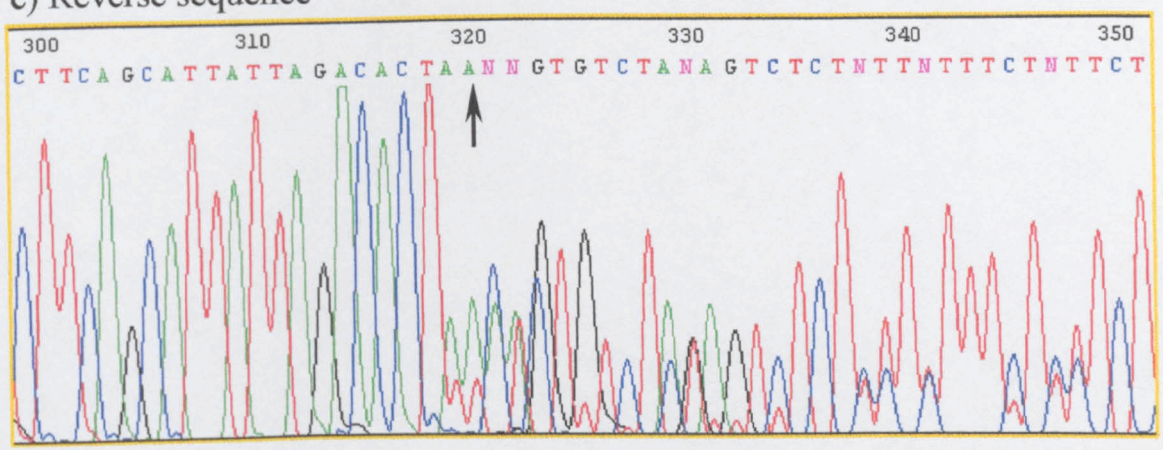
a) Forward sequence



b) SDS-PAGE image of BRCA1 exon 11



c) Reverse sequence



A part of wild type sequence of BRCA1 exon 11

```
1   gaa aaa gaa gag aaa cta gaa aca gtt aaa gtg tct
      E   K   E   E   K   L   E   T   V   K   V   S

37  aat aat gct gaa gac ccc aaa gat ctc atg tta agt
      N   N   A   E   D   P   K   D   L   M   L   S

73  gga gaa agg gtt ttg caa act gaa aga tct gta gag
      G   E   R   V   L   Q   T   E   R   S   V   E

109 agt agc agt att tca ttg gta cct ggt act gat tat
      S   S   S   I   S   L   V   P   G   T   D   Y

145 ggc act cag gaa agt atc tcg tta ctg gaa gtt agc
      G   T   Q   E   S   I   S   L   L   E   V   S

181 act cta ggg aag gca aaa aca gaa
      T   L   G   K   A   K   T   E
```

Mutant sequence of a part of BRCA1 exon11 [2336-2337 del AA]

```
1   gaa aaa gaa gag aaa cta gaa aca gtt agt gtc taa
      E   K   E   E   K   L   E   T   V   S   V   -
                  Site for deletion AA

37  taa tgc tga aga ccc caa aga tct cat gtt aag tgg
      -   C   -   R   P   Q   R   S   H   V   K   W

73  aga aag ggt ttt gca aac tga aag atc tgt aga gag
      R   K   G   F   A   N   -   K   I   C   R   E

109 tag cag tat ttc att ggt acc tgg tac tga tta tgg
      -   Q   Y   F   I   G   T   W   Y   -   L   W

145 cac tca gga aag tat ctc gtt act gga agt tag cac
      H   S   G   K   Y   L   V   T   G   S   -   H

181 tct agg gaa ggc aaa aac aga a
      S   R   E   G   K   N   R
```

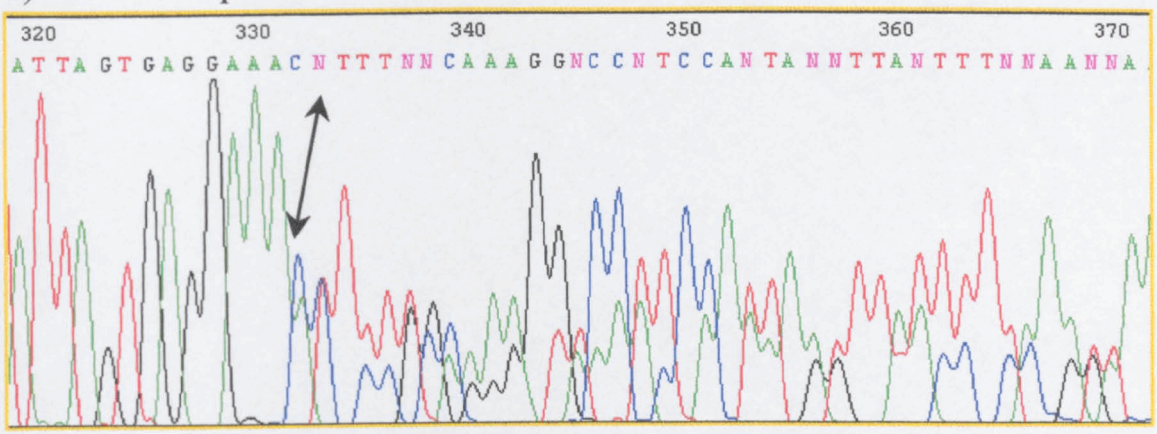
Blue colour =Coding sequence
Underlined Blue colour = two base pair (aa) that is deleted in a mutant case
Upper case letter (red colour) = Amino acid sequence
Upper case letter (black colour) = Not translated in mutant sequence

Note: Figure 4.4 (b) lanes 1-8 and 10 show the normal pattern of entire translated protein of BRCA1 exon 11(3429bp ~ 127kDa) and lane 9 depicts the truncated protein (1551bp ~ 57kDa), which run faster than normal one on the SDS-PAGE.

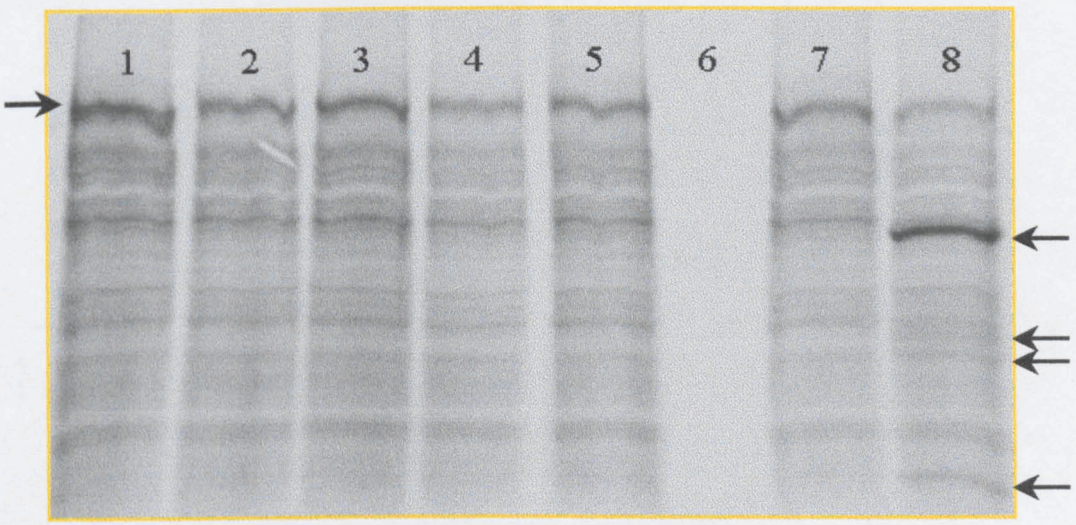
Direct sequencing revealed (in both directions figure 4.4, a and c) a mutation in sample lane 9 and characterised two base pair deletion (AA) in BRCA1 gene exon 11 at nucleotides 2336 and 2337 that leads to a TAA (stop codon) at codon 741 on complete BRCA1cDNA.

Figure 4.5: Electropherograms and SDS-PAGE image of a mutant sequence of BRCA2 exon11 detected by PTT

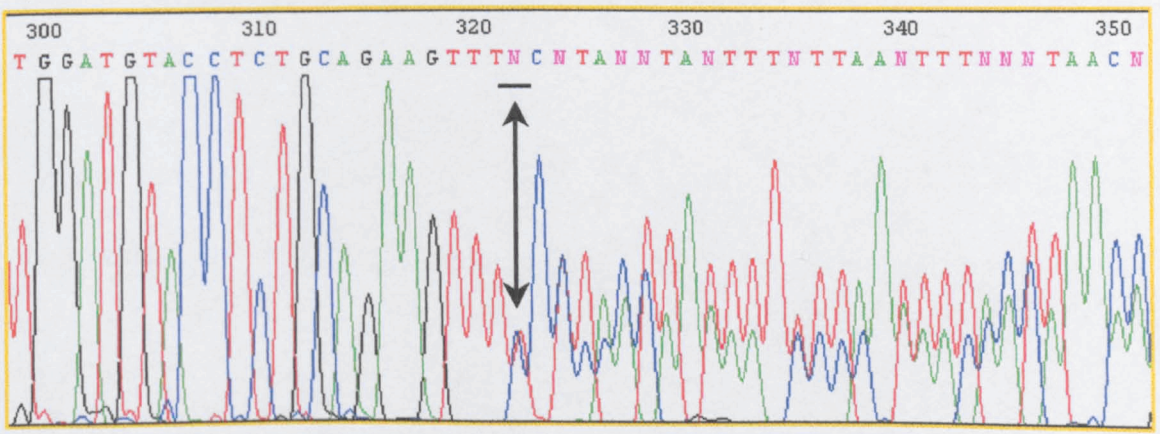
a) Forward sequence



b) SDS-PAGE image of BRCA2 exon 11 (segment one)



c) Reverse sequence



A part of wild type sequence of BRCA2 exon 11

```
1   gag aat att agt gag gaa act tct gca gag gta cat
      E   N   I   S   E   E   T   S   A   E   V   H

37  cca ata agt tta tct tca agt aaa tgt cat gat tct
      P   I   S   L   S   S   S   K   C   H   D   S

73  gtt gtt tca atg ttt aag ata gaa aat cat aat gat
      V   V   S   M   F   K   I   E   N   H   N   D

109 aaa act gta agt gaa aaa aat aat aaa tgc caa ctg
      K   T   V   S   E   K   N   N   K   C   Q   L

145 ata tta caa aat aat att
      I   L   Q   N   N   I
```

Mutant sequence of a part of BRCA2 exon11 [3979-3980 ins A]

```
1   gag aat att agt gag gaa aac ttc tgc aga ggt aca
      E   N   I   S   E   E   N   F   C   R   G   T
        Site for insertion A ↑

37  tcc aat aag ttt atc ttc aag taa atg tca tga ttc
      S   N   K   F   I   F   K   -   M   S   -   F

73  tgt tgt ttc aat gtt taa gat aga aaa tca taa tga
      C   C   F   N   V   -   D   R   K   S   -   -

109 taa aac tgt aag tga aaa aaa taa taa atg cca act
      -   N   C   K   -   K   K   -   -   M   P   T

145 gat att aca aaa taa tat t
      D   I   T   K   -   Y
```

Blue colour =Coding sequence
Underlined Blue colour = A one base pair that is inserted in a mutant case
Upper case letter (red colour) = Amino acid sequence
Upper case letter (black colour) = Not translated in mutant sequence

Note: Figure 4.5,b shows an image of SDS-PAGE (6% acrylamide) that reveal the pattern of a normal and the truncated protein of BRCA2 exon 11 segment one.

A one base pair (A) insertion between nucleotides 3979 and 3980 (on BRCA2 cDNA) leads to a TAA (stop codon) at codon 1246 and made a truncated protein (1904~71kDa) that seen on lane 8. Arrows in lane 8 show different positions of the truncated protein.

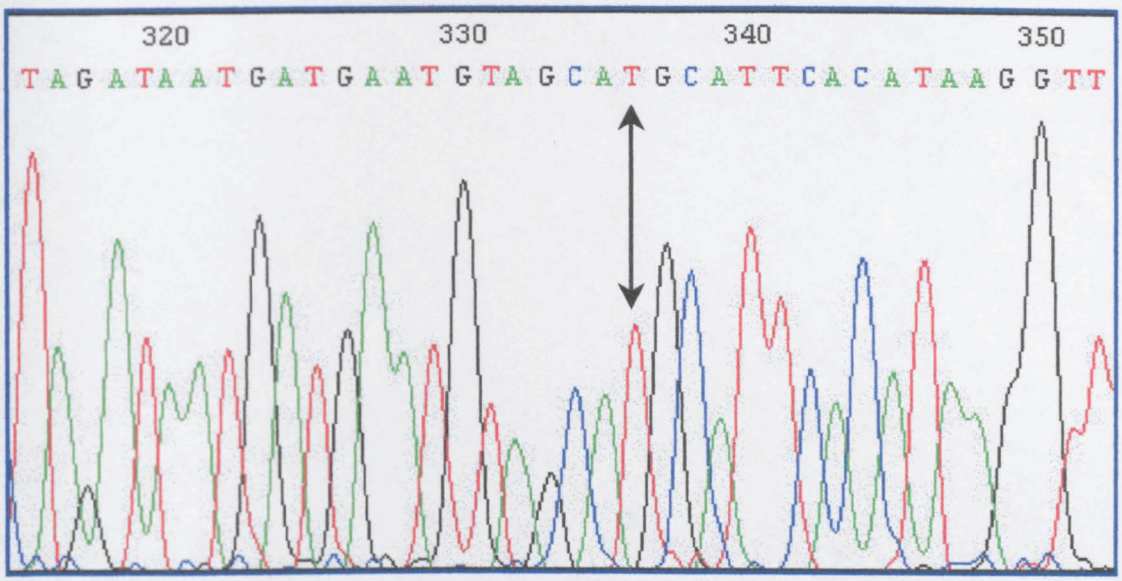
Lanes 1-5 and 7 show normal pattern of the full-length translated protein of BRCA2 exon 11 segment one (2799bp~111kDa).

A negative control reaction containing no added template was used to allow measurement of any background incorporation (lane 6).

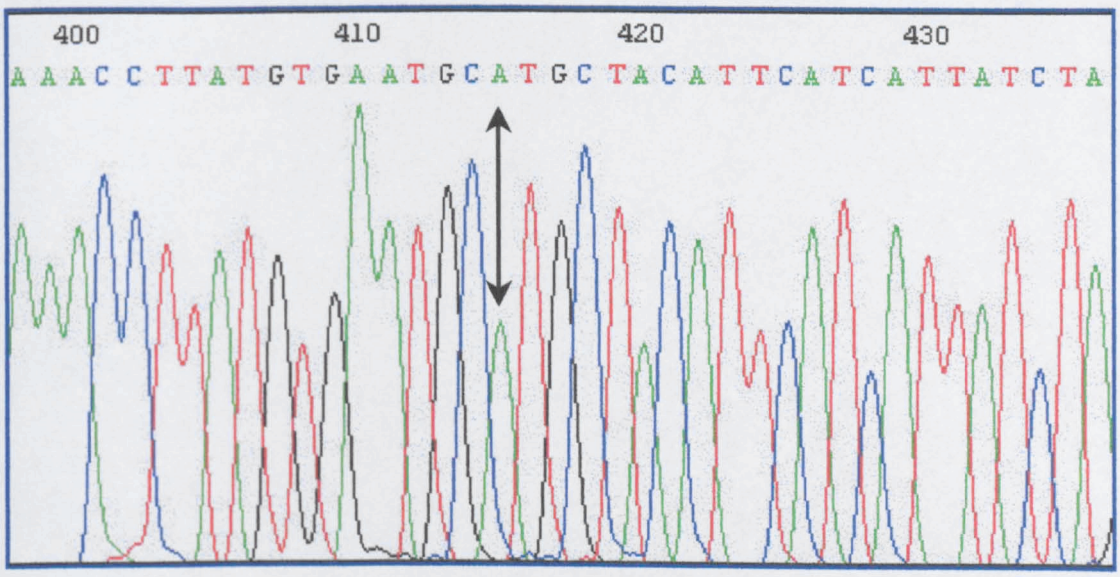
Direct sequencing revealed (in both directions) a mutation in this sample (lane 6) and characterised a one base pair (A) insertion. Figures 4.5,a and c show this alteration.

Figure 4.6: Electropherograms of a homozygote variant in BRCA2 exon 11 detected by direct sequencing

a) Forward sequence



b) Reverse sequence



The wild type sequence of BRCA2 exon 11

```
3763  gca ggt tgt tac gag gca ttg gat gat tca gag
        A   G   C   Y   E   A   L   D   D   S   E

3796  gat att ctt cat aac tct cta gat aat gat gaa
        D   I   L   H   N   S   L   D   N   D   E

3829  tgt agc acg cat tca cat aag gtt ttt gct gac
        C   S   T   H   S   H   K   V   F   A   D

3862  att cag agt gaa gaa att tta caa cat aac caa
        I   Q   S   E   E   I   L   Q   H   N   Q
```

The sequence variant of BRCA2 exon 11

5972 C>T (T1915M) Thr>Met

```
3763  gca ggt tgt tac gag gca ttg gat gat tca gag
        A   G   C   Y   E   A   L   D   D   S   E

3796  gat att ctt cat aac tct cta gat aat gat gaa
        D   I   L   H   N   S   L   D   N   D   E

3829  tgt agc atg cat tca cat aag gtt ttt gct gac
        C   S   M   H   S   H   K   V   F   A   D

3862  att cag agt gaa gaa att tta caa cat aac caa
        I   Q   S   E   E   I   L   Q   H   N   Q
```

Lower case letter (blue colour) = Exonic segment

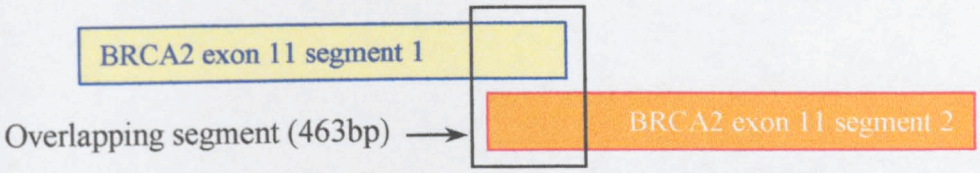
Underlined Bold Italic blue colour= A base pair substitution in sequence variant sample.

Upper case letter (red colour) = Amino acid sequence

Note: In BRCA2 gene at nucleotide 5972 (exon23), a base pair homozygote variant (C>T) substitute Threonine to Methionine amino acid. Effect of this sequence variant on BRCA2 gene expression is unclear.

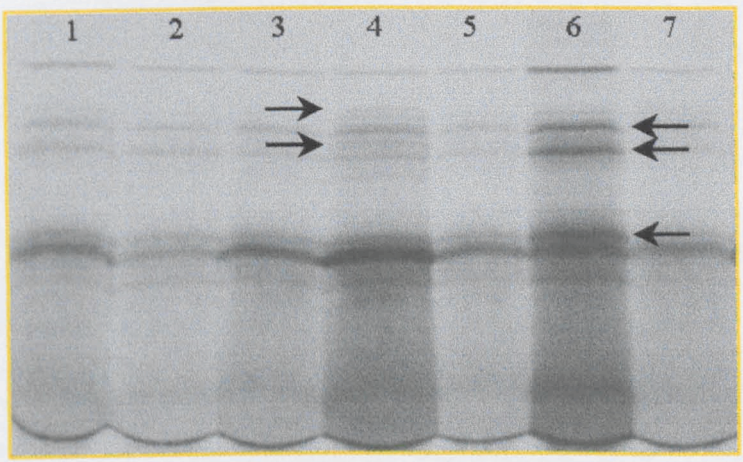
This segment was analysed by PTT and obviously no abnormal pattern (truncated protein) was observed.

At the time of analysing of the BRCA2 exon 11 by approaching two overlapping segments, several samples were suspected as contain mutations (truncated protein) from the autoradiography results. To test, the suspected samples were amplified for the overlapping segment of two fragments (BRCA2 exon11 fragments one and two = 463bp) and were used as a template for PTT.



The proteins were separated on SDS-PAGE (15% acrylamide), transferred to nitrocellulose and visualised by autoradiography (Picture below).

Figure 4.7: SDS-PAGE image of BRCA2 exon 11- overlapping segments

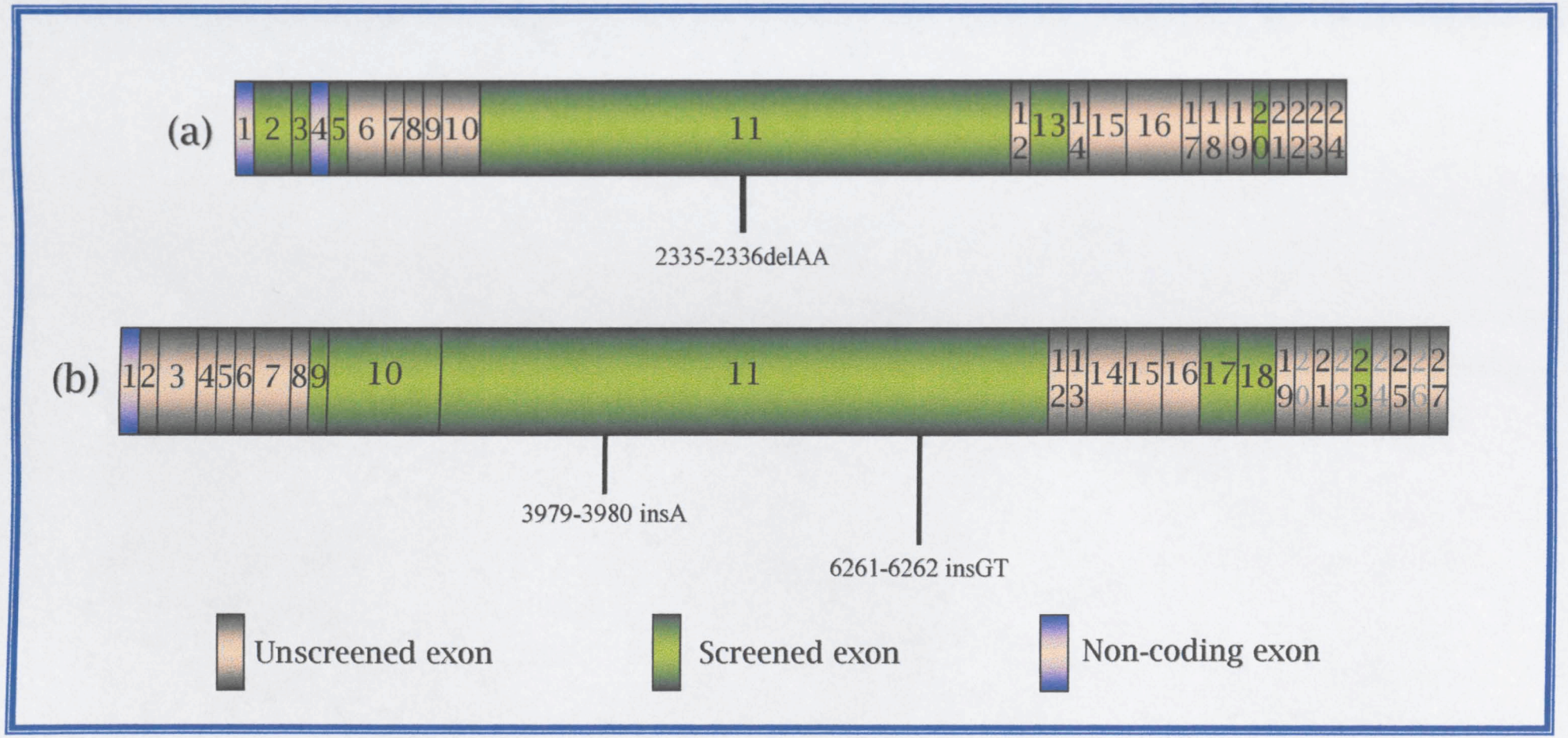


Lanes 1-3, 5 and 7 show the normal pattern of proteins BRCA2 exon11 from the overlapping fragment (463bp ~17kDa polypeptides). The pattern of bands in lanes 4 and 6 for the same protein were appeared to be abnormal (arrows). However, nucleotide sequencing of the corresponding region revealed a normal DNA sequence.

Note: The well separated ~17kDa protein on SDS-PAGE 15% revealed the capacity of PTT and SDS-PAGE for small amplicons used for *in-vitro* coupled transcription-translation reactions.

Although these samples were found normal, the analysing of short PCR products by PTT might have an advantage when combined with the Meta-PCR technique in which short Meta-PCR products are assembled (See chapter 6 for more detail).

Figure 4.8: Mutations characterised by PTT in the context of the BRCA1(a) and BRCA2(b) cDNA



4.5 Multiplex PTT

New variations on exiting mutation detection techniques are rapidly emerging to reduce the cost of genetic testing while maintaining specificity and sensitivity. The PTT is one of the common methods used in genetic laboratories to analyse breast cancer predisposing genes. There are different approaches for the analysing of exon 10 and 11 of the BRCA genes. Some analyse exon 11 in two segments in separate reactions and others screen in three or four segments.

In this study different approaches were applied to scan exon 11 of the BRCA genes and to investigate the advantages and disadvantages of each method. Finally, I made significant improvements to the method called Multiplex PTT that leads to a reduction in the time and cost (by up to 50%) for analysing most (exon 11) of the BRCA1 and BRCA2 genes.

Basically, the assay is similar to the conventional PTT. Some modifications were performed to achieve stronger signals and through an increase in the yield of *de novo* protein.

It was anticipated that at least two problems might be encountered: The first, synthesis of more amounts of artificial protein and the second is obtaining the clear separation and visualisation.

Using two big PCR products (mixture of PCR products of exon 11 of the both BRCA1 and BRCA2 gene ~ 8.5kb in total) in a coupled transcription-translation reaction at the same time may reduce *in-vitro* protein yields.

In this study small amounts of TNT® T7 Quick for PCR DNA Mater Mix (from Promega, Southampton, UK; catalogue no. L5540) were used for coupled transcription-translation reaction (1/4 of the manufacturer's recommended), see chapter two section 2.1.7 and appendix XI for more details. This reduction in the concentration of reaction components did not

compromise product yield. This means that the cost of the test has been previously reduced, exploiting multiplex PTT assay might reduce the test expenses even further.

Gradient SDS-PAGE (4%-20% acrylamide) has been shown to achieve better resolution. Additional amounts (0.5µl) of T_7 RNA polymerase may lead to more *de novo* proteins production. Increasing the incubation time (90 minutes in 30°C) did not effect on yield.

In order to ensure correct interpretation of the results, a batch of samples including normal, and negative control should be analysed alongside of samples tested which lead to simplify the comparison of mobility patterns of normal and truncated proteins.

In this experiment the *de novo* protein were separated on SDS-PAGE (6% acrylamide), transferred to nitrocellulose and visualised by autoradiography (Figures 4.9 and 4.10a, b).

Figure 4.9: SDS-PAGE image of the Multiplex PTT

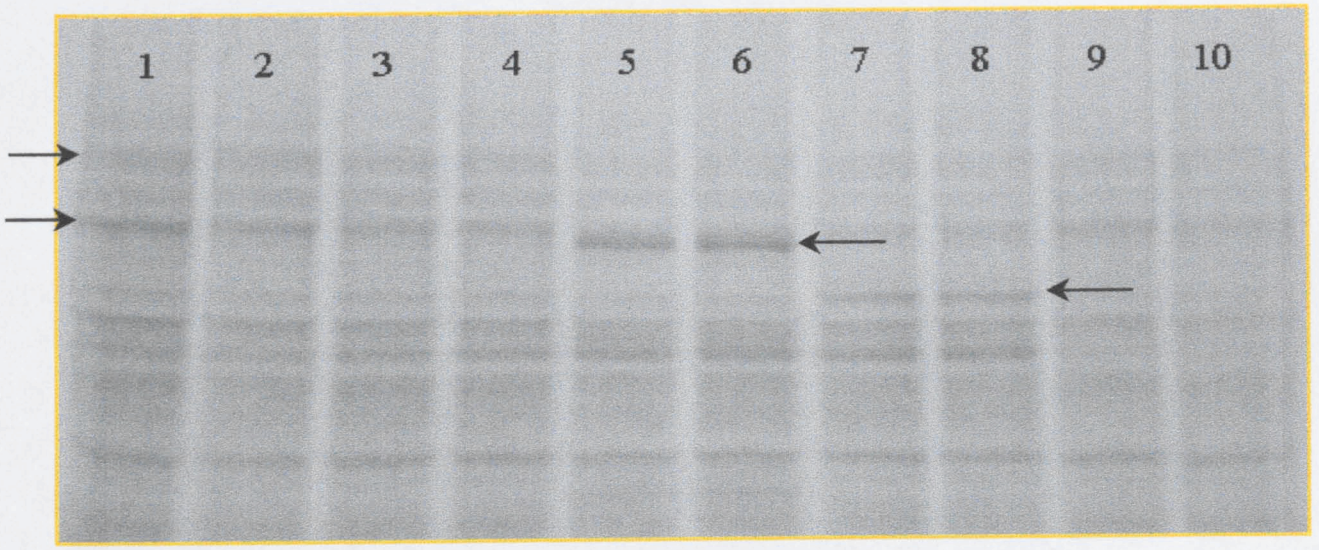


Figure 4.9: Lanes 1-4 and 9,10 depicted normal pattern of mixed full length of translated proteins of BRCA1 (lane 1 bottom arrow) and BRCA2 exon 11 (lane 1 top arrow).*

Lanes 5 and 6 shown previously ascertained truncated protein of BRCA2 exon 11 (arrow) with background of full length of protein of BRCA1 exon 11.

Lanes 7 and 8 shown previously ascertained truncated protein of BRCA1 exon 11 (arrow) with background of full length of protein of BRCA2 exon 11.

*In this experiment PCR products from the entire length of exon 11of both BRCA1 and BRCA2 gene, ~3.5kb and ~5kb respectively were used as templates for coupled transcription and translation in single reaction.

Note: Any given suspected samples should be targeted for conventional PTT to avoid misinterpretation of abnormal patterns. With more experience one become familiar with the normal pattern of *in-vitro* synthesized protein resulting from the co-translation from specific PCR products and discrimination of abnormal patterns is readily observed.

The following SDS-PAGE images show the patterns of the normal and previously ascertained truncated protein of different sizes. These additional images were only produced to show the capacity of multiplex PTT in application of various sizes of PCR products.

Figure 4.10,a: SDS-PAGE image of the Multiplex PTT

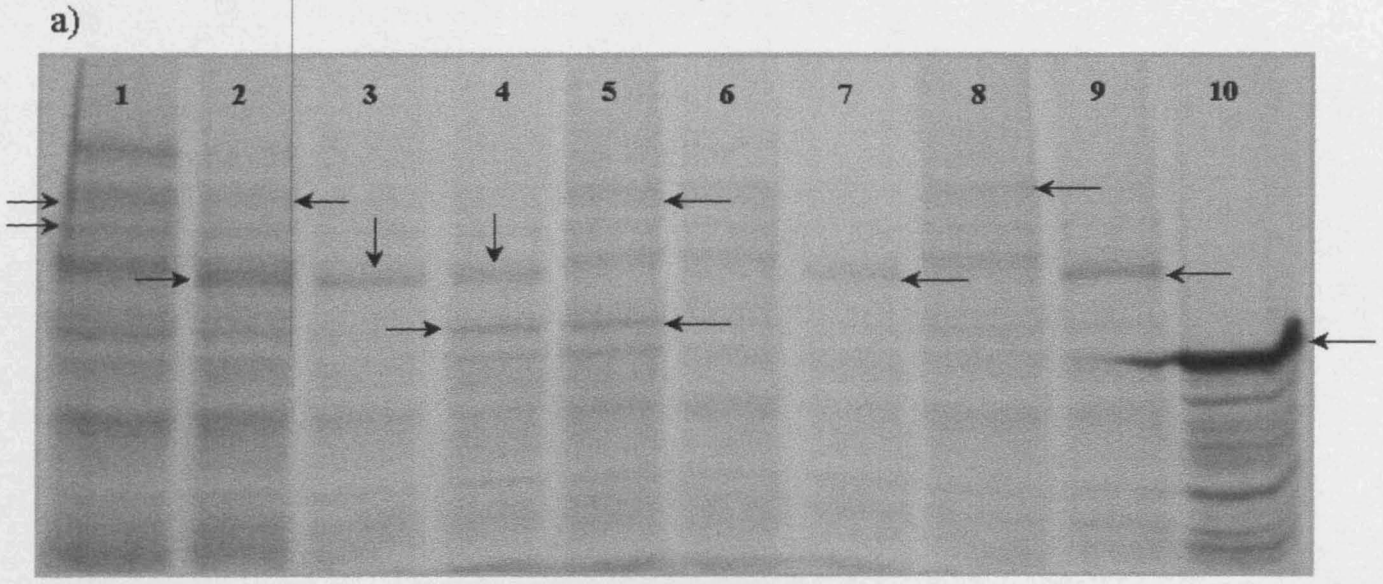


Figure 4.10,a

Templates in lane 1: Mixture of a wild type protein of BRCA2 exon 11 segment one (2799bp~111kDa-bottom arrow) and wild type protein of the BRCA2 exon 11 full-length protein (4959bp~183kDa-top arrow).

Templates in lane 2: Mixture of a truncated protein of BRCA2 exon 11 segment one (1904bp~71kDa-bottom arrow) and wild type of the BRCA2 exon 11 full-length protein (4959bp~183kDa-top arrow).

Templates in lane 3: Mixture of a truncated protein of BRCA2 exon 11 segment one (1904bp~71kDa- arrow) and a truncated protein of the BRCA2 exon 11(1881bp~69kDa- disguising).

Templates in lane 4: Mixture of a truncated protein of BRCA2 exon 11 segment one (1904bp~71kDa-top arrow) and a truncated protein of the BRCA1 exon 11 (1551bp~57kDa- bottom arrow).

Templates in lane 5: Mixture of a wild type protein of the BRCA2 exon 11 full-length protein (4959bp~183kDa-top arrow) and a truncated protein of the BRCA1 exon 11 (1551bp~57kDa- bottom arrow).

Templates in lane 6: A wild type protein of BRCA2 exon 11 segment one (2799bp~111kDa), poor protein product.

Templates in lane 7: A truncated protein of BRCA2 exon 11 segment one (1904bp~71kDa- arrow)

Templates in lane 8: A wild type protein of the BRCA2 exon 11 full-length protein (4959bp~183kDa).

Templates in lane 9: A truncated protein of the BRCA2 exon 11(1881bp~ 69kDa).

Templates in lane 10: A wild type of Luciferase protein (T_7 Control DNA)

Figure 4.10,b: SDS-PAGE image of the Multiplex PTT

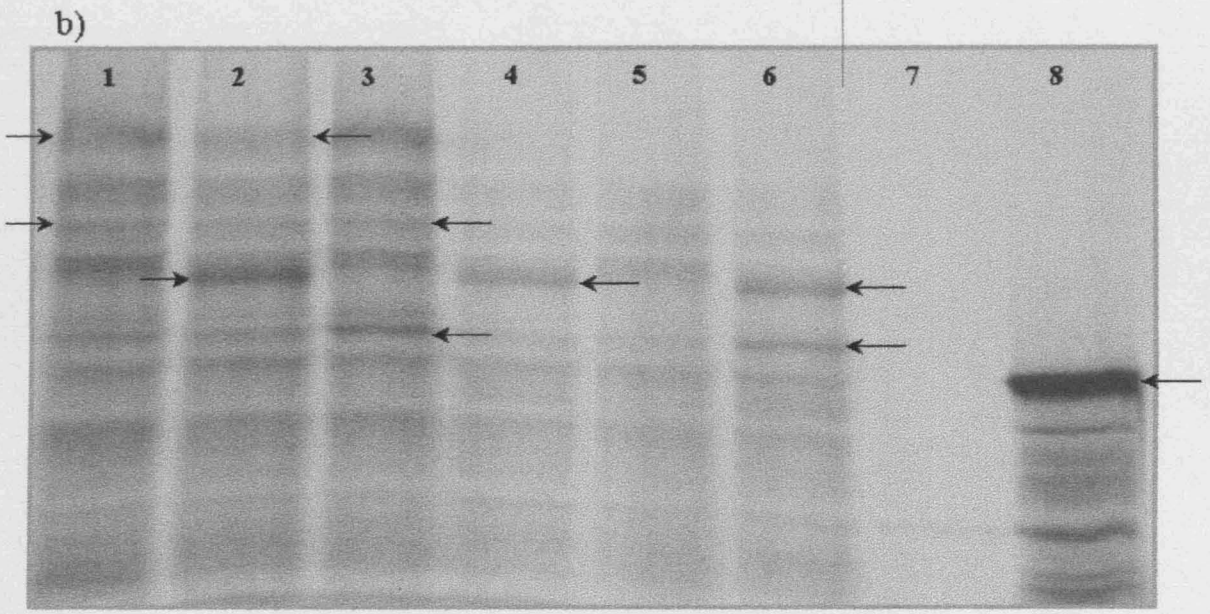


Figure 4.10,b

Templates in lane 1: Mixture of a wild type protein of BRCA2 exon 11 segment one (2799bp~111kDa-bottom arrow) and wild type protein of the BRCA1 exon 11 full-length protein (3429bp~127kDa-top arrow).

Templates in lane 2: Mixture of a truncated protein of BRCA2 exon 11 segment one (1904bp~71kDa-bottom arrow) and wild type protein of the BRCA1 exon 11 full-length protein (3429bp~127kDa-top arrow).

Templates in lane 3: Mixture of a wild type protein of BRCA2 exon 11 segment one (2799bp~111kDa-top arrow) and a truncated protein of the BRCA1 exon 11 (1551bp~57kDa- bottom arrow).

Templates in lane 4: Mixture of a truncated protein of the BRCA2 exon 11(1881bp~69kDa-arrow) and wild type protein of the BRCA1 exon 11 full-length protein (3429bp~127kDa-unregonisable because of poor protein producing).

Templates in lane 5: Mixture of a wild type protein of the BRCA2 exon 11 full-length protein (4959bp~183Da) and wild type protein of the BRCA1 exon 11 full-length protein (3429bp~127kDa). Both protein product are poor and unrecognisable.

Templates in lane 6: Mixture of a truncated protein of the BRCA2 exon 11(1881bp~69kDa-top arrow) and a truncated protein of the BRCA1 exon 11 (1551bp~57kDa- bottom arrow).

Templates in lane 7:A negative control reaction containing no added template was used to allow measurement of any background incorporation.

Templates in lane 8: A wild type of Luciferase protein (T_7 Control DNA)

4.6 Discussion

PTT is one of the most commonly used methods in genetics laboratory worldwide. The specificity of the test is 100% and the sensitivity although not yet defined but in a systematic study seems to approach 90%-95%, because of insensitivity of the method in detection of mutation at close to the 5' and 3' end of the DNA specific of interest.

1) To date only PTT kit from Promega ™, Southampton, UK is available, it can produce polypeptide chain from up to 5kb artificial transcript [[20]].

In this study to assess the capability of the PTT and the utility of the TNT® T7 Quick for PCR DNA kit (from Promega ™, Southampton, UK) in producing of a range of artificial protein and to observe the clarity of those visualisation by autoradiography, different DNA specific fragments from range of ~470bp to ~5kb in sizes (Figure 4.2 - 4.7) were tested.

I have observed no significant effect of the length of the PCR products on the efficiency of the translation/transcription reaction, however, with increasing the size of the translated products, gel resolution will diminish the minimum truncation event that will be detectable. It is strongly recommend that, at this DNA fragment size (around 5kb), the 5' and 3' end of the region of interest should be analyse by another mutation detection technique to avoid missing any mutation due to insensitivity of the test.

However, in my experience, translation/transcription of PCR products up to 4-5kb is not difficult, best results are obtained when transcription/ translation amplification fragments of up to 2kb from genomic DNA are analysed by PTT.

2) I found Multiplex-PTT to be a very cost effective approach for the analysis of large fragments such as BRCA genes exon 11 and might be

applicable in genetic laboratories to reduce expenses and time of genetic tests.

Although, reticulocyte lysate (PTT kit from Promega ™, Southampton, UK) contains the cellular components necessary for protein synthesis (tRNA, ribosomes, amino acids, translational factors, further optimisation for mRNA translation by adding an energy-generating system consisting of phosphocreatine kinase and phosphocreatine, a mixture of tRNAs to expand the range of mRNAs, potassium acetate and magnesium acetate) but it has not been reported with the same promoters in the same reaction multiple proteins can be expressed [21].

3) For the BRCA1 exon 11, the first translation site (ATG) appears at codon 74 (nucleotides 222) and the second one is at codon 308 (nucleotides 924) when a mutation occurred upstream to the codon 74, the synthesized polypeptide is very small and/or no radioactive signal from newly synthesized polypeptide can be detected. Therefore, when a mutation occurs upstream of the codon 308 there are only two ATG sites in the PCR products (one in the forward primer- Kozak sequence, see chapter 2 section 2.1.8 - and the next one is at the codon 74) to facilitate radiolabelling; the chance to detect a mutation is still low. But in the middle and towards to the end of this segment there are sufficient ATGs to enable radiolabelling, thus there is still a little chance to miss a mutation close to the 3' end.

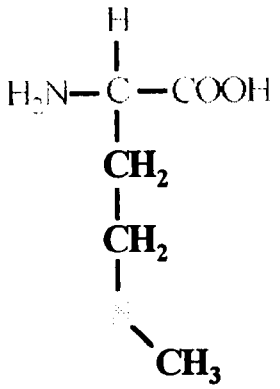
For the BRCA2 exon 11, the first translation site (ATG) appears at the codon 148 (nucleotides 444) when a mutation occurred upstream to the that, only one ATG site in the PCR products is remain (Kozak sequence) to produce signal, so that the band close to the bottom of the gel may appear very faint and can be easily missed.

However, using cysteine (TCT) labelling may overcome these problems due to enough number sites of TCT throughout the BRCA1 and BRCA2 exon 11 to make signals. Therefore, it has been strongly recommended by Promega™ to use [^{35}S] Methionine because it prevents the background labelling of a rabbit reticulocyte lysate 42kDa protein, which can occur using other labels [22].

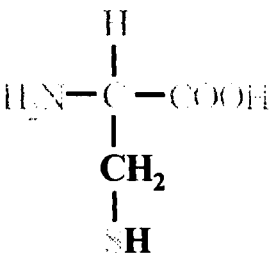
In addition, designing a forward primer (Kozak sequence) includes more than one ATG site may useful to enhance [^{35}S] Methionine incorporation and lead to better visualisation by autoradiography where mutation occur close to 5' end.

Note:

1) Among twenty amino acids only Methionine and Cysteine have sulfhydryl group to be radiolabeled.



(a) Methionine



(b) Cysteine

2) The above codon numbers is accounted based on BRCA genes exon 11 not cDNA.

4.7 References

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Chapter 5

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5 Meta-PCR technique

5.1 Introduction

Conventional methods used to clone nucleotide sequences encoding open reading frames or to detect gene mutations' rely on either the preparation of cDNA or the isolation of each exon of a gene individually from genomic DNA [1, 2].

For genes that have several exons, often spread over large distances in genomic DNA, preparation of cDNA or fragments of each exon can be a tedious process. In some cases, original tissue is not available; leaving genomic DNA as the source of genetic material as the most convenient choice for most laboratories due to the simplicity of its preparation and its intrinsic stability.

Yet working with genomic DNA leaves the exon as the unit of analysis for mutation scanning. Because the average higher eukaryote exon is only 140bp in size [3] the sheer number of tests involved scanning for the complete of genes for point mutations means that the more sensitive, labour-intensive methods impose severe constraints on throughput.

A variety of scanning methods are available to identify unknown point mutations. The choice of technique being determined by several competing factors, two of the most important factors is the sensitivity of the method

and the complexity of the procedure. Some widely used mutation detection methods such as SSCP/HA are technically simple but are only capable of scanning small DNA fragments and even than with variable sensitivity [1, 4, 5]. Other mutation-scanning techniques like PTT are capable of locating mutations in DNA fragments much larger than the average exon [6].

There has been an increasing demand for rapid and accurate methods for scanning of point mutations in large multi-exon genes. Recently a method called Lexon has been described (Tuohy and Groden, 1998) for the production of in-frame concatamers of PCR products of APC gene exons 3-9A for downstream analysis in the Protein Truncation Test [7]. Following a new technique named Meta-PCR have been developed by Wallace et al (1999) for creating chimaeric DNA molecules using a modified PCR reaction for downstream analysis by direct nucleotide sequencing [8].

Meta-PCR is a simple and very convenient method for linking small DNA fragments from large multi-exon genes that can maximize the length of sequence scanned by downstream techniques. Through Meta-PCR small PCR amplicons are assembled into a single large chimaeric DNA molecule in two coupled stages carried out in a single reaction vessel. The order of fragments is predetermined by primer design. Currently, up to five PCR amplifiable fragments can be combined to form a single linear amplicon [8].

In this study BRCA genes were chosen as the target to evaluate feasibility of the Meta-PCR technique. RNA as a source of genetic material is not suitable for analysing and scanning of each exon of BRCA1 and BRCA2 genes individually is an onerous task (Table 5.1); this technique might circumvent these problems using genomic DNA.

Three distinct sets of BRCA gene fragments were selected to assemble with different approach for downstream analysis. The first set consisted of BRCA1 exons 2, 20 and the BRCA2 exon 18 and their subsequent analysis by Protein Truncation Test (See chapter 4 for great detail) [2, 6, 9, 10], the

second set comprised BRCA1 exons 2, 20,23 and 24 and their subsequent analysis by direct sequencing and the last one contained six significant coding regions from the BRCA genes, the 5' and 3' termini of exon 11 from both BRCA1 and BRCA2 gene, exons 2 and 20 from BRCA1, which most likely to harbour a mutation. Downstream analysis of Meta-PCR products by Protein Truncation Test was used rather than direct sequencing because the total assembled above fragments size (~2.8kb) is sufficiently big to ignore analysing by the latter approach.

5.2 Principle of Meta-PCR

The Meta-PCR process involves two coupled steps: the main phases of the primary Meta-PCR reaction are illustrated in Figures 5.1 A and B.

During this phase, genomic DNA is amplified with limiting quantities of primers (Tables 5.2, 5.3 and 5.4). In the initial cycles, the reaction behaves like a simple multiplex PCR. However, as the amplimers begin to increase in concentration, there are **insufficient amount of each primer** to hybridise to and re-extend the entire single-stranded DNA. This "**encourages hybridisation to occur between the linker segments of the linker primers**" (now incorporated into the PCR products) forming two types of intermediate (Fig. 5.1A). One of these intermediates (intermediate I in Fig. 5.1A) is capable of being extended by *Taq* polymerase, leading to a fusion product comprising the two fragments. As progressively more extendable intermediates form, longer fragments accumulate, until at some point, probably late in the primary Meta-PCR reaction, the full-length product arises (Fig.5.1B). Reamplification of a small aliquot of the primary Meta-PCR in a secondary reaction (secondary Meta-PCR) with an excess of internal nested primers reamplifies the full-length product with high specificity, producing sufficient product for downstream analysis (Fig. 5.1B).

Table 5.1: BRCA1 and BRCA2 coding regions sizes

BRCA1 exons sizes/bp		BRCA2 exons sizes/bp
Exon 2 (exon size=99)		Exon 2 (exon size=106)
Exon 3 (exon size=54)		Exon 3 (exon size= 249)
Exon 5 (exon size=78)		Exon 4 (exon size=109)
Exon 6 (exon size=89)		Exon 5 (exon size= 51)
Exon 7 (exon size=140)		Exon 6 (exon size= 41)
Exon 8 (exon size=108)		Exon 7 (exon size= 115)
Exon 9 (exon size=46)		Exon 8 (exon size= 50)
Exon 10 (exon size=77)		Exon 9 (exon size= 112)
Exon 11 (exon size=3426)		Exon 10 (exon size= 1116)
Exon 12 (exon size=89)		Exon 11 (exon size= 4931)
Exon 13 (exon size=172		Exon 12 (exon size= 99)
Exon 14 (exon size=127)		Exon 13 (exon size= 73)
Exon 15 (exon size=191)		Exon 14 (exon size= 428)
Exon 16 (exon size=311)		Exon 15 (exon size= 182)
Exon 17 (exon size=88)		Exon 16 (exon size= 188)
Exon 18 (exon size=71)		Exon 17 (exon size= 171)
Exon 19 (exon size=41)		Exon 18 (exon size= 355)
Exon 20 (exon size=84)		Exon 19 (exon size= 156)
Exon 21 (exon size=55)		Exon 20 (exon size= 145)
Exon 22 (exon size=74)		Exon 21 (exon size= 122)
Exon 23 (exon size=61)		Exon 22 (exon size= 199)
Exon 24 (exon size=325)		Exon 23 (exon size= 164)
		Exon 24 (exon size= 139)
		Exon 25 (exon size= 305)
		Exon 26 (exon size= 147)
		Exon 27 (exon size= 609)

Figure 5.1 A

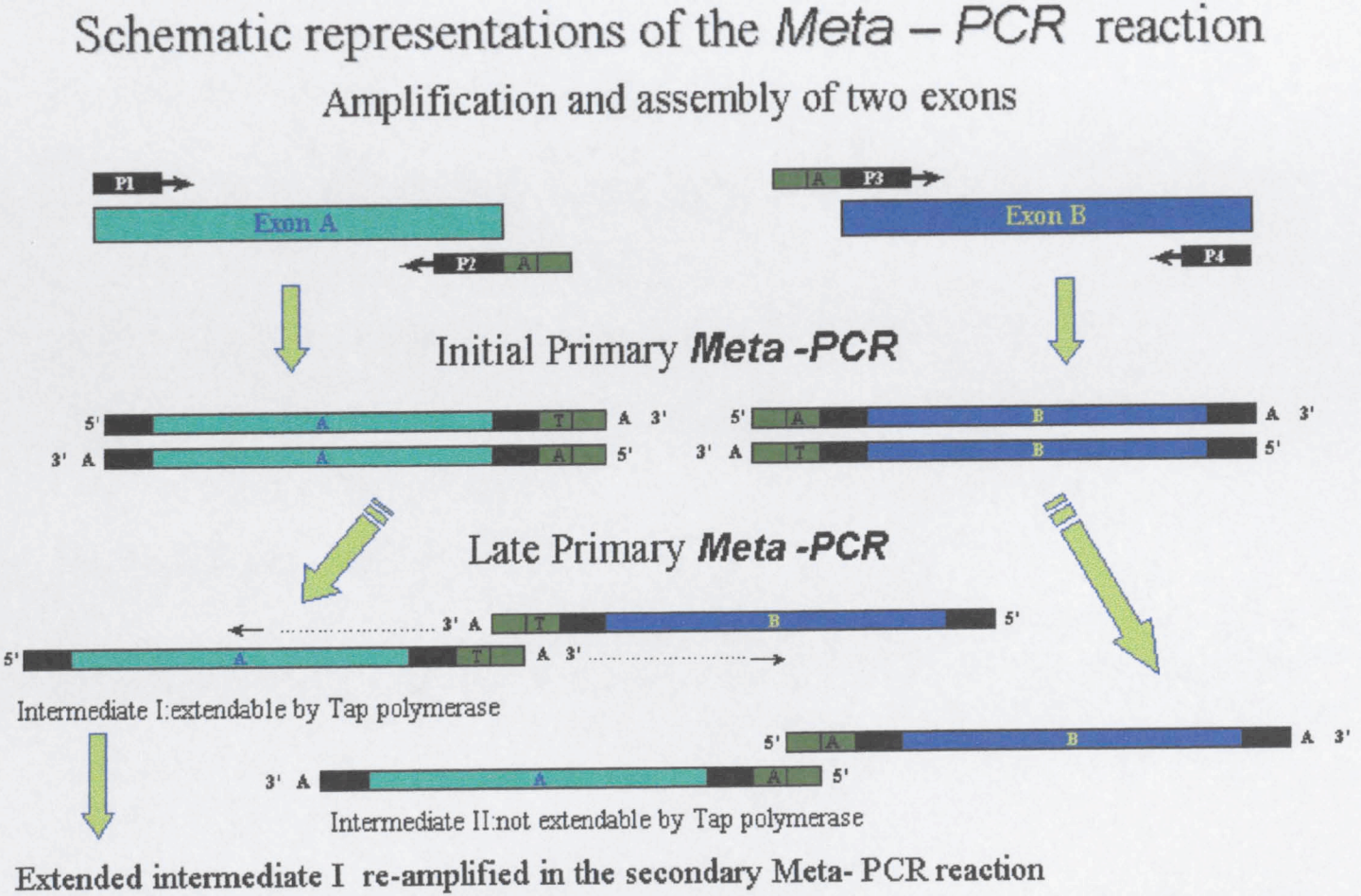
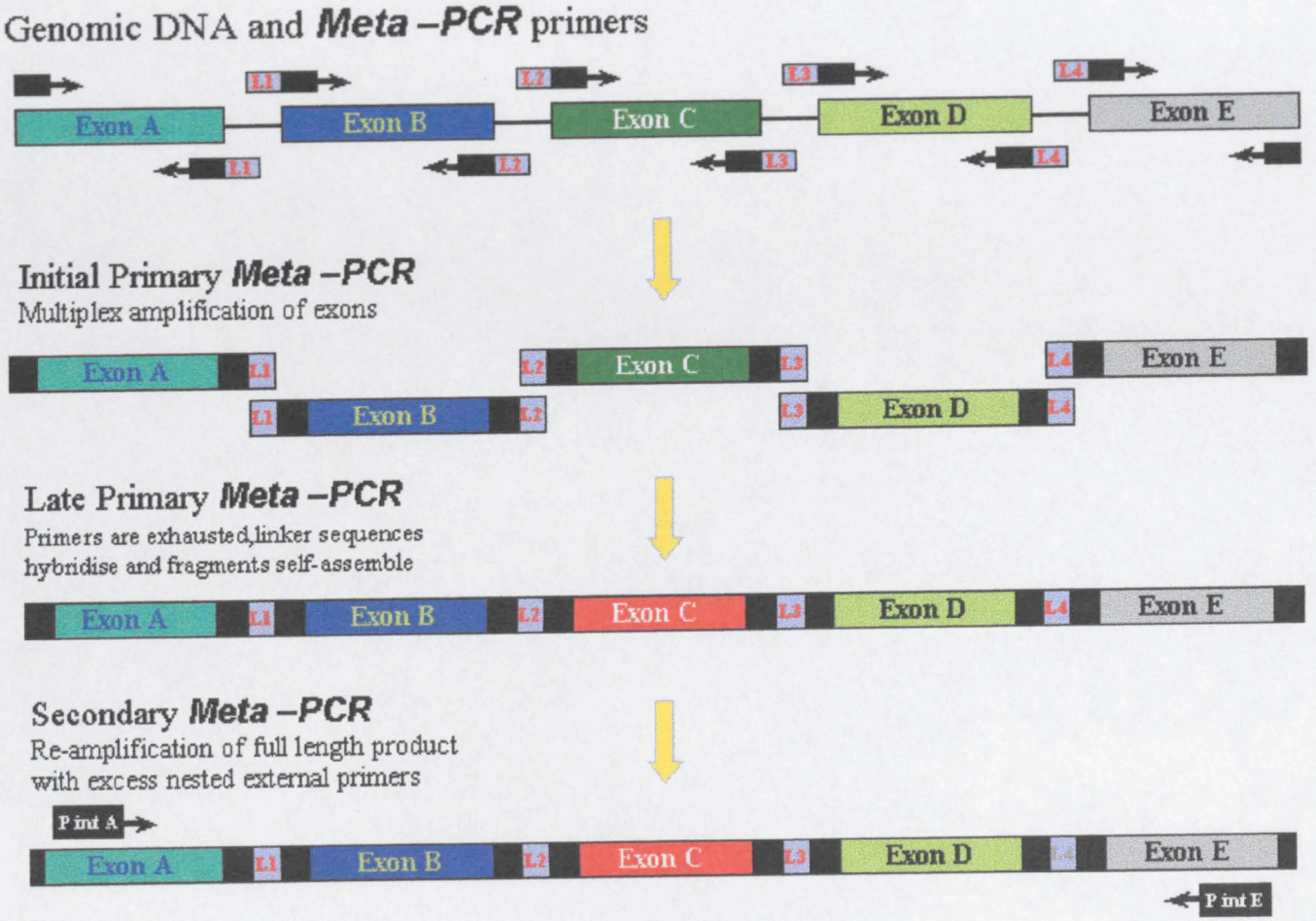

Schematic representations of the Meta – PCR reaction
Amplification and assembly of two exons
P1
Exon A
P2
A
P3
Exon B
P4
Initial Primary Meta -PCR
Late Primary Meta -PCR
Intermediate I:extendable by Tap polymerase
Intermediate II:not extendable by Tap polymerase
Extended intermediate I re-amplified in the secondary Meta- PCR reaction

Figure 5.1 B


Genomic DNA and Meta –PCR primers
L1
L2
L3
L4
Exon A
Exon B
Exon C
Exon D
Exon E
L1
L2
L3
L4
Initial Primary Meta –PCR
Multiplex amplification of exons
Exon A
L1
L2
Exon C
L3
L4
Exon E
L1
Exon B
L2
L3
Exon D
L4
Late Primary Meta –PCR
Primers are exhausted,linker sequences
hybridise and fragments self-assemble
Exon A
L1
Exon B
L2
Exon C
L3
Exon D
L4
Exon E
Secondary Meta –PCR
Re-amplification of full length product
with excess nested external primers
P int A
Exon A
L1
Exon B
L2
Exon C
L3
Exon D
L4
Exon E
P int E

5.3 Meta-PCR in practice

In order to evaluate the capability of the Meta-PCR technique for overcoming difficulties of analysis of complex genetic disorders, the combination of PTT or direct sequencing methods was approached as a downstream analysis. Different approaches have a significant influence on primer design and maximising the length of sequence scanned.

For PTT analysis, special care has to be taken to ensure that the primers designed maintain open reading frame, this leads to some inflexibility in primer designing.

For direct sequencing approach, many parts of the target genes (Intron and/or exon) can be selected, allow flexibility in the positioning of the primer, and make it relatively easy to determine the most suitable annealing temperatures (T_m) which is critical for the assembly of DNA fragments.

5.3.1 General considerations for primer design

The genomic specific segments were selected to have closely equivalent T_m values for the positioning of the primers. The complementary 5' termini, or linker segments of the Meta-PCR primers, were designed typically with approximately 50% GC content, avoiding any internal secondary structure, and to have T_m values that exceeded those of the genomic specific segments to favour self-assembly. A supplementary unmatched adenine residue was inserted between the genomic and 5' linker segments of the self-assembling primers to accommodate the 3' adenine overhangs added by Taq polymerase to the nascent DNA strand.

It should be noted that adding a supplementary unmatched adenine (A) residue at the sense sequence causes an insertion of a supplementary thymine (T) residue at the 3' end in the antisense DNA fragments assembled into PCR products. This must be accounted for where maintenance of an open reading frame is desired.

All primers were checked to avoid homology to Alu repeat sequences using the BLAST analysis program available at:
http://www.ncbi.nlm.gov/cgi-bin/BLAST/nph-blast

5.3.2 Advantages of Meta-PCR technique

1) The Lexon method has weakness relative to Meta-PCR technique; it does not accommodate an additional 3' adenine overhang by *Taq* polymerase in the primer design. Failure to do this leaves only a subset of overlap intermediate being extendable by *Taq* polymerase, since *Taq* lacks a 3' $\longrightarrow$ 5' exonuclease activity, and is thus incapable of resolving mismatched 3' terminal bases. This leads to insufficient overlap extension and results in a low yield of the spliced product. Meta-PCR intercalates the junction between the genomic-specific primer segments and linker-specific primer segments with an additional unmatched adenine residue. The sole purpose of the extra adenine is to facilitate extension of intermediates that form between fragments that have undergone the 3' adenine addition. This gives efficient overlap extension and high yield of the desired product [8].

2) The linker specific primer segments in Meta-PCR are designed with higher T_m values than the genomic specific segments, unlike those used in the Lexon method. This encourages the formation of overlap intermediates, further improving the yield and specificity of the final desired product [8].

3) Meta-PCR also improves upon the Lexon method. The Lexon method involve a primary amplification step where the component fragments are amplified in separate reaction vessels with non-limiting primer concentrations. This primary reaction is then combined multiple fragments in the secondary reactions to create the recombinant products. In contrast, the primary amplification step of Meta-PCR is a multiplex reaction with limiting concentrations of primers. Direct coupling of primary and

secondary PCR reactions give Meta-PCR considerable time and cost saving over the Lexon method.

In view of the nature of this experiment described in this chapter, I have included details of the methodology as they pertain Meta-PCR, in order to clarify the results and their interpretation.

5.3.3 Meta-PCR amplification

Genomic DNA was used as a template for amplification. Primers were synthesized on a 0.2μM scale and were purified by high-performance liquid chromatography (HPLC) at Oswel Ltd (Southampton-UK) for direct sequencing approach and on a 0.05μM scale which were provided as high-purity and salt free (HPSF) by MWG (Milton Keynes-UK) for PTT analysis. Meta-PCR amplification was performed in two stages. The primary reactions were performed in 20μl volumes using 100ng of genomic DNA, 1μl of each primer (40nM/L) (Table 5.2, 5.3 and 5.4), 2μl of mixture of dNTP (5mM), 2μl Buffer B x10 [20mM Tris-HCl (pH 8.0 at 25°C), 100mM KCl, 0.1mM EDTA, 1mM DTT, 50% glycerol, 0.5% Tween® 20 and 0.5% Nonidet®-P40], 3μl Mg25mM, 1μl *Taq* DNA polymerase (Promega, Southampton, UK; catalogue no. M1665) in accordance with manufacturer's recommendations, and distilled water was added to a final volume of 20μl.

For amplification, each sample was denatured at 94°C for 3 minute and followed by 30 cycles of PCR (at 94°C for 1minute, at 60°C-65°C for 1 minute and extending at 72°C for 2 minutes on an Applied Biosystems DNA thermal cycler, Applera Europe BV, Cheshire, UK) were followed by a final synthesis 72°C for 10 minutes.

The secondary Meta-PCR were performed in separate 20μl volumes using 3-5μl of the primary PCR product, 2μl of each internal (nested) primer (5μM/L), 2μl of mixture of dNTP (2.5mM), 2μl Buffer B x10 [20mM Tris-

HCl (pH 8.0 at 25°C), 100mM KCl, 0.1mM EDTA, 1mM DTT, 50% glycerol, 0.5% Tween® 20 and 0.5% Nonidet®-P40], 2μl Mg25mM, 1μl *Taq* DNA polymerase (Promega, Southampton, UK; catalogue no. M1665) in accordance with manufacturer's recommendations, and distilled water was added to a final volume of 20μl.

The thermal cycling conditions were identical to those used for the primary amplification. On completion of the secondary PCR, a 3μl portion of the PCR products were checked on a 0.7%-1.2% agarose gel to confirm the presence of the expected fragments sizes.

The above formula was applied for Meta-PCR amplification in both PTT and direct sequencing approaches.

5.4 Downstream analysis of Meta-PCR products by Protein Truncation Test

In this study Meta-PCR technique was applied to assemble BRCA2 exon 18 and BRCA1 exons 2 and 20 and was followed by downstream PTT analysis. The BRCA1 exons 2 and 20 are hot spot regions that are known to harbour particularly deleterious mutations. In order to avoid missing any mutation in these two exons, the above three exons were assembled in the following order of preferences: 18, 20 and 2. However, the order of fragments is reproducible and can be predetermined by primer design.

5.4.1 Primer design

For maintenance of the open reading frame demanded that the exon-to-exon connections constitute an integral number of codons, and that in-frame stop codons within the intron sequence be mutated to sense codons.

This scheme facilitates mini-gene construction in translation based mutation detection methods, such as PTT.

In order to maintain assembled fragments of the BRCA2 exon2, BRCA1 exon20 and 2 in an open reading frame format, three site directed mutation were introduced, as follow:

1) For the first fragment, the BRCA2 exon 18, the sequence of aat **taa** ttt gca ctc ttg gta aaa at was changed to the sequence of aat **tac** ttt gca ctc ttg gta aaa at

2) For the second fragment, the BRCA1 exon 20, the sequence of cc tca agt **tga** caa aaa tct ca was changed to the sequence of cc tca agt **tgt** caa aaa tct ca

3) For the third fragment, the BRCA1 exon 2, the sequence of tgg **taa** gtc agc aca aga gtg tat was changed to the sequence of tgg **tat** gtc agc aca aga gtg tat

These site direct mutations were introduced to the above sequences 1 to 3 relative to the relevant primers named Ex18R-L4F, Ex20R-L1F, Ex2R (int) respectively (See also table 5.2 and section 5.4.2).

Two linker specific primer segments (each 24bp) were used to produce a chimaeric DNA molecule for above three specific gene fragments (Table 5.2).

Note: Adding a supplementary unmatched adenine (A) residue at the sense sequence leads to insertion of a supplementary thymine (T) residue at the 3' end in the antisense sequence of the DNA fragments assembled in the PCR products. It is important to ensure that the PCR products are in frame for PTT analysis

Two nested primers including an internal forward primer (comprising a T_7 promoter and a perfect Kozak consensus sequence to facilitate coupled transcription-translation) and a conventional reverse primer were designed to produce sufficient product for PTT analysis and to facilitate priming a few residues upstream and downstream of the external primers (Table 5.2).

Table 5.2: Details of linker specific primer segments and genomic specific fragments used for amplification and assembling of BRCA1 exons 2 and 20 and BRCA2 exon18 by Meta-PCR

Name	Length	Sequence 5' → 3'	Genomic segment Tm /°C	Linker Tm /°C
Ex18F (ext)	27	tct cag tat ttc tta gat aaa ttc agt	58	--
Ex18R-L4F	51	atg ctc act gaa cct gcc tac ctt a at ttt tac caa gag tgc aaa gta att	58	63.7
Ex20F-L4R	47	aag gta ggc agg ttc agt gag cat a ga tgg gtt gtg ttt ggt ttc tt	59	63.7
Ex20R-L1F	47	agc cac tac cca aac tcc tgt cat a tg aga ttt ttg aca act tga gg	58	61.2
Ex2F-L1R	49	atg aca gga gtt tgg gta gtg gct a gt tca ttg gaa cag aaa gaa atg g	60	61.2
Ex2R-(ext)	23	gga atc cca aag taa tac act ct	59	--
Ex2R (int)	24	*ata cac tct tgt gct gac ata cca*	60	--
Ex18F-(T7 promoter + int)	24	gct aat acg act cac tat agg aac aga cca cca tg*a gag tca cac ttc cta aaa tat gc*	60	--

Gene of interest sequence= Blue colour Linker = Black colour

a = supplementary unmatched adenine located between 3' end of the linker specific primer segment and 5' end of the genomic specific fragment

Italic lower case = internal primer sequence (Black colour)

External (total) assembling DNA fragments size = 825bp

Internal amplification PCR product size= 782bp (747bp + 35bp T_7 promoter)

5.4.2 The complete assembled sequences of BRCA2 exon18 and BRCA1 exons 20 and 2 (Intron-exon boundaries)

(T_7 promoter flanking sequence)
gct aat acg act cac tat agg aac aga cca cca tg

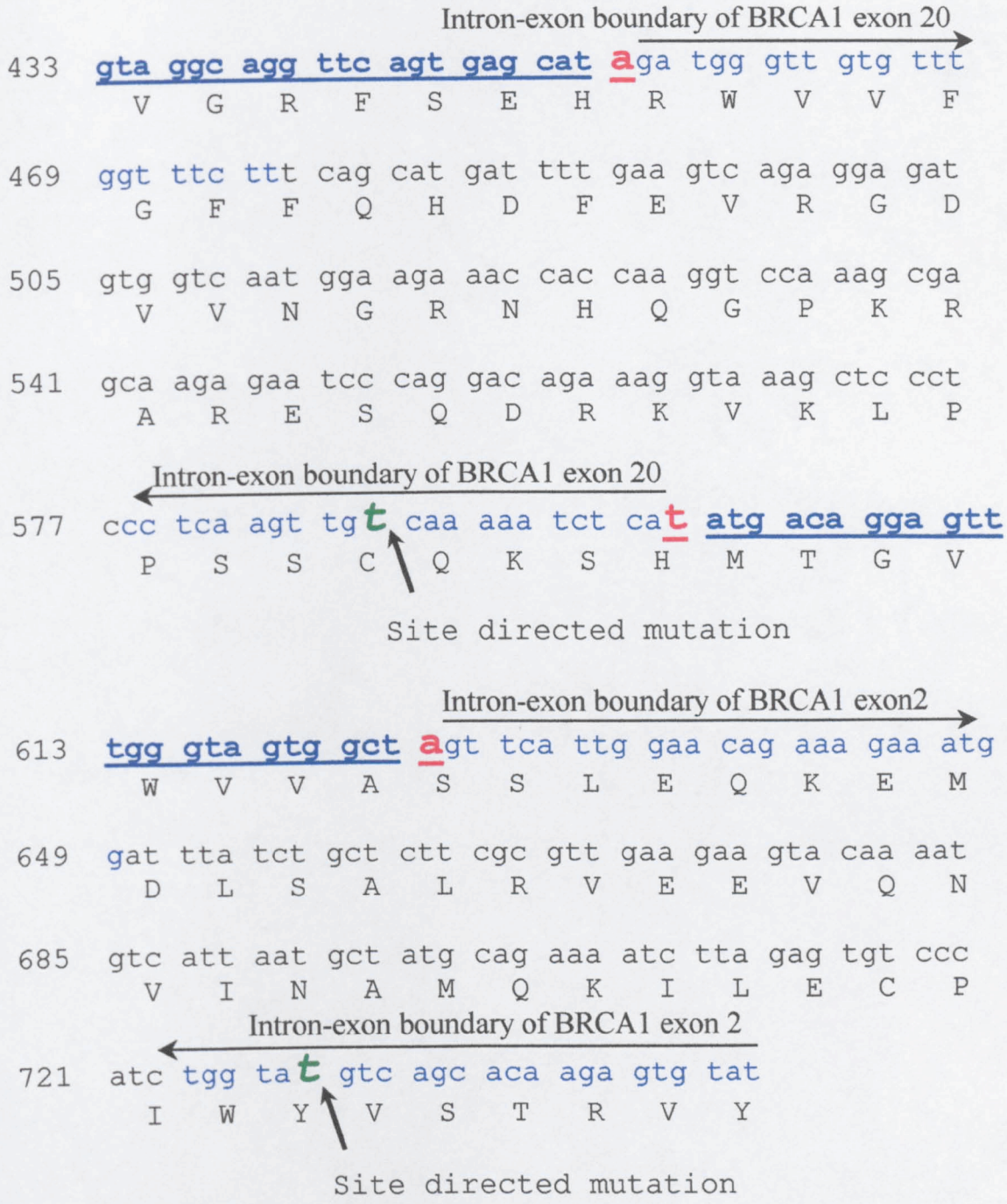
Intron-exon boundary of BRCA2 exon 18

```
  1  aga gtc aca ctt cct aaa ata tgc att ttt gtt ttc
       R   V   T   L   P   K   I   C   I   F   V   F

 37  act ttt aga tat gat acg gaa att gat aga agc aga
       T   F   R   Y   D   T   E   I   D   R   S   R

 73  aga tcg gct ata aaa aag ata atg gaa agg gat gac
       R   S   A   I   K   K   I   M   E   R   D   D

109  aca gct gca aaa aca ctt gtt ctc tgt gtt tct gac
       T   A   A   K   T   L   V   L   C   V   S   D

145  ata att tca ttg agc gca aat ata tct gaa act tct
       I   I   S   L   S   A   N   I   S   E   T   S

181  agc aat aaa act agt agt gca gat acc caa aaa gtg
       S   N   K   T   S   S   A   D   T   Q   K   V

217  gcc att att gaa ctt aca gat ggg tgg tat gct gtt
       A   I   I   E   L   T   D   G   W   Y   A   V

253  aag gcc cag tta gat cct ccc ctc tta gct gtc tta
       K   A   Q   L   D   P   P   L   L   A   V   L

289  aag aat ggc aga ctg aca gtt ggt cag aag att att
       K   N   G   R   L   T   V   G   Q   K   I   I

325  ctt cat gga gca gaa ctg gtg ggc tct cct gat gcc
       L   H   G   A   E   L   V   G   S   P   D   A

361  tgt aca cct ctt gaa gcc cca gaa tct ctt atg tta
       C   T   P   L   E   A   P   E   S   L   M   L
```

Intron-exon boundary of BRCA2 exon 18

```
397  aag gta aat tac ttt gca ctc ttg gta aaa att aag
       K   V   N   Y   F   A   L   L   V   K   I   K
```

Site directed mutation



*The black/blue colour lower letter case= Intron-exon boundaries 18,20 and 2

The bold italic green colour lower letter case = site direct mutagenesis

***The blue colour lower letter case = Sequence of gene of interest for position of primer a primer (Forward or Reverse)**

***The bold red colour underlined a or t=A supplementary unmatched adenine or thiamine residues was inserted between the genomic and 5'linker segments**

***The bold blue colour underlined lower letter case = Linkers sequences**

Upper case = One letter amino acid code

In order to maintain assembled fragment in an open reading frame format, three site directed mutation were introduced, as follow:

In BRCA2 exon 18 **taa** (stop codon) is changed to **tac** (tyrosine) at nucleotide 408*

In BRCA1 exon 20 **tga** (stop codon) is changed to **tgt** (cysteine) at nucleotide 588*

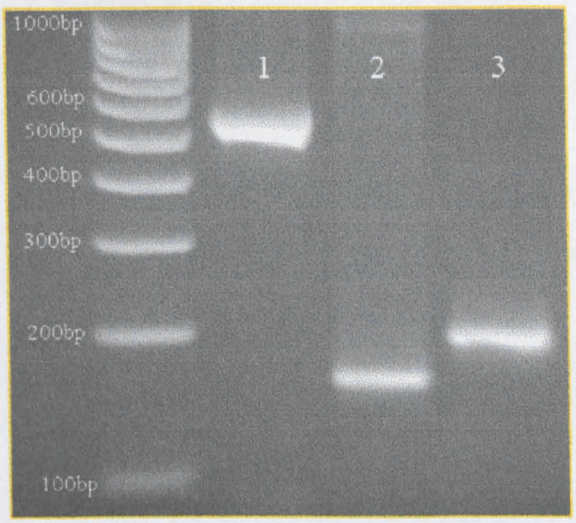
In BRCA1 exon 2 **taa** (stop codon) is changed to **tat** (tyrosine) at nucleotide 729*

*Nucleotide number is presented at the position within assembled fragment.

5.4.3 Results

PCR amplification of each exon under common amplification conditions gave a single fragment of the expected size, confirming the integrity of the primer syntheses and the compatibility of the primer pairs (Fig. 5.2).

Figure 5.2 : PCR products from BRCA2 exon 18 and BRCA1 exons 2 and 20 individually



PCR products were analysed on 1% agarose gel.

Lane 1, PCR products of BRCA2 exon18 =525bp
Lane 2, PCR products of BRCA1 exon2 =161bp
Lane 3, PCR products of BRCA1 exon20 =195bp

The size of each DNA specific fragment when amplified individually is larger than when assembled, because the PCR products of each specific DNA fragment includes a "hanging" linker specific segment either at the tails or at one side. (See sections 5.4.2 and 5.4.3.1)

5.4.3.1 The sequences and sizes of each DNA specific fragment when amplified individually

Blue lower case = Primer pairs
Bold underlined lower case= Linker specific segment

a) BRCA2 Exon 18 PCR product size for check gel = 525bp

tct cag tat ttc tta gat aaa ttc agt ttt tat tct cag tta ttc agt gac ttg ttt aaa cag tgg aat tct aga gtc aca ctt cct aaa ata tgc att ttt gtt ttc act ttt agA TAT GAT ACGGAAATTGATAGAAGCAGAAGATCGGCTATAAAAAAGATAA TGGAAAGGGATGACACAGCTGCAAAAACACTTGTTCTCTGTGTT TCTGACATAATTTCATTGAGCGCAAATATATCTGAAACTTCTAGC AATAAAACTAGTAGTGCAGATACCCAAAAAGTGGCCATTATTGA ACTTACAGATGGGTGGTATGCTGTTAAGGCCCAGTTAGATCCTC CCCTCTTAGCTGTCTTAAAGAATGGCAGACTGACAGTTGGTCAG AAGATTATTCTTCATGGAGCAGAACTGGTGGGCTCTCCT GAT GCC TGT ACA CCT CTT GAA GCC CCA GAA TCT CTT ATG TTA AAG gta aat **taa** ttt gca ctc ttg gta aaa at **atgctcactgaacctgcctaccttt a**

BRCA1 Exon 20 PCR product size for check gel = 195bp

aag gta ggc agg ttc agt gag cat a **ga** tgg gtt gtg ttt ggt ttc ttt cag CAT GAT TTT GAA GTC AGA GGA GAT GTG GTC AAT GGA AGA AAC CAC CAA GGT CCA AAG CGA GCA AGA GAA TCC CAG GAC AGA AAG gta aag ctc cct ccc tca agt **tga** caa aaa tct ca **agc cac tac cca aac tcc tgt cat a**

BRCA1 Exon 2 PCR product size for check gel = 161bp

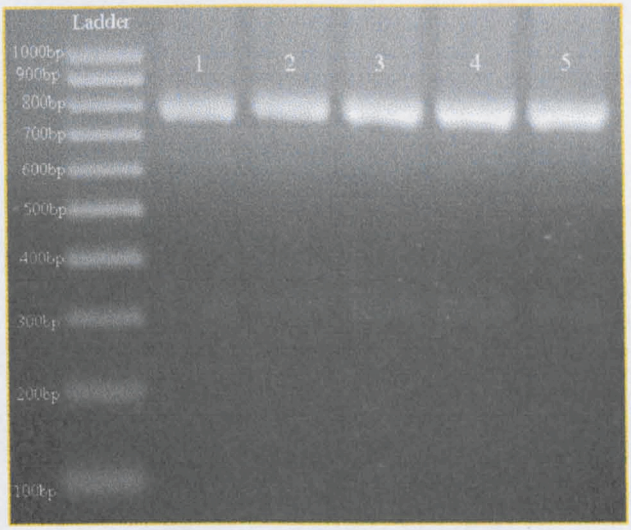
atg aca gga gtt tgg gta gtg gct a

gT TCA TTG GAA CAG AAA GAA ATG GAT TTA TCT GCT CTT CGC GTT GAA GAA GTA CAA AAT GTC ATT AAT GCT ATG CAG AAA ATC TTA GAG TGT CCC ATC TGg **taa** gtc agc aca **aga gtg tat taa ttt ggg att cc**

5.4.3.2 The secondary Meta-PCR products

The products of secondary Meta-PCR assays consistently yielded fragments of the expected size when was performed on a range of genomic DNA samples. The product yields were generally high with low backgrounds of non-specific products. For downstream analysis by PTT and direct sequencing the secondary Meta-PCR products were analysed on a 1.2% agarose gel confirming the integrity of PCR products (Fig. 5.3).

Figure 5.3: The secondary Meta-PCR products assembled of the BRCA2 exon 18 and the BRCA1 exons 20 and 2



Lanes 1-5 show the assembled fragments analysed on 1.2% agarose gel.
PCR products sizes = 782bp (Includes T_7 promoter sequence size-35bp)

The size of assembled fragments is smaller than when those amplified individually (782bp versus 881bp respectively), because the PCR products of each specific DNA fragment includes hanging linker specific segment either at the both tail or at the one side.

5.4.3.3 Recovery of secondary Meta-PCR products following agarose gel electrophoresis

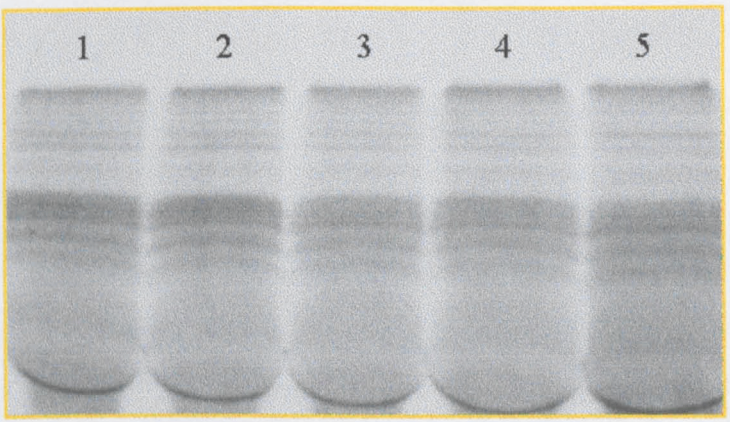
The secondary Meta-PCR products were recovered from agarose gel slices using a Jetsorb™ gel extraction kit (Genomed Inc.) according to the manufacturer's instruction. A total of 150ng of recovered Meta-PCR products was used as a template for downstream PTT analysis.

5.4.3.4 PTT analysis

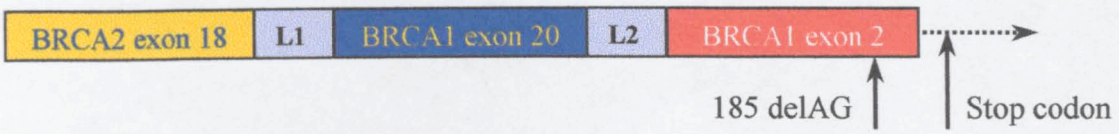
The secondary Meta-PCR products from two previously ascertained heterozygotes for BRCA1 mutations in exon 2 (185del AG and 181ins T- see chapter 3 for more detail) were used as a template for PTT following conventional procedures. The proteins were revealed by SDS-PAGE (10% acrylamide), treated in solution 3% glycerol, 20% methanol for an hour, dry down for one hour at 80°C by auto dryer and visualized by autoradiography for 24 hours.

Templates: Lane 1, 2 and 5 are wild type translated protein; lane 3 (185delAG) and lane 4 (181-182 insT) are mutant samples (See chapter 3) but lead to no truncated proteins in this experiment (Figure 5.4).

Figure 5.4: SDS-PAGE image of assembled the BRCA1 and BRCA2 genes



This result was obtained because of the ordering of fragments during assembly (See picture below).



As can be seen in the above schematic picture, exon 2 was assembled as the latest fragment in the concatamer DNA molecule. In an unassembled BRCA1 exon2, 185delAG leads to TGA at 51 nucleotides downstream from position where the mutation occurs. However, within the assembled DNA molecule, the mutant sample (185delAG in exon2) does not produce a truncating mutation (frameshift or stop codon) because there are only 36 nucleotides downstream to that position. Another mutant sample (181-182 insT within exon 2) showed almost the same pattern, and resulted in no truncated proteins in both samples in Figure 5.4 (See chapter 3 section 3.4, Figures 3.5 and 3.6 for more detail).

Therefore, there were no mutant samples with truncating mutations within exon 18 or exon 20 examined in this experiment.

However, these experiments showed that the correct size protein products had been generated at high fidelity from the Meta-PCR products via in-vitro coupled transcription-translation.

5.5 Downstream analysis of Meta-PCR products by direct sequencing

In this approach the strategy was modified by increasing the number of segments to be linked, which are the BRCA1 exons 2, 20, 23 and 24 and using optimised linker specific primer segments (designed shorter in size from 24bp to 13bp).

Once again due to the significance of exons 2 and 20 in BRCA1 protein function and in order to avoid missing any mutation in these two exons, the above four exons were assembled in the following order of preferences: 23 20, 2 and 24.

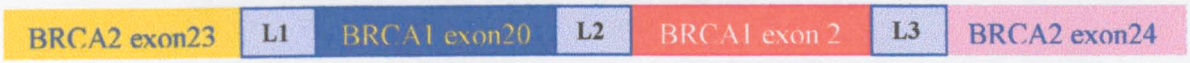
5.5.1 Primer design

Three linker fragments (each 13bp) were used to produce a chimaeric DNA molecule from the above four specific gene fragments (Table 5.3).

These linkers are much shorter (~50%) and richer in GC content than those used for the PTT approach. This new design significantly promotes the formation of overlap intermediates, further improving the yield and specificity of the final desired product (See section 5.5.2).

Two nested primers were designed to produce sufficient product for direct sequencing analysis and prime few residues upstream and downstream of external primers (Table 5.3).

Table 5.3: Details of linkers specific primer segments and genomic specific fragments used for amplification and assembling of BRCA1 exons 23, 20, 2 and 24 by Meta-PCR



Name	Length	Sequence 5 '→ 3'	Genomic segment Tm /°C	Linker Tm/°C
Ex 23F (ext.)	25	atg atg aag tga cag ttc cag tag t	59.9	---
Ex 23R-L1F	35	**ctg cgc ggc ggc tA** caa aag gac ccc ata tag cac	59.8	65
Ex 20F-L1R	35	**agc cgc cgc gca gA** gtg tct gct cca ctt cca ttg	59.3	65
Ex20R-L2F	38	**tcg ggc ccg cgg aA** cct gtg tga aag tat cta gca ctg	59.7	64
Ex2F-L2R	38	**tcc gcg ggc ccg aA** tgt gtt aaa gtt cat tgg aac aga	60.3	64
Ex 2R-L3F	37	**agg ccg ggc cgc tA** ccc aaa tta ata cac tct tgt gc	59.6	65
Ex 24F-L3R	36	**agc ggc ccg gcc tA** cga ttg att aga gcc tag tcc a	59.9	65
Ex 24R (ext.)	23	agt agc cag gac agt aga agg ac	59.7	---
Ex 23F (Int.)	24	TCC TAC TTT GAC ACT TTG AAT GCT	61.1	---
Ex 24R (Int.)	21	GTC CTG TGG CTC TGT ACC TGT	59.8	---

Linker = Blue colour + A
Gene of interest sequence= Black colour
Internal primer sequence= BLACK UPEER CASE
Internal amplification PCR product size: 850bp
A = supplementary unmatched adenine located between 3' end of the linker specific primer segment and 5' end of the genomic specific fragment

5.5.2 The complete assembled sequences of BRCA1 intron-exon boundaries 23, 20, 2 and 24

Intron-exon boundary of BRCA1 exon 23

```
  1  tcctactttg acactttgaa tgctctttcc ttcctgggga
 41  tccagggtgt ccacccaatt gtggttgtgc agccagatgc
 81  ctggacagag gacaatggct tccatggtaa ggtgcctgca
```

Intron-exon boundary of BRCA1 exon 23 — Linker 1

```
121  tgtacctgtg ctatatgggg tccttttgtagccgccgcgca
```

Intron-exon boundary of BRCA1 exon 20

```
161  gagtgtctg ctccacttcc attgaaggaa gcttctcttt
201  ctcttatcct gatgggttgt gtttggtttc tttcagcatg
241  attttgaagt cagaggagat gtggtcaatg gaagaaacca
281  ccaaggtcca aagcgagcaa gagaatccca ggacagaaag
321  gtaaagctcc ctccctcaag ttgacaaaaa tctcacccca
361  ccactctgta ttccactccc ctttgcagag atgggccgct
401  tcattttgta agacttatta catacataca cagtgctaga
```

Intron-exon boundary of BRCA1 exon20 — Linker 2 — Intron-exon oundary boundary of BRCA1-exon2

```
441  tactttcaca caggttccgc gggcccgaat gtgttaaagt
481  tcattggaac agaaagaaat ggatttatct gctcttcgcg
521  ttgaagaagt acaaaatgtc attaatgcta tgcagaaaat
```

Intron-exon boundary of BRCA1 exon2

```
561  cttagagtgt cccatctggt aagtcagcac aagagtgtat
```

Linker 3 — Intron-exon boundary of BRCA1 exon24

```
601  taatttgggt agcggcccgg cctacgattg attagagcct
641  agtccaggag aatgaattga cactaatctc tgcttgtgtt
```

681 ctctgtctcc agcaattggg cagatgtgtg aggcacctgt

721 ggtgacccga gagtgggtgt tggacagtgt agcactctac

761 cagtgccagg agctggacac ctacctgata ccccagatcc

Intron-exon boundary of BRCA1 exon24

801 cccacagcca ctactgactg cagccagcca caggtacaga

841 gccacaggac

*The black/blue colour lower letter case= Intron-exon boundaries 23, 20, 2 and 24

***The blue colour lower letter case = Sequence of gene of interest for position of primer a primer (Forward or Reverse)**

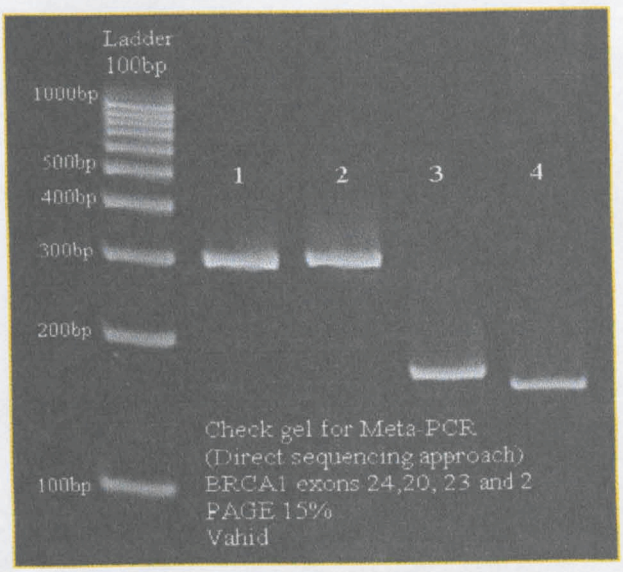
***The bold blue colour underlined lower letter case= Linkers sequences**

***The bold red colour underlined a or t=A supplementary unmatched adenine or thiamine residues was inserted between the genomic and 5′ linker segments**

5.5.3 Results

PCR amplification of each exon under common amplification conditions shows no primer, confirming the quality and the quantity of PCR products as expected (Fig. 5.5).

Figure 5.5: PCR products of BRCA1 exon 23, 20, 2 and 24 individually



The size of each DNA specific fragment when amplified individually is larger than when those assembled, because the PCR products of each specific DNA fragment is included hanging linker specific segment either at the both tail or at the one side. (See the sections 5.5.2 and 5.5.3.1)

PCR products were monitored on 15% PAGE.

Lane 1, PCR products of BRCA1 exon24 =322 bp
Lane 2, PCR products of BRCA1 exon20 =320 bp
Lane 3, PCR products of BRCA1 exon23 =187 bp
Lane 4, PCR products of BRCA1 exon2 =169 bp

5.5.3.1 The sequences and sizes of each DNA specific fragment when amplified individually

Bold blue cases = Primer pairs
Bold underlined red lower case= Linker specific segment

BRCA1 exon 23 PCR product size for check gel = 187bp

atgatgaagtgacagttccagtagtcctactttgacactttgaatgctctttccttcctggggatccag
GGTGTCCACCCAATTGTGGTTGTGCAGCCAGATGCCTGGACAGA
GGACAATGGCTTCCATG gtaaggtgcctgcatgtacct**gtgctatatggg gtc ctt ttg**
tagc cgc cgc gca ga

BRCA1 exon 20 PCR product size for check gel = 320bp

agc cgc cgc gca ga
gtg tct gct cca ctt cca ttg aaggaagcttctctttctcttatcctgatgggttgtgtttggtttctttcag
CATGATTTTGAAGTCAGAGGAGATGTGGTCAATGGAAGAAACC
ACCAAGGTCCAAAGCGAGCAAGAGAATCCCAGGACAGAAAG
gtaaagctccctccctcaagttgacaaaaatctcaccccaccactctgtattccactccccttgcagagat
gggccgcttcattttgtaagacttattacatacataca**cag tgc tag ata ctt tca cac agg**
ttcc gcg ggc ccg aa

BRCA1 exon 2 PCR product size for check gel = 169bp

tcc gcg ggc ccg aa
tgtgttaaagTTCATTGGAACAGAAAGAAATGGATTTATCTGCTCTT
CGCGTTGAAGAAGTACAAAATGTCATTAATGCTATGCAGAAAAT
CTTAGAGTGTCCCATCTGgtaagt ca**g cac aag agt gta tta att tgg g**
tagc ggc ccg gcc ta

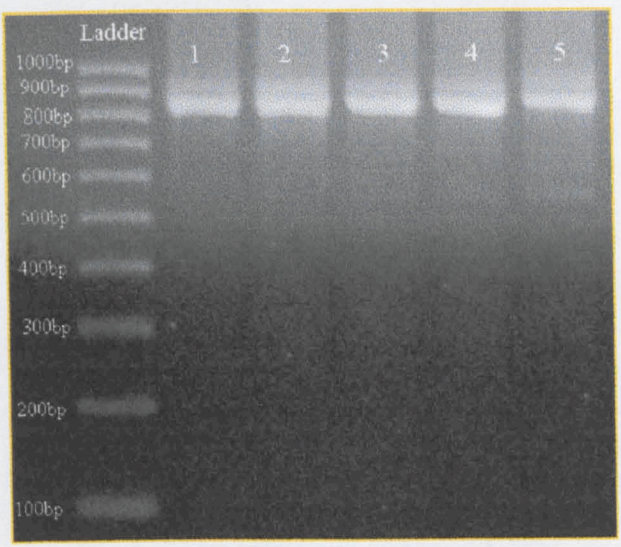
BRCA1 exon 24 PCR product size for check gel = 322bp

agc ggc ccg gcc ta
cga ttg att aga gcc tag tcc aggagaatgaattgacactaatctctgcttgtgttctctgtctccag
CAATTGGGCAGATGTGTGAGGCACCTGTGGTGACCCGAGAGTG
GGTGTTGGACAGTGTAGCACTCTACCAGTGCCAGGAGCTGGAC
ACCTACCTGATACCCCAGATCCCCCACAGCCACTACTGACTGC
AGCCAGCCAC AGG TAC AGA GCC ACA GGA CCCCAAGAATG
AGCTTACAAAGTGGCCTTTCCAGGCCCTGGGAGCTCCTCTCACT
CTTCA**GT CCT TCT ACT GTC CTG GCT ACT**

5.5.3.2 The secondary Meta-PCR products

The products of secondary Meta-PCR assays consistently yielded fragments of the expected size when was performed on a range of genomic DNA samples. The reaction yields were generally high with low backgrounds. For downstream analysis by direct sequencing the secondary Meta-PCR products were monitored on a 1.2% agarose gel confirming the integrity of the PCR products (Fig. 5.6).

Figure 5.6: The secondary Meta-PCR products assembled from BRCA1 exons 23, 20, 2 and 24



Lanes 1-5 show the assembled fragments analysed on 1.2% agarose gel. Meta-PCR products (assembled) sizes = 850bp

The size of assembled fragments is smaller than when those amplified individually (850bp versus 998bp respectively), because the PCR products of each specific DNA fragment includes hanging linker specific segment either at the both tail or at the one side.

5.5.3.3 Recovery of secondary Meta-PCR products following agarose gel electrophoresis

The secondary Meta-PCR products were recovered from agarose gel slices using a Jetsorb™ gel extraction kit (Genomed Inc.) according to the manufacturer's instruction. A total of 60-80ng of recovered Meta-PCR products was sequenced in both orientation using internal nested primers with BigDye terminator cycle sequencing kits (Applied Biosystems). The manufacturer's protocols were followed with the exception that the annealing temperature for the cycle sequencing reaction was increased from 50°C to 55°C to reflect the high T_m of internal primers.

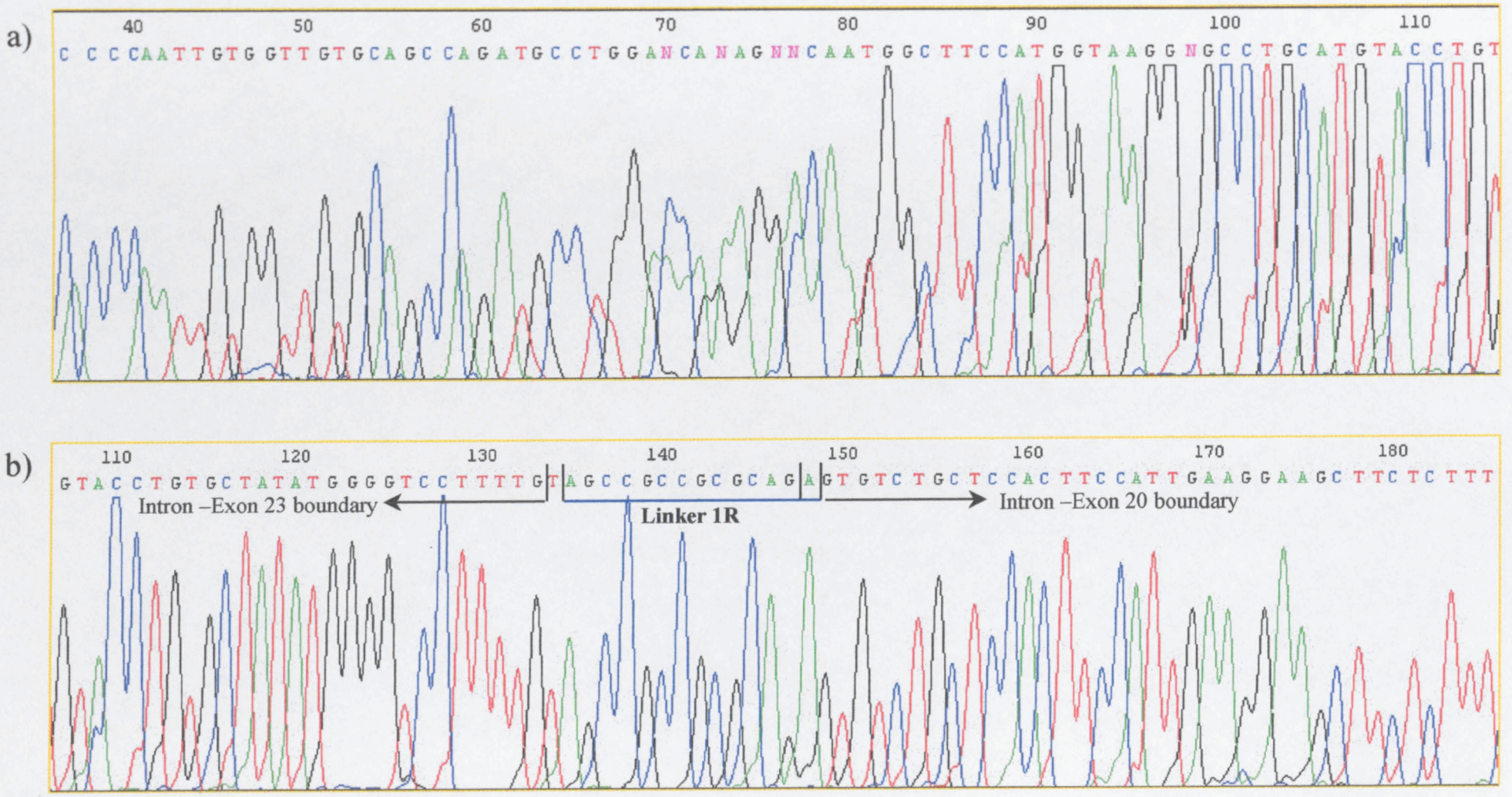
5.5.3.4 Determination of the nucleotide sequence of wild type Meta-PCR products

Direct sequencing confirmed that Meta-PCR products comprised the expected exons in the correct orientation.

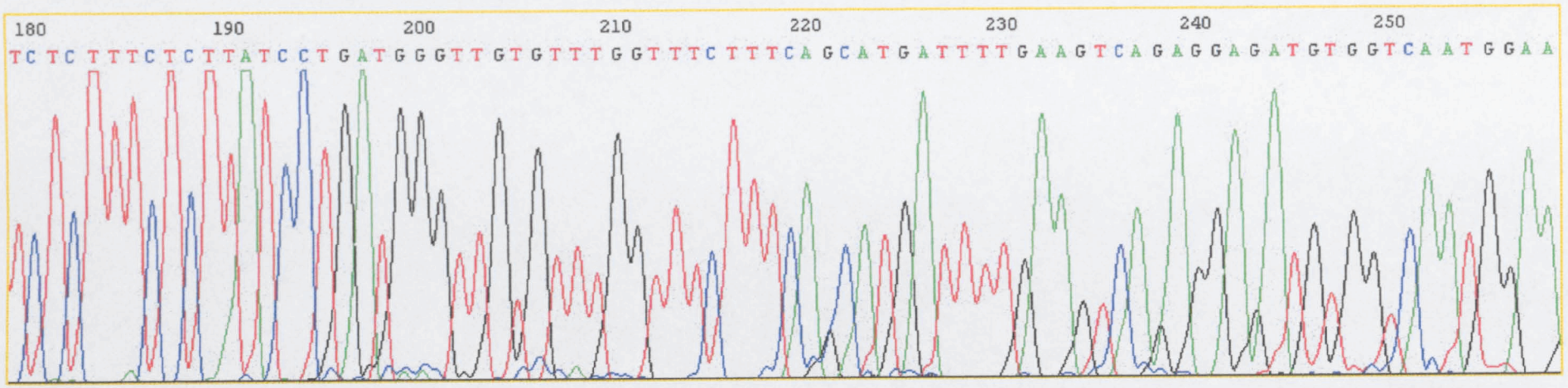
Figures 5.7a-i and 5.8a-j depict the full length of wild type Meta-PCR products sequenced from both termini in single reaction. No degradation of sequence was observed across the transitions from one component to the next.

Figures 5.9. - 5.11 show the nucleotide sequences (Forward and reverse direction) from wild type Meta-PCR products using several samples to confirm reproducibility of the Meta-PCR reaction by direct sequencing analysis and to show linkers junction areas more clearly.

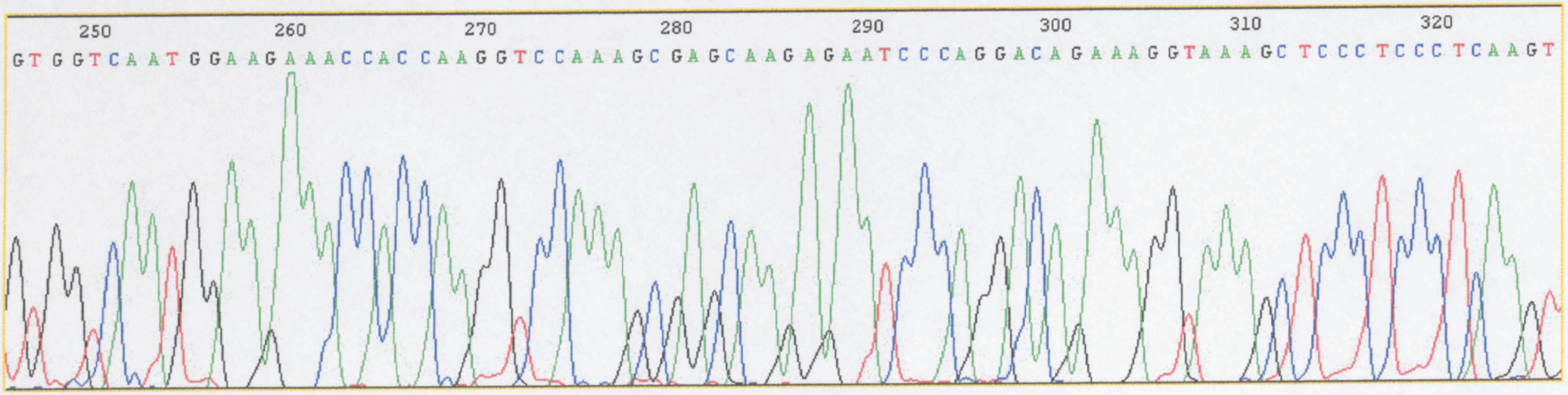
Figure 5.7: Electropherograms from analysis of full-length forward sequences of wild type Meta-PCR products (DS approach, size= 850bp)



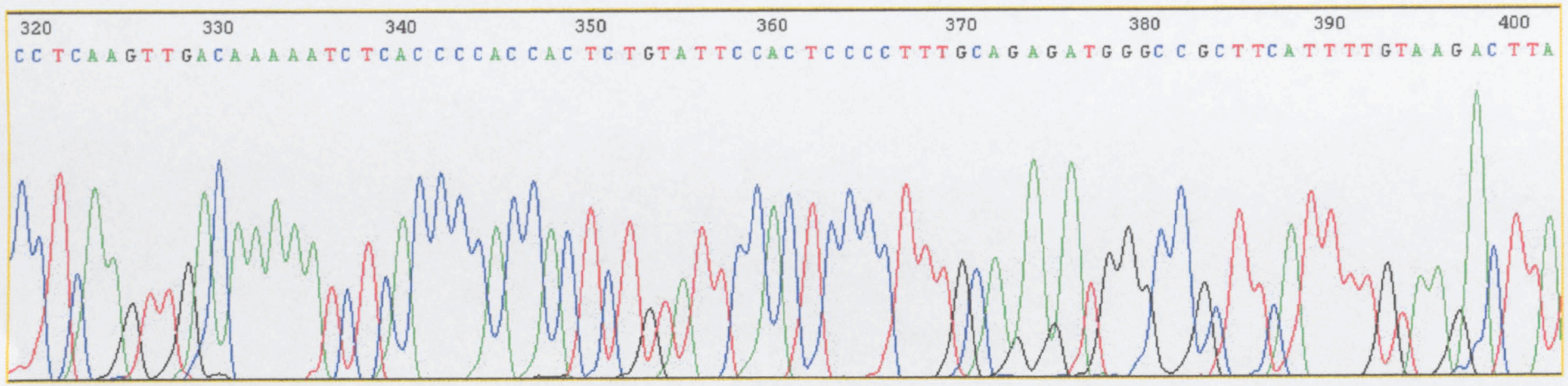
c)


180
190
200
210
220
230
240
250
TC TC TTTCTCTTATCCTGATGGGTTGTGTTTGGTTTCTTTCAGCATGATTTTGAAGTCAGAGGAGATGTGGTCAATGGAA

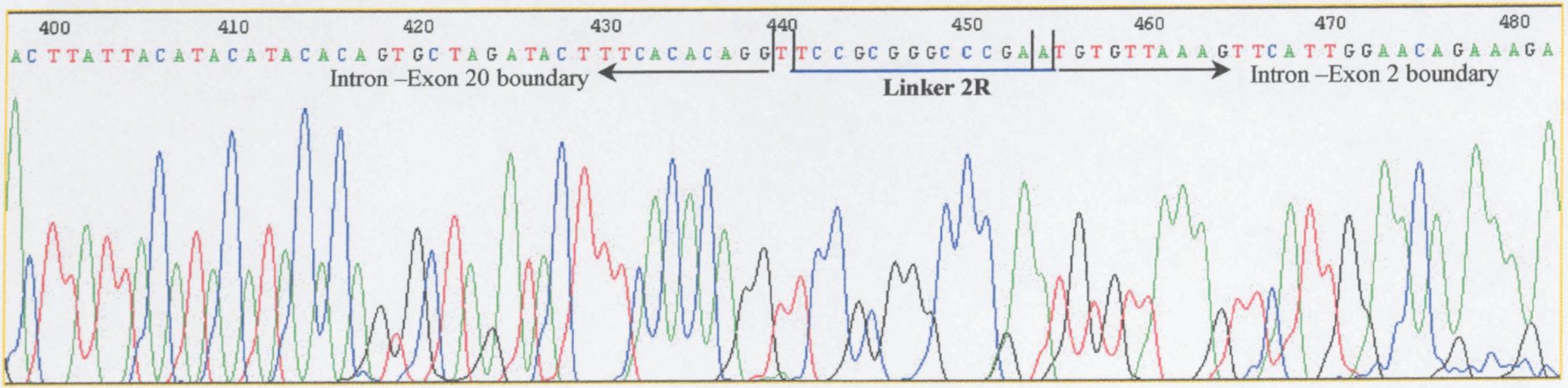
d)


250
260
270
280
290
300
310
320
GTGGTCAATGGAAGAAACCACCAAGGTCCAAAGCGAGCAAGAGAATCCCAGGACAGAAAGGTAAAGCTCCCTCCCTCAAGT

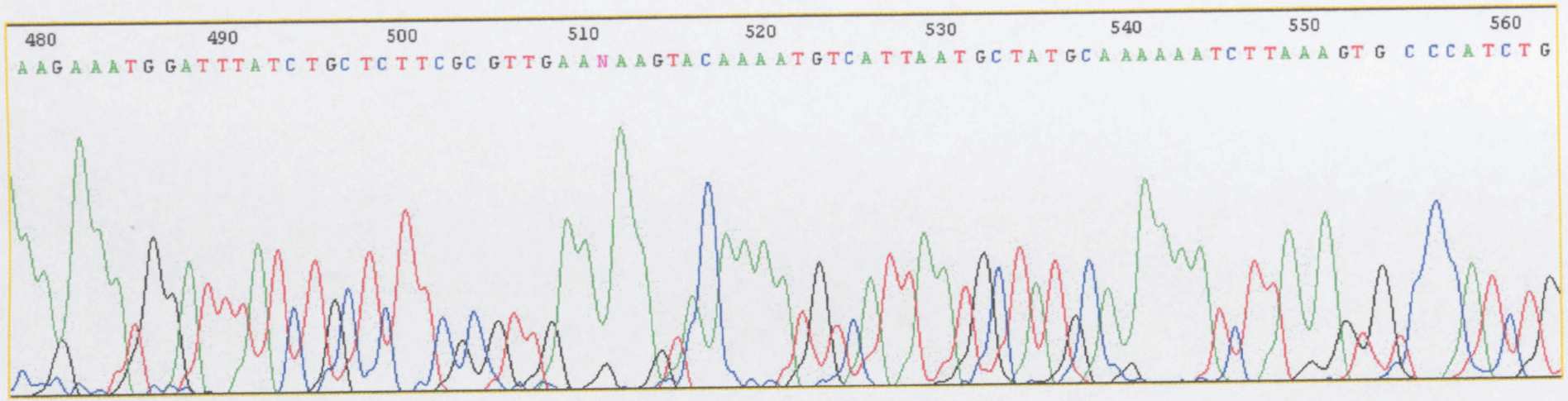
e)


320 330 340 350 360 370 380 390 400
CC TCAAGTTGACAAAAATC TCAC CCCAC CAC TC TGTATTC CAC TC CCC TTTGCAGAGATGGGC C GCTTCATTTTGTAAGAC TTA

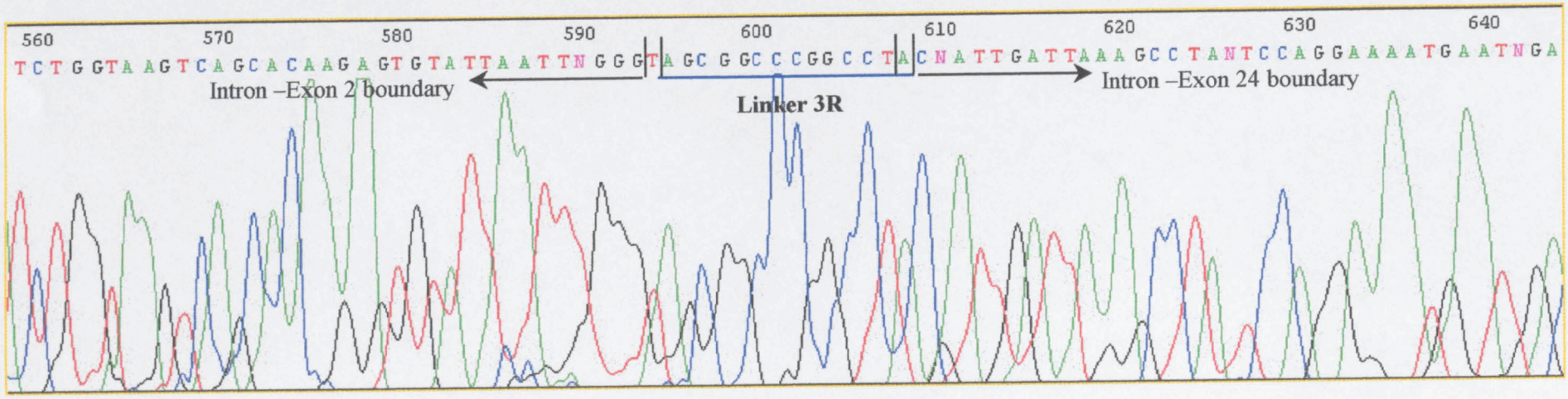
f)


400 410 420 430 440 450 460 470 480
AC TTATTACATACATACACA GT GC TA GATAC T TTCACACAG GT TC C GC G GGC C C GA AT GT GTTAAA GT TCA TTGGAACA GAAAGA
Intron –Exon 20 boundary
Linker 2R
Intron –Exon 2 boundary

g)


480 490 500 510 520 530 540 550 560
AAGAAATG GATTTATC TGC TCTTCGC GTTGAANAAGTACAAAATGTCATTAATGC TATGCAAAAAATCTTAAAGT G C CCATCTG

h)


560 570 580 590 600 610 620 630 640
TCT G GTA AG TCA GCA CAAG A GT G TA TTA A TT TN G G GTA GC G GC C CGG C C TA C NA TT GA TTAAA GC C TANTC CA G GA AAAA TG A A TNGA
Intron –Exon 2 boundary
Linker 3R
Intron –Exon 24 boundary

i)

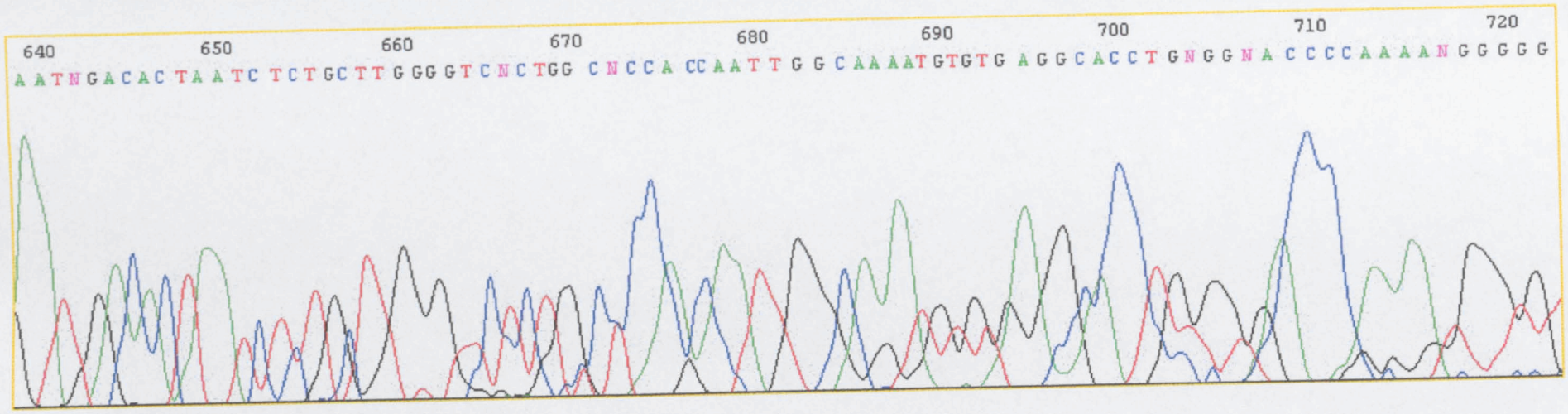
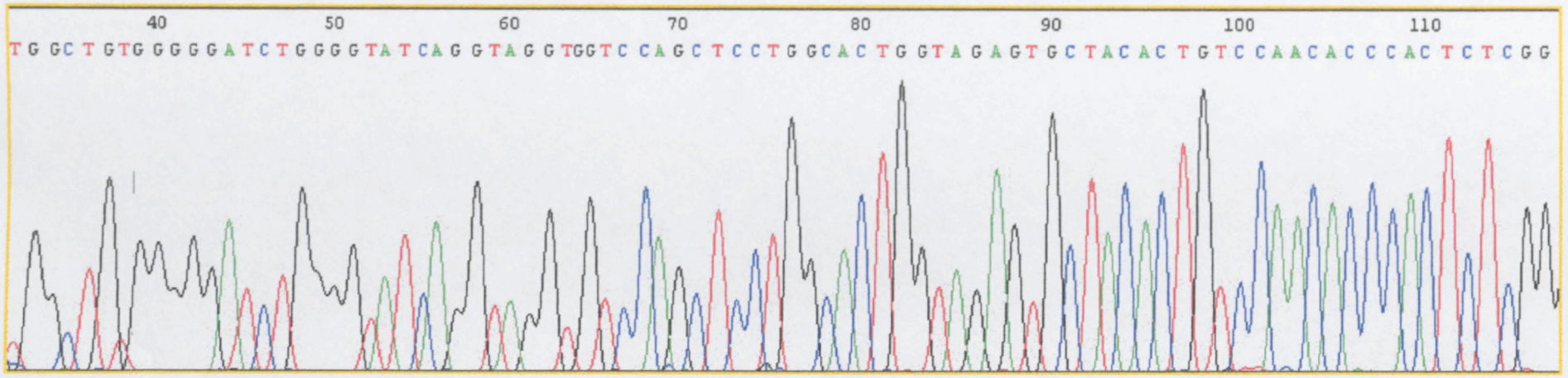
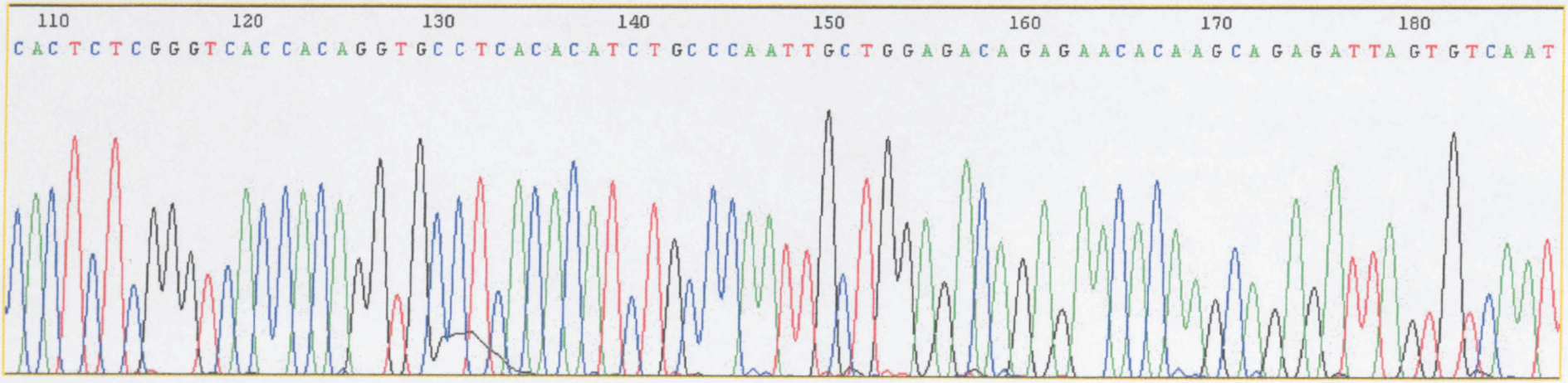

640
650
660
670
680
690
700
710
720
A A T N G A C A C T A A T C T C T G C T T G G G G T C N C T G G C N C C A C C A A T T G G C A A A A T G T G T G A G G C A C C T G N G G N A C C C C A A A A N G G G G G

Figure 5.8: Electropherograms from analysis of full length reverse sequences of wild type Meta-PCR products (DS approach, size= 850bp)

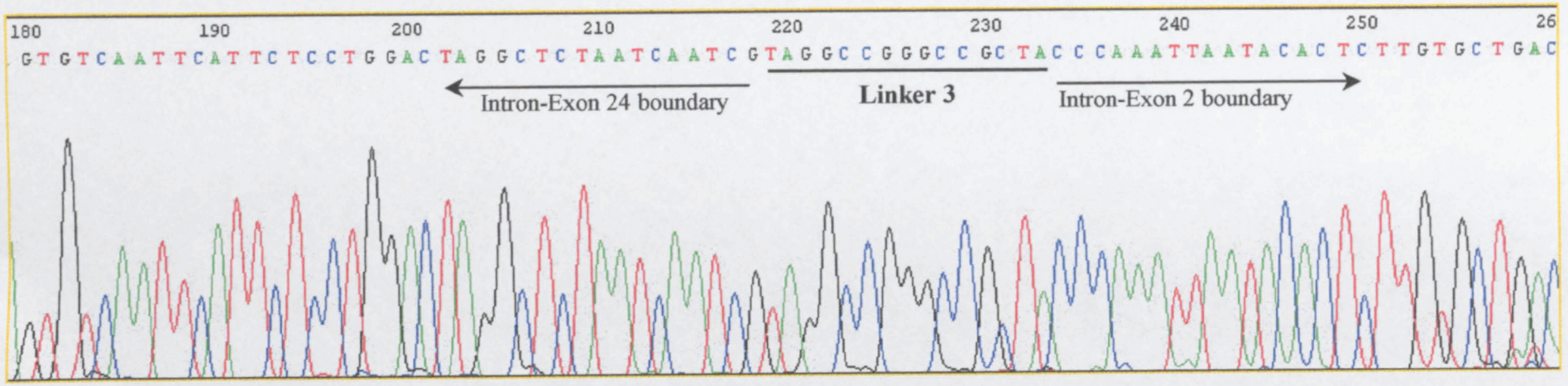
a)



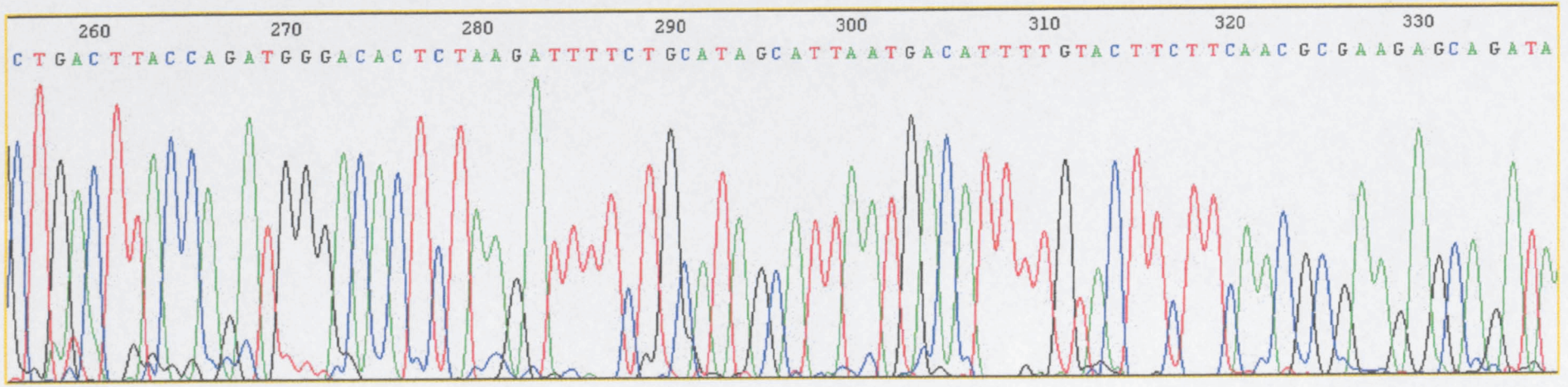
b)



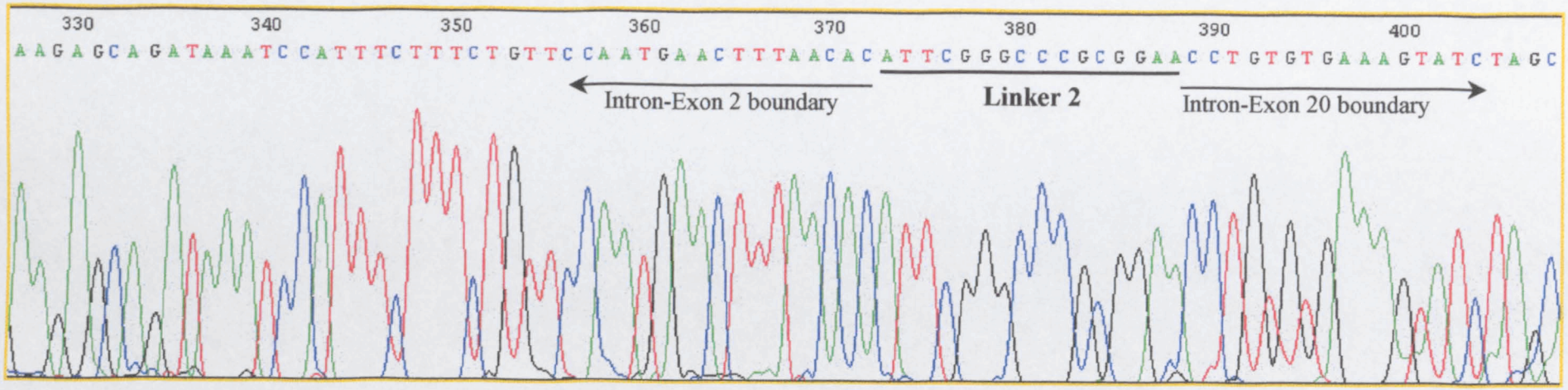
c)


180
190
200
210
220
230
240
250
GTGTCAATTCATTCTCCTGGACTAGGCTCTAATCAATCGTAGGCCGGGCCGCTACCCAAATTAATACACTCTTGTGCTGAC
Intron-Exon 24 boundary
Linker 3
Intron-Exon 2 boundary

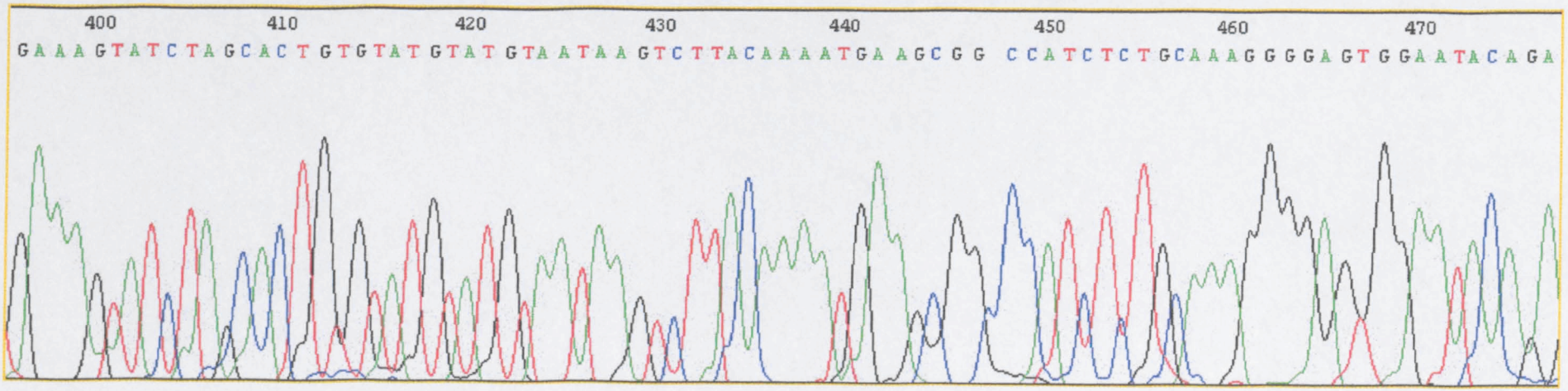
d)


260
270
280
290
300
310
320
330
CTGACTTACCAGATGGGACACTCTAAGATTTTCTGCATAGCATTAATGACATTTTGTACTTCTTCAACGCGAAGAGCAGATA

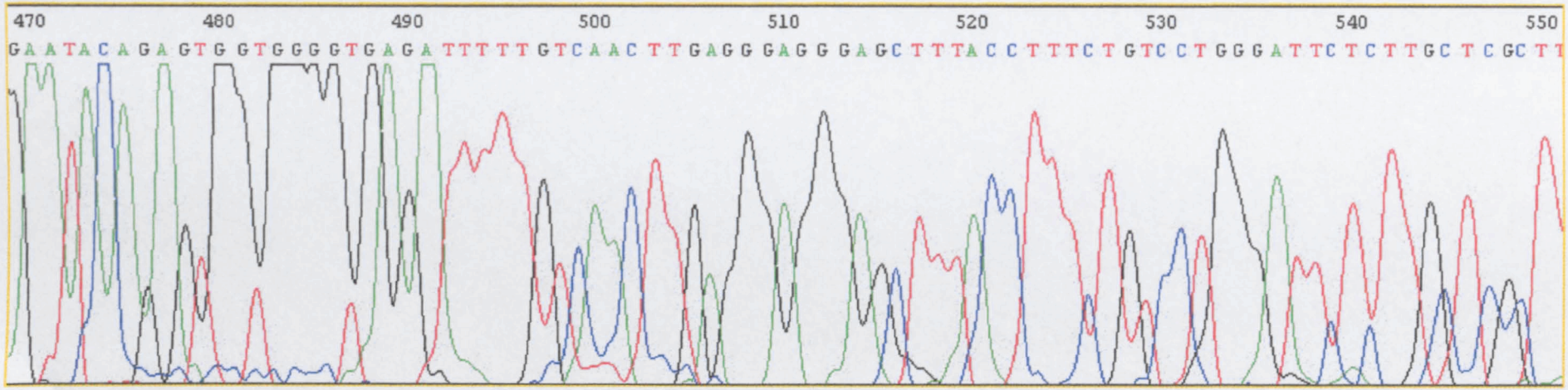
e)


330
340
350
360
370
380
390
400
A A G A G C A G A T A A A T C C A T T T C T T T C T G T T C C A A T G A A C T T T A A C A C A T T C G G G C C C G C G G A A C C T G T G T G A A A G T A T C T A G C
Intron-Exon 2 boundary
Linker 2
Intron-Exon 20 boundary

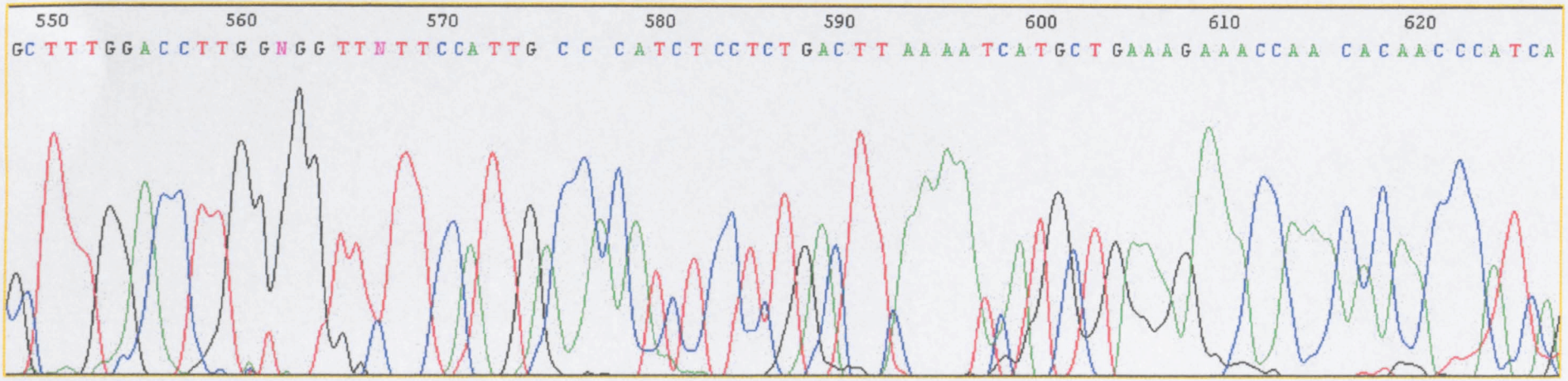
f)


400
410
420
430
440
450
460
470
G A A A G T A T C T A G C A C T G T G T A T G T A T G T A A T A A G T C T T A C A A A A T G A A G C G G C C A T C T C T G C A A A G G G G A G T G G A A T A C A G A

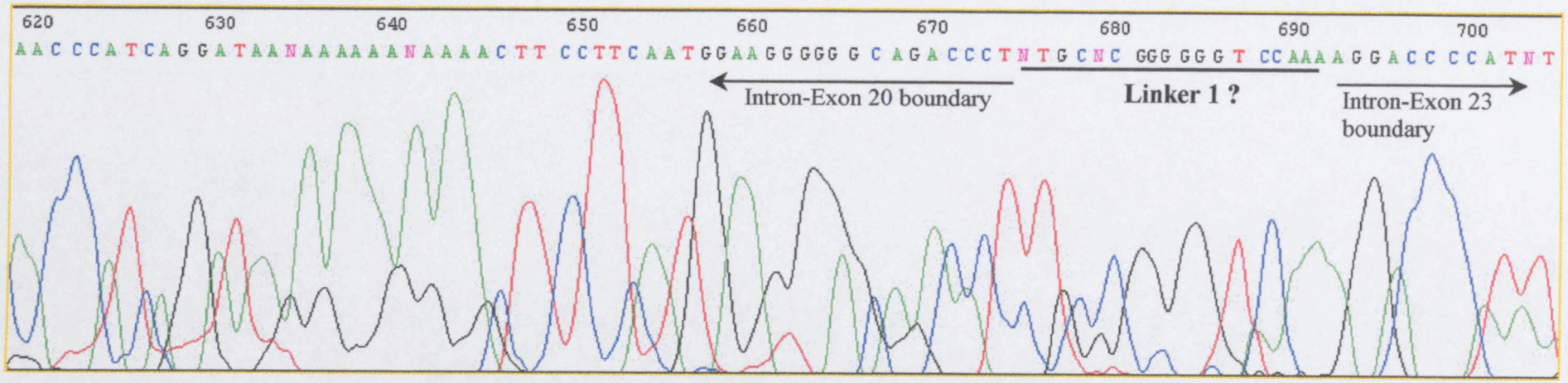
g)


470 480 490 500 510 520 530 540 550
GAATACAGAGTGGTGGGGTGAGATTTTTGTCAACTTGAGGGAGGGAGCTTTACCTTTCTGTCCTGGGATTCTCTTGCTCGCTT

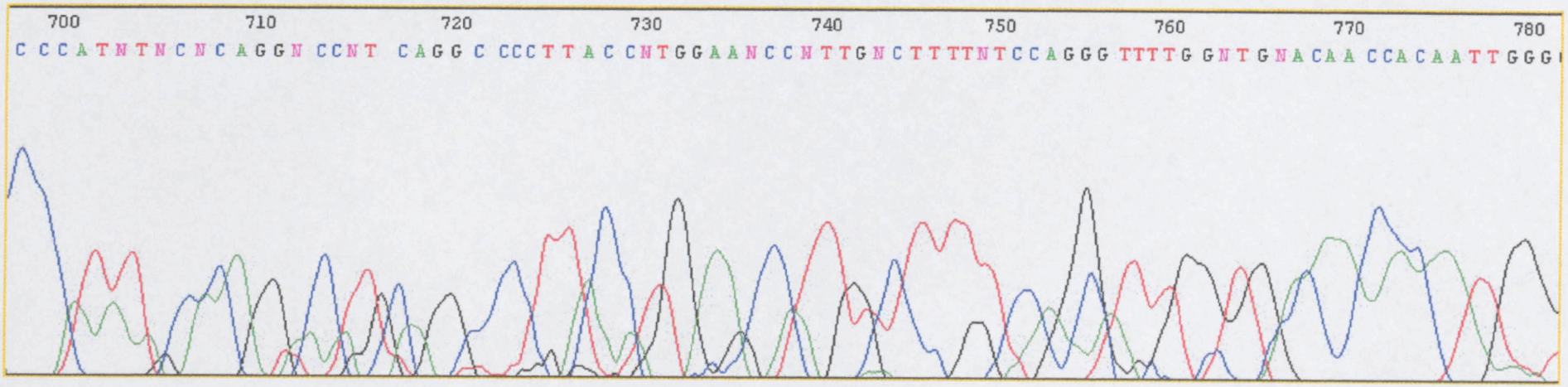
h)


550 560 570 580 590 600 610 620
GCTTTGGACCTTGGNGGTTNTTCCATTG CC CATCTCCTCTGACTT AAAATCATGCTGAAAGAAACCAA CACAACCCATCA

i)

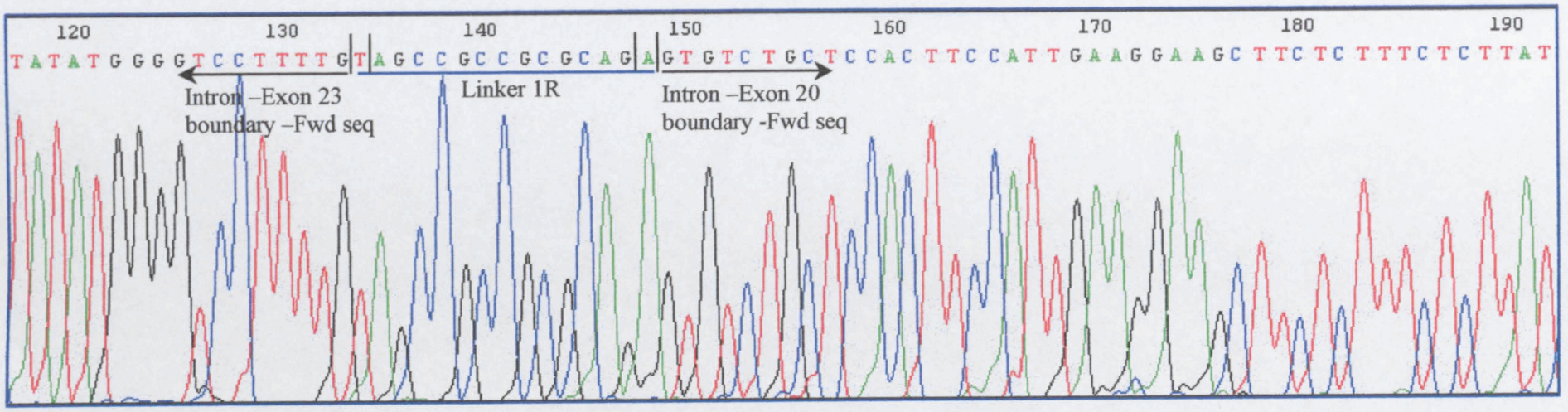


j)



Note: The location of linker 1 and junction boundaries in reverse sequence direction is not clear, however it was characterised with forward sequencing (See figure 5.7b).

Figure 5.9: Electropherograms of normal sequences of assembled exon 23 to exon 20 of BRCA1 gene



Note: Forward sequences show assembling E23 to E20 with linker 1 and for reverse electropherogram see Figure 5.8. (i)

Figure 5.10: Electropherogram of normal sequences of assembled exon20 to exon2 of BRCA1 gene

[Forward (a) and reverse (b) sequences show assembling E20 to E2 with linker 2]

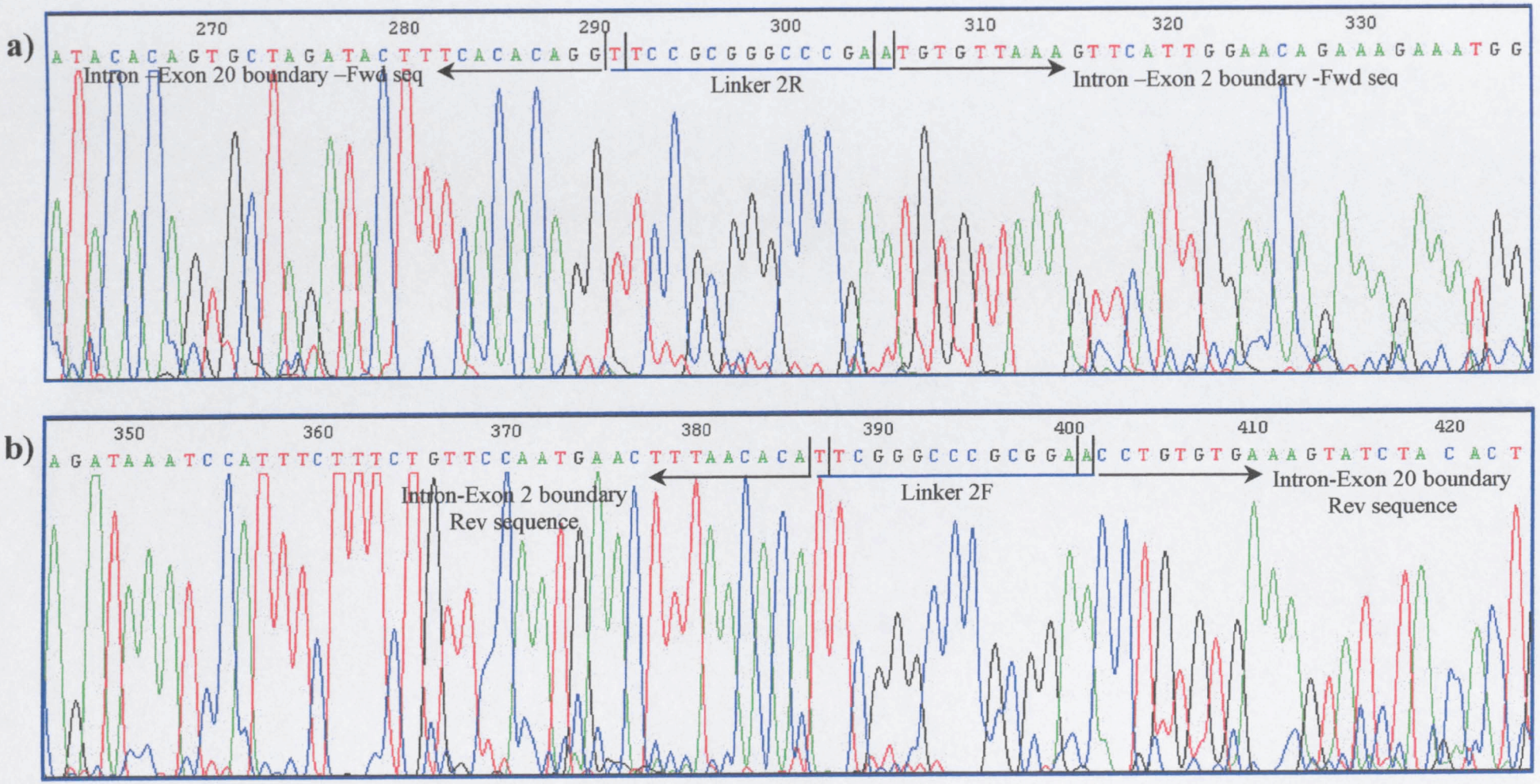
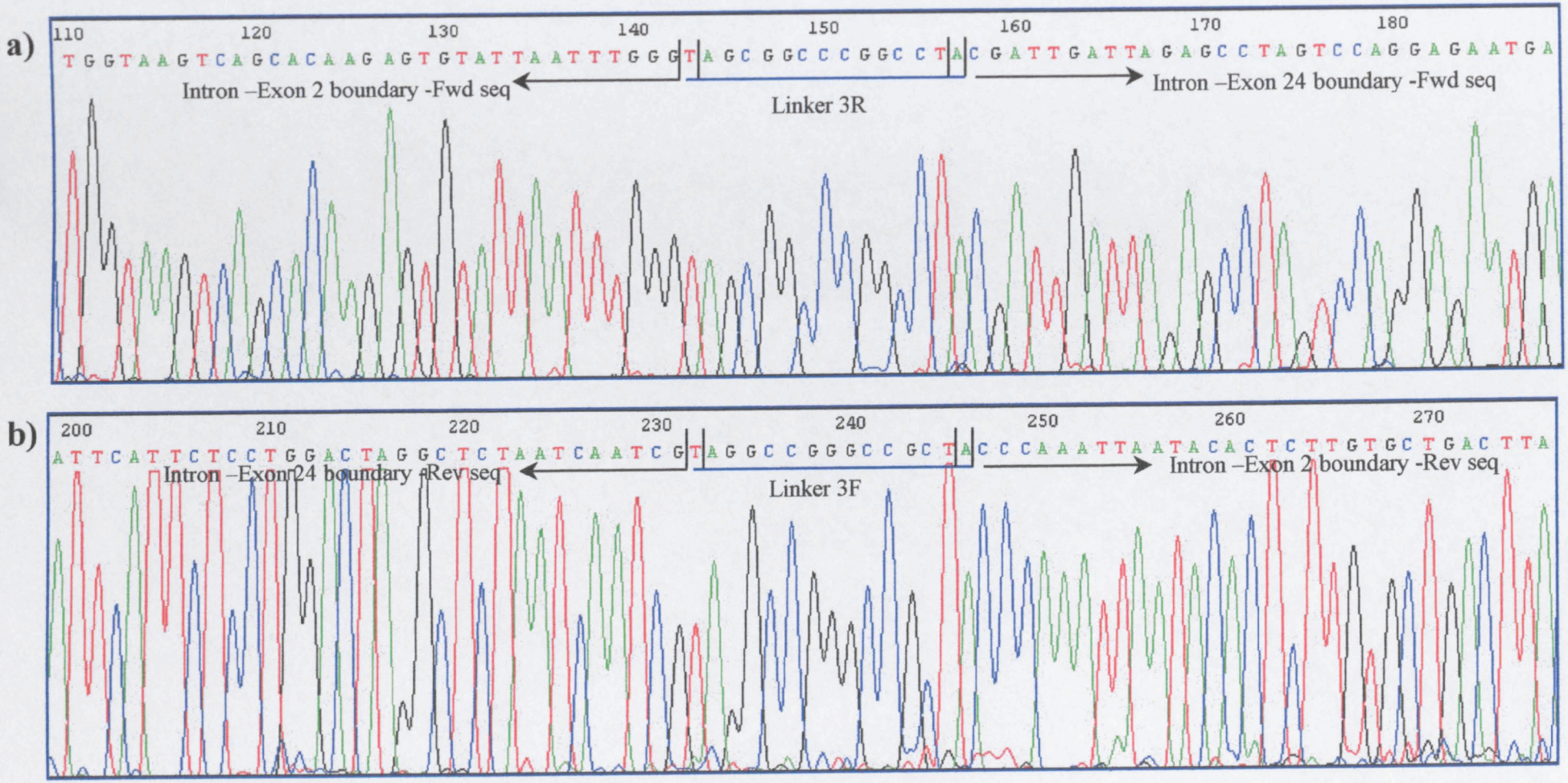


Figure 5.11: Electropherogram of normal sequences of assembled exon2 to exon24 of BRCA1 gene

[Forward (a) and Reverse (b) sequences show assembling E2 to E24 with linker 3]



5.5.3.5 Detection of three previously ascertained heterozygous BRCA1 mutations from Meta-PCR products

To test the suitability of the Meta-PCR products for downstream application such as direct sequencing, Meta-PCR products from three previously determined heterozygous BRCA1 mutations in exon 2 and exon 20, were directly sequenced to confirm that the genotype present in genomic DNA was correctly represent in the Meta-PCR products. The three mutant heterozygotes were BRCA1; 181-182 insT, 185 delAG and a 12bp duplication [BRCA1, IVS20+48]. These were clearly visible in the heterozygotes in figures 5.12 - 5.14.

Figure 5.12: Electropherogram of assembled the BRCA1 exon2 to exon24 Meta-PCR products from mutant heterozygote of **BRCA1 in exon2 (181-182 ins T)**

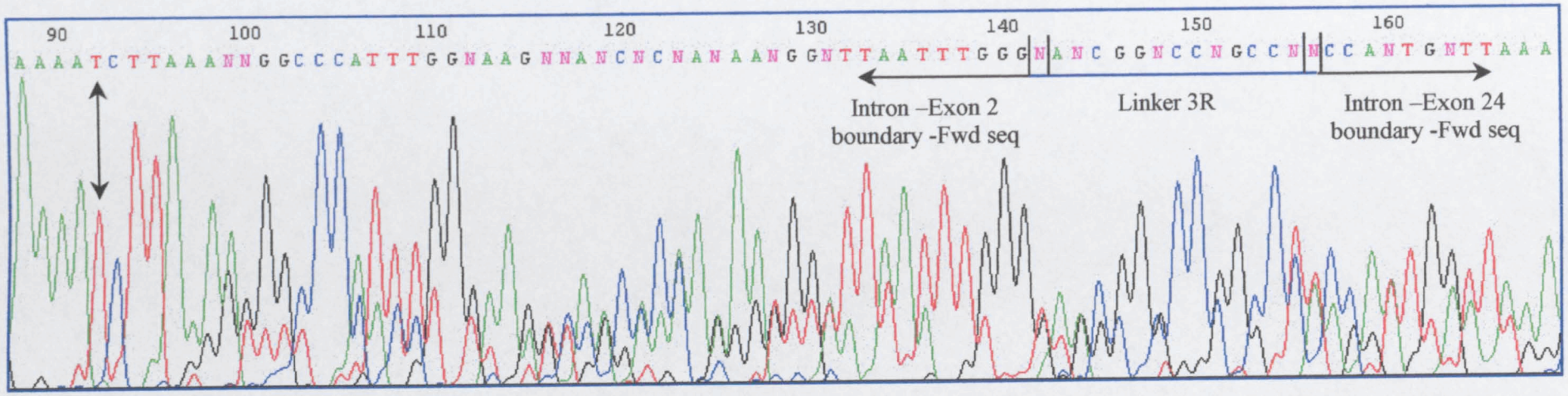


Figure 5.12: Forward sequences show the mutant heterozygote of BRCA1 exon2 (181-182 ins T) within the assembled the BRCA1 exon2 to exon24 by Meta-PCR technique. Arrow depicts the location of [T] insertion and frameshift sequence consequently (See chapter 3 figure 3.6 for the same mutant heterozygote sequence pattern without assembled sequence).

Figure 5.13: Electropherogram of assembled the BRCA1 exon20 to exon2 Meta-PCR products from mutant heterozygote of **BRCA1 in exon2 (185del AG)**

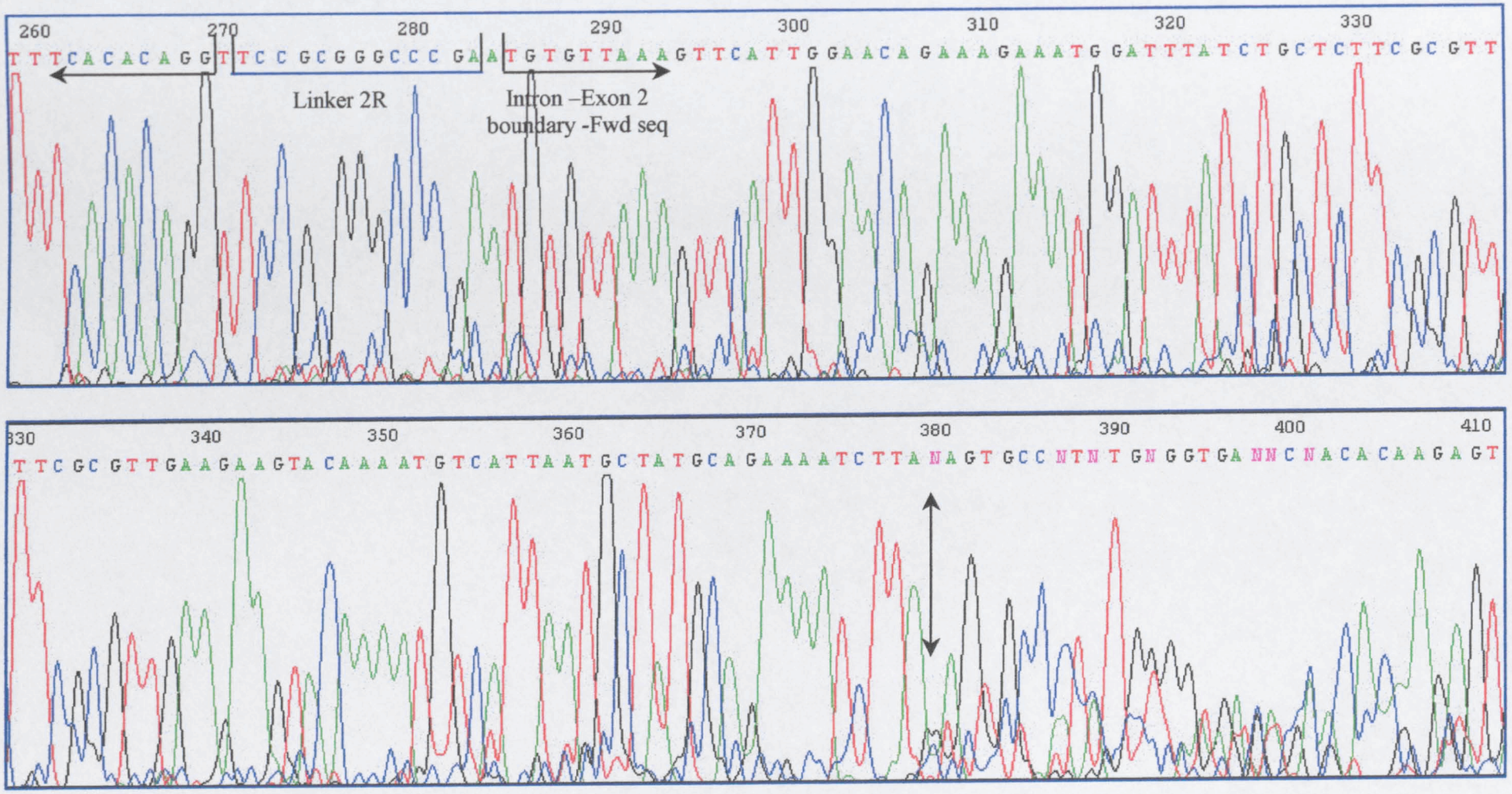


Figure 5.14: Electropherogram of assembled the BRCA1 exon20 to exon2 Meta-PCR products from mutant heterozygote of **BRCA1, IVS20+48 [12bp duplication gtattccactcc]**

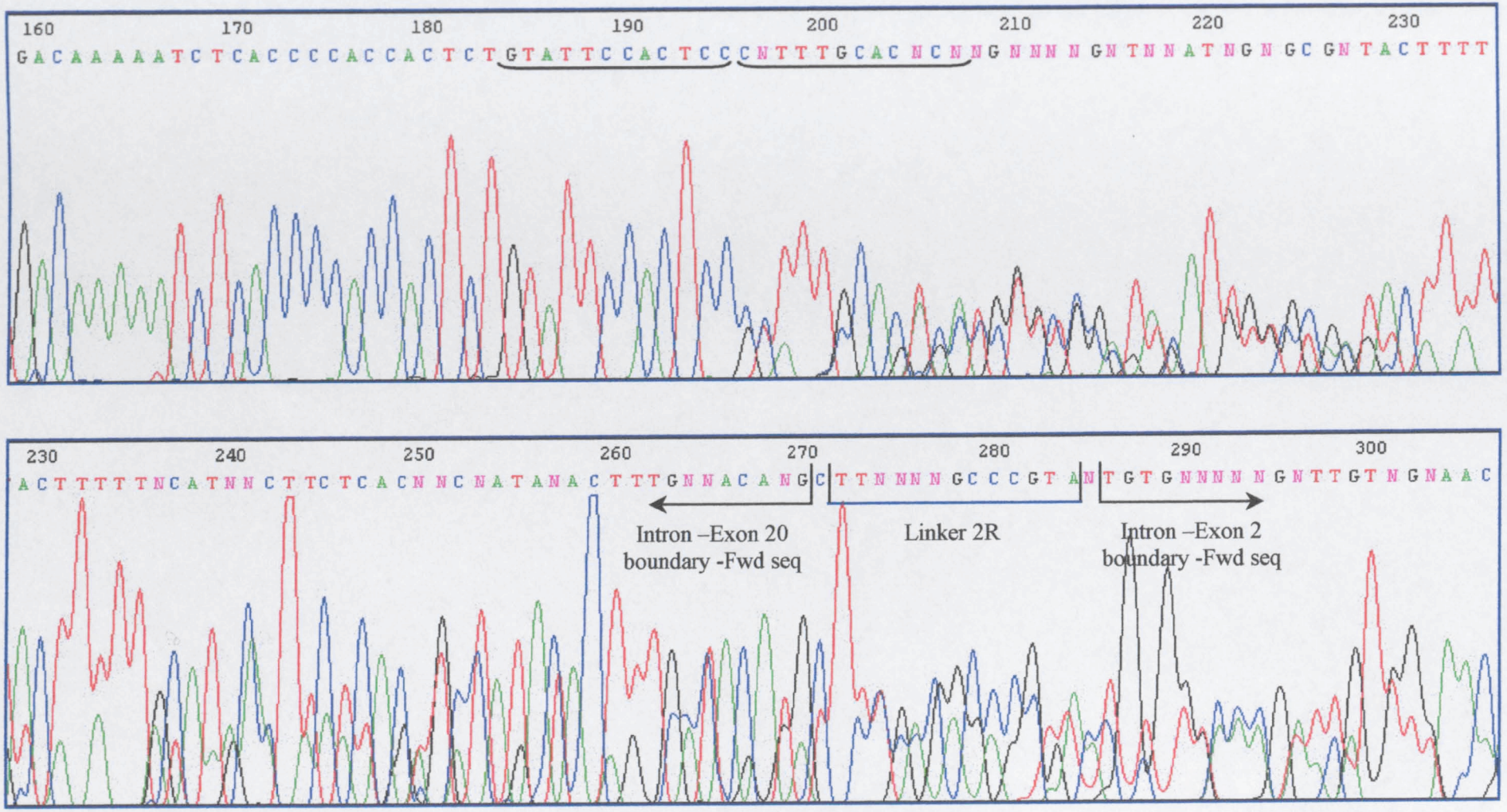


Figure 5.13: Forward electropherogram show the nucleotide sequence of the mutant heterozygote of BRCA1 exon2 (185-186 delAG) within the assembled the BRCA1 exon20 to exon2 by Meta-PCR technique. Arrow depicts the location of [AG] deletion and frameshift sequence consequently (See chapter 3 figure 3.5 for the same mutant heterozygote sequence pattern without assembled sequences).

Figure 5.14: Forward electropherogram show the nucleotide sequence of the mutant heterozygote of BRCA1, IVS20+48 [12bp duplication gtattccactcc] within the assembled the BRCA1 exon20 to exon2 by Meta-PCR technique. Down brackets depict the location of 12bp duplication gtattccactcc and frameshift sequence consequently occurred. (See chapter 3 Figure 3.4 for the same mutant heterozygote sequence pattern without assembled sequences).

5.6 Do the size and/or the segment numbers matter?

In the two previous trials, it was shown that three and four segments could be assembled with different approaches (sections 5.4 and 5.5 respectively). Here, I am trying to answer to the significant question with respect to Meta-PCR. "What is the size limit for gene assembly?"

To date, up to five PCR amplifiable fragments can be combined to form a single linear amplicon [8]. I attempted to assemble six key coding regions from the BRCA genes, the 5' and 3' termini of exon 11 from both BRCA1 and BRCA2 genes and exons 2 and 20 from BRCA1.

Downstream analysis of Meta-PCR products by Protein Truncation Test was used rather than direct nucleotide sequencing because the total assembled above fragments size (~2.8kb) is sufficiently big to ignore analysing by the latter approach.

5.6.1 Primer design

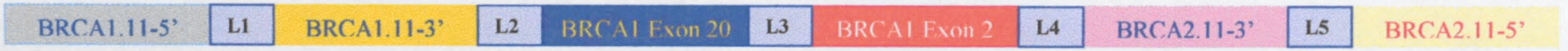
The various complete combinations of linker specific primer segments and genomic specific fragments were designed as shown in Table 5.4.

Five linker specific primer segments (each 13-14bp) were used to produce a chimaeric DNA molecule for above six specific gene fragments (Table 5.4). Also, one site directed mutation was introduced into BRCA1 exon 2 segment, in which the sequence of **taa** gtc agc aca aga gtg tat was changed to the **tat** gtc agc aca aga gtg tat to maintain an open reading frame in the final assembled sequences (See section 5.6.2).

To obtain the best results in PTT and SDS-PAGE separation the order of the fragments was determined preferentially.

Two nested primers including an internal forward primer (comprising a T_7 promoter and a perfect Kozak consensus sequence to facilitate coupled transcription-translation) and a conventional reverse primer were designed to produce sufficient product for PTT analysis and to facilitate priming a few residues upstream and downstream of the external primers (Table 5.4).

Table 5.4: Details of linker specific primer segments and genomic specific fragments used for amplification and assembling of BRCA1 and BRCA2 exon 11 (5' and 3'ends) and BRCA1exons 2 and 20 by Meta-PCR



Number	Name	Length	Sequence 5 ' → 3'	PCR pro. size/bp	Genomic segment T_m / °C	Linker T_m / °C
1	1.11 5' -f (ext.)	19	agc cag ttg gtt gat ttc c	668	68	---
2	1.11 5' -r-L1F	36	tcg gcc gcc ggc aAc ttt ggc att tga ttc aga ctc	---	70	75
3	1.11 3' -f-L1R	35	tgc cgg cgg ccg aAa gct tag cag gag tcc tag cc	587	70	75
4	1.11 3' -r-L2F	34	gct ccg cgg cgc gtA ggg caa aca caa aaa cct g	---	69	76
5	E20-f-L2R	37	acg cgc cgc gga gcA gat ggg ttg tgt ttg gtt tct t	145	71	76
6	E20-r-L3F	35	agg ccg ggc cgg gaA ctt gag gga ggg agc ttt ac	---	69	76

7	E2-f-L3R	36	tcc cgg ccc ggc ctA tca ttg gaa cag aaa gaa atg	134	70	76
8	E2-r-L4F	38	tgg ccg gcg gcg aAa tac act ctt gtg ctg aca tac ca	---	70	75
9	2.11 3' -f-L4R	36	tcg ccg ccg gcc aAc ctc gtg ttg ata aga gaa acc	575	69	75
10	2.11 3' -r-L5F	34	tcg ggc gcg cgg tAa gtg att ggc aac acg aaa g	---	69	74
11	2.11 5' -f-L5R	38	acc gcg cgc ccg aAt tag gtt tat tgc att ctt ctg tg	868	69	74
12	2.11 5' -R (ext.)	23	ggt tcg ttt aca caa gtc aag tc	---	69	---
13	1.11 5' -f (int.)	56	*gct aat acg act cac tat agg aac aga cca cca tgt cag ctg ctt gtg aat ttt ct*	2823	68	---
14	2.11 5' -R (int.)	24	*cct ttc att agc tac ttg gaa gac*	---	70	---

Gene interest sequence = Black colour
Linker = Blue colour + A
Internal primer sequence = Italic lower case (Red colour flanking by 35bp T_7 promoter)
Secondary Meta-PCR sequences size (Internal amplification)= 2823bp (excluding 35bp T_7 promoter)
Annealing temperature used = 65 °C

5.6.2 The complete assembled sequences of BRCA1 and BRCA2 exon 11 (5' and 3'ends) and BRCA1 e20, e2 (Intron-exon boundaries)

(T_7 polymerase+spacer+ATG flanking sequence)
gct aat acg act cac tat agg aac aga cca cca tg

Intron-exon boundary of BRCA1.11 5' end →

```
  1  tca gct gct tgt gaa ttt tct gag acg gat gta
       S   A   A   C   E   F   S   E   T   D   V

 34  aca aat act gaa cat cat caa ccc agt aat aat
       T   N   T   E   H   H   Q   P   S   N   N

 67  gat ttg aac acc act gag aag cgt gca gct gag
       D   L   N   T   T   E   K   R   A   A   E

100  agg cat cca gaa aag tat cag ggt agt tct gtt
       R   H   P   E   K   Y   Q   G   S   S   V

133  tca aac ttg cat gtg gag cca tgt ggc aca aat
       S   N   L   H   V   E   P   C   G   T   N

166  act cat gcc agc tca tta cag cat gag aac agc
       T   H   A   S   S   L   Q   H   E   N   S

199  agt tta tta ctc act aaa gac aga atg aat gta
       S   L   L   L   T   K   D   R   M   N   V

232  gaa aag gct gaa ttc tgt aat aaa agc aaa cag
       E   K   A   E   F   C   N   K   S   K   Q

265  cct ggc tta gca agg agc caa cat aac aga tgg
       P   G   L   A   R   S   Q   H   N   R   W

298  gct gga agt aag gaa aca tgt aat gat agg cgg
       A   G   S   K   E   T   C   N   D   R   R

331  act ccc agc aca gaa aaa aag gta gat ctg aat
       T   P   S   T   E   K   K   V   D   L   N

364  gct gat ccc ctg tgt gag aga aaa gaa tgg aat
       A   D   P   L   C   E   R   K   E   W   N

397  aag cag aaa ctg cca tgc tca gag aat cct aga
```

```
        K   Q   K   L   P   C   S   E   N   P   R

430  gat act gaa gat gtt cct tgg ata aca cta aat
       D   T   E   D   V   P   W   I   T   L   N

463  agc agc att cag aaa gtt aat gag tgg ttt tcc
       S   S   I   Q   K   V   N   E   W   F   S

496  aga agt gat gaa ctg tta ggt tct gat gac tca
       R   S   D   E   L   L   G   S   D   D   S
```

Exon boundary of BRCA1.11 5' end

```
529  cat gat ggg gag tct gaa tca aat gcc aaa gtt
       H   D   G   E   S   E   S   N   A   K   V
```

Exon boundary of BRCA1.11 3' end

```
562  cgg ccg ccg gca aag ctt agc agg agt cct agc
       R   P   P   A   K   L   S   R   S   P   S
     —
595  cct ttc acc cat aca cat ttg gct cag ggt tac
       P   F   T   H   T   H   L   A   Q   G   Y

628  cga aga ggg gcc aag aaa tta gag tcc tca gaa
       R   R   G   A   K   K   L   E   S   S   E

661  gag aac tta tct agt gag gat gaa gag ctt ccc
       E   N   L   S   S   E   D   E   E   L   P

694  tgc ttc caa cac ttg tta ttt ggt aaa gta aac
       C   F   Q   H   L   L   F   G   K   V   N

727  aat ata cct tct cag tct act agg cat agc acc
       N   I   P   S   Q   S   T   R   H   S   T

760  gtt gct acc gag tgt ctg tct aag aac aca gag
       V   A   T   E   C   L   S   K   N   T   E

793  gag aat tta tta tca ttg aag aat agc tta aat
       E   N   L   L   S   L   K   N   S   L   N

826  gac tgc agt aac cag gta ata ttg gca aag gca
       D   C   S   N   Q   V   I   L   A   K   A
```

```
859   tct cag gaa cat cac ctt agt gag gaa aca aaa
        S   Q   E   H   H   L   S   E   E   T   K

892   tgt tct gct agc ttg ttt tct tca cag tgc agt
        C   S   A   S   L   F   S   S   Q   C   S

925   gaa ttg gaa gac ttg act gca aat aca aac acc
        E   L   E   D   L   T   A   N   T   N   T

958   cag gat cct ttc ttg att ggt tct tcc aaa caa
        Q   D   P   F   L   I   G   S   S   K   Q

991   atg agg cat cag tct gaa agc cag gga gtt ggt
        M   R   H   Q   S   E   S   Q   G   V   G

1024  ctg agt gac aag gaa ttg gtt tca gat gat gaa
        L   S   D   K   E   L   V   S   D   D   E

1057  gaa aga gga acg ggc ttg gaa gaa aat aat caa
        E   R   G   T   G   L   E   E   N   N   Q

1090  gaa gag caa agc atg gat tca aac tta ggt att
        E   E   Q   S   M   D   S   N   L   G   I
```

Intron-exon boundary of BRCA1.11 3' end

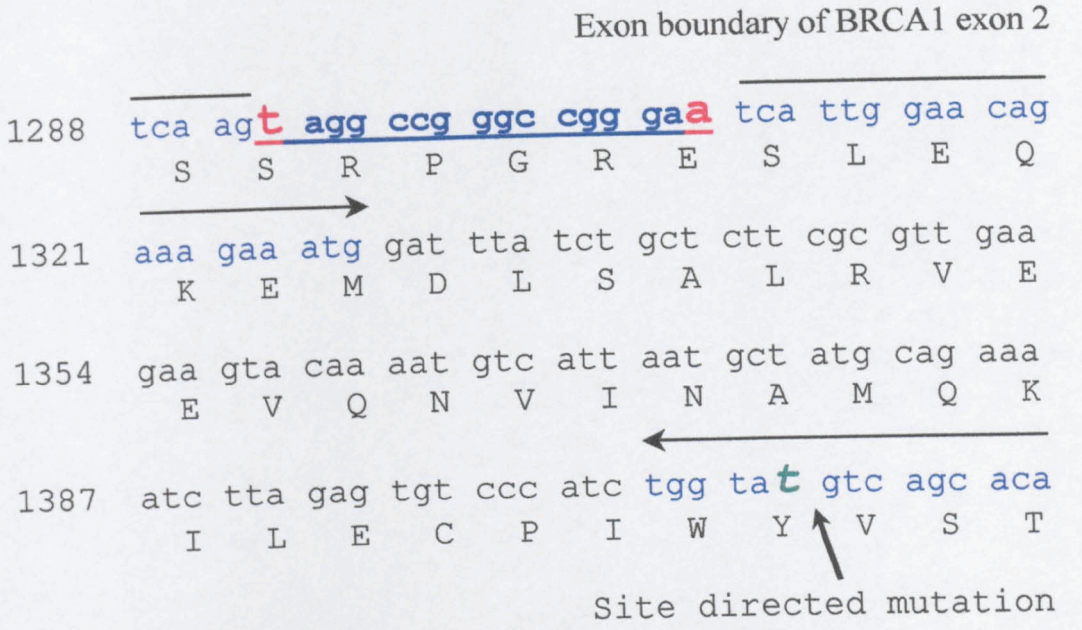
```
1123  gga acc agg ttt ttg tgt ttg ccc tgc tcc gcg
        G   T   R   F   L   C   L   P   C   S   A
```

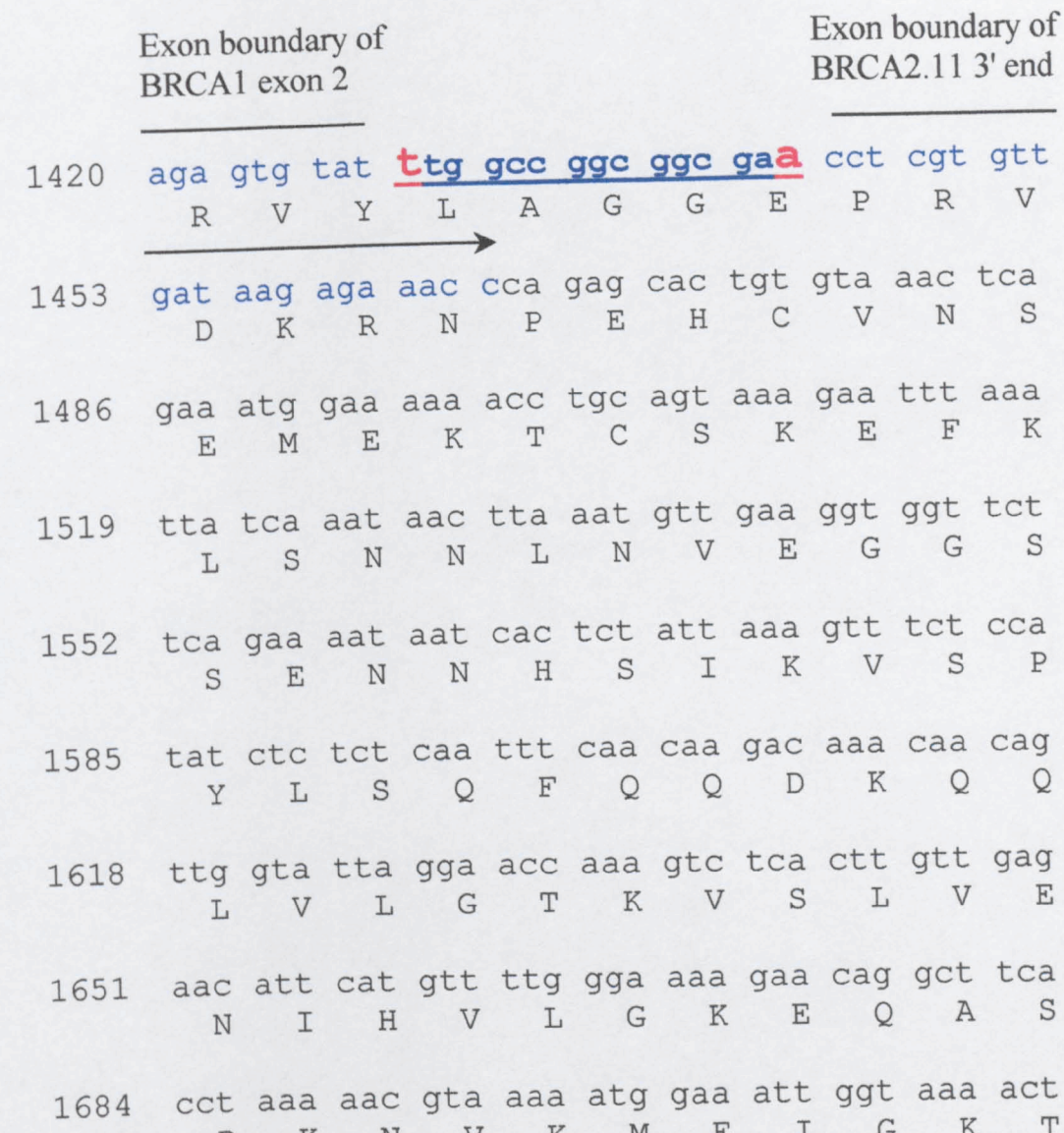
Intron-exon boundary of BRCA1 exon 20

```
1156  gcg cgt aga tgg gtt gtg ttt ggt ttc ttt cag
        A   R   R   W   V   V   F   G   F   F   Q

1189  cat gat ttt gaa gtc aga gga gat gtg gtc aat
        H   D   F   E   V   R   G   D   V   V   N

1222  gga aga aac cac caa ggt cca aag cga gca aga
        G   R   N   H   Q   G   P   K   R   A   R
```

Intron-exon boundary of BRCA1 exon 20

```
1255  gaa tcc cag gac aga aag gta aag ctc cct ccc
        E   S   Q   D   R   K   V   K   L   P   P
```


Exon boundary of BRCA1 exon 2
1288 tca agt agg ccg ggc cgg gaa tca ttg gaa cag
S S R P G R E S L E Q
1321 aaa gaa atg gat tta tct gct ctt cgc gtt gaa
K E M D L S A L R V E
1354 gaa gta caa aat gtc att aat gct atg cag aaa
E V Q N V I N A M Q K
1387 atc tta gag tgt ccc atc tgg tat gtc agc aca
I L E C P I W Y V S T
Site directed mutation


Exon boundary of BRCA1 exon 2
Exon boundary of BRCA2.11 3' end
1420 aga gtg tat ttg gcc ggc ggc gaa cct cgt gtt
R V Y L A G G E P R V
1453 gat aag aga aac cca gag cac tgt gta aac tca
D K R N P E H C V N S
1486 gaa atg gaa aaa acc tgc agt aaa gaa ttt aaa
E M E K T C S K E F K
1519 tta tca aat aac tta aat gtt gaa ggt ggt tct
L S N N L N V E G G S
1552 tca gaa aat aat cac tct att aaa gtt tct cca
S E N N H S I K V S P
1585 tat ctc tct caa ttt caa caa gac aaa caa cag
Y L S Q F Q Q D K Q Q
1618 ttg gta tta gga acc aaa gtc tca ctt gtt gag
L V L G T K V S L V E
1651 aac att cat gtt ttg gga aaa gaa cag gct tca
N I H V L G K E Q A S
1684 cct aaa aac gta aaa atg gaa att ggt aaa act
P K N V K M E I G K T

```
1717  gaa act ttt tct gat gtt cct gtg aaa aca aat
        E   T   F   S   D   V   P   V   K   T   N

1750  ata gaa gtt tgt tct act tac tcc aaa gat tca
        I   E   V   C   S   T   Y   S   K   D   S

1783  gaa aac tac ttt gaa aca gaa gca gta gaa att
        E   N   Y   F   E   T   E   A   V   E   I

1816  gct aaa gct ttt atg gaa gat gat gaa ctg aca
        A   K   A   F   M   E   D   D   E   L   T

1849  gat tct aaa ctg cca agt cat gcc aca cat tct
        D   S   K   L   P   S   H   A   T   H   S

1882  ctt ttt aca tgt ccc gaa aat gag gaa atg gtt
        L   F   T   C   P   E   N   E   E   M   V

1915  ttg tca aat tca aga att gga aaa aga aga gga
        L   S   N   S   R   I   G   K   R   R   G

1948  gag ccc ctt atc tta gtg ggt aag tgt tca ttt
        E   P   L   I   L   V   G   K   C   S   F
```

Intron-exon boundary of BRCA2.11 3' end

```
1981  tta cct ttc gtg ttg cca atc act ttc ggg cgc
        L   P   F   V   L   P   I   T   F   G   R
```

Intron-exon boundary of BRCA2.11 5' end

```
2014  gcg gta tta ggt tta ttg cat tct tct gtg aaa
        A   V   L   G   L   L   H   S   S   V   K

2047  aga agc tgt tca cag aat gat tct gaa gaa cca
        R   S   C   S   Q   N   D   S   E   E   P

2080  act ttg tcc tta act agc tct ttt ggg aca att
        T   L   S   L   T   S   S   F   G   T   I

2113  ctg agg aaa tgt tct aga aat gaa aca tgt tct
        L   R   K   C   S   R   N   E   T   C   S

2146  aat aat aca gta atc tct cag gat ctt gat tat
        N   N   T   V   I   S   Q   D   L   D   Y
```

```
2179  aaa gaa gca aaa tgt aat aag gaa aaa cta cag
        K   E   A   K   C   N   K   E   K   L   Q

2212  tta ttt att acc cca gaa gct gat tct ctg tca
        L   F   I   T   P   E   A   D   S   L   S

2245  tgc ctg cag gaa gga cag tgt gaa aat gat cca
        C   L   Q   E   G   Q   C   E   N   D   P

2278  aaa agc aaa aaa gtt tca gat ata aaa gaa gag
        K   S   K   K   V   S   D   I   K   E   E

2311  gtc ttg gct gca gca tgt cac cca gta caa cat
        V   L   A   A   A   C   H   P   V   Q   H

2344  tca aaa gtg gaa tac agt gat act gac ttt caa
        S   K   V   E   Y   S   D   T   D   F   Q

2377  tcc cag aaa agt ctt tta tat gat cat gaa aat
        S   Q   K   S   L   L   Y   D   H   E   N

2410  gcc agc act ctt att tta act cct act tcc aag
        A   S   T   L   I   L   T   P   T   S   K

2443  gat gtt ctg tca aac cta gtc atg att tct aga
        D   V   L   S   N   L   V   M   I   S   R

2476  ggc aaa gaa tca tac aaa atg tca gac aag ctc
        G   K   E   S   Y   K   M   S   D   K   L

2509  aaa ggt aac aat tat gaa tct gat gtt gaa tta
        K   G   N   N   Y   E   S   D   V   E   L

2542  acc aaa aat att ccc atg gaa aag aat caa gat
        T   K   N   I   P   M   E   K   N   Q   D

2575  gta tgt gct tta aat gaa aat tat aaa aac gtt
        V   C   A   L   N   E   N   Y   K   N   V

2608  gag ctg ttg cca cct gaa aaa tac atg aga gta
        E   L   L   P   P   E   K   Y   M   R   V

2641  gca tca cct tca aga aag gta caa ttc aac caa
        A   S   P   S   R   K   V   Q   F   N   Q

2674  aac aca aat cta aga gta atc caa aaa aat caa
        N   T   N   L   R   V   I   Q   K   N   Q
```

```
2707  gaa gaa act act tca att tca aaa ata act gtc
        E   E   T   T   S   I   S   K   I   T   V

2740  aat cca gac tct gaa gaa ctt ttc tca gac aat
        N   P   D   S   E   E   L   F   S   D   N
```

Exon boundary of BRCA2.11 5' end

```
2773  gag aat aat ttt gtc ttc caa gta gct aat gaa
        E   N   N   F   V   F   Q   V   A   N   E

2806  agg
        R
```

*The black/blue colour lower letter case= Intron-exon boundaries of six fragments

The bold italic green colour lower letter case= site direct mutagenesis

***The blue colour lower letter case = Sequence of gene of interest for position of a primer (Forward or Reverse)**

***The bold blue colour underlined lower letter case= Linkers sequences**

***The bold red colour underlined a or t= A supplementary unmatched adenine or thiamine residues was inserted between the genomic and 5′ linker segments**

Upper case = One letter amino acid code

In order to maintain assembled fragment in an open reading frame format, one site directed mutation were introduced, as follow:

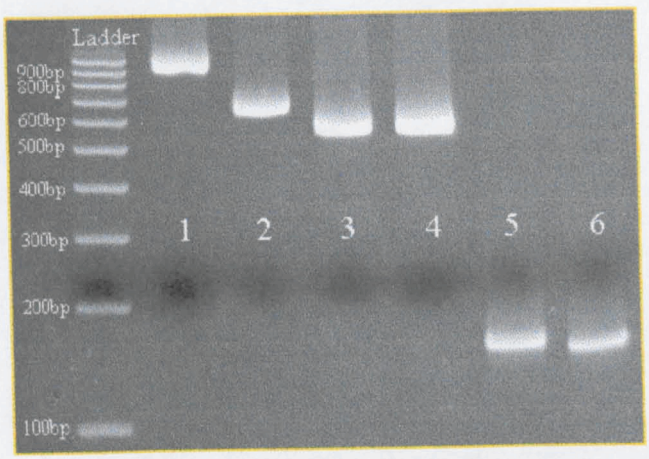
In BRCA1 exon 2 segment **taa** (stop codon) is changed to **tat** (tyrosine) at nucleotide 1410*

*Nucleotide number is presented at the position within assembled fragment.

5.6.3 Results

PCR amplification of each exon under common amplification conditions gave a single fragment of the expected size, confirming the integrity of the primer syntheses and the compatibility of the primer pairs (Fig. 5.15).

Figure 5.15: PCR products from BRCA1 and BRCA2 exon 11 (5' and 3' ends) and BRCA1 exons 20 and 2



PCR products were analysed on 15% Polyacrylamide gel.

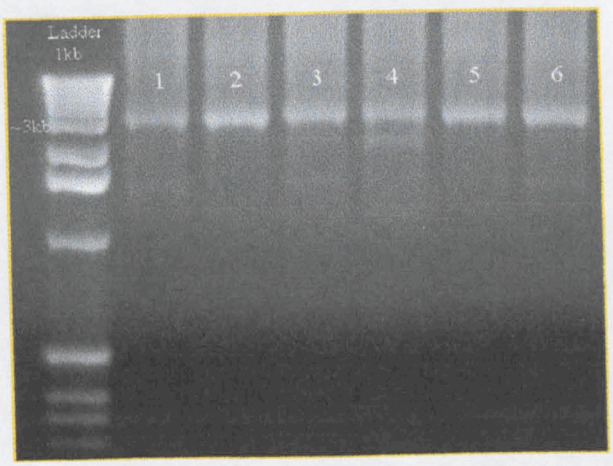
Lane 1, PCR products of **BRCA2 exon11 5' end** =868bp
Lane 2, PCR products of **BRCA1 exon11 5' end** =668bp
Lane 3, PCR products of **BRCA1 exon11 3' end** =587bp
Lane 4, PCR products of **BRCA2 exon11 3' end** =575bp
Lane 5, PCR products of **BRCA1 exon20** =145bp
Lane 6, PCR products of **BRCA1 exon2** =134bp

The size of each DNA specific fragment when amplified individually is larger than when assembled, because the PCR products of each specific DNA fragment includes a "hanging" linker specific segment either at the tails or at one side.

5.6.3.1 The secondary Meta-PCR products

The products of secondary Meta-PCR assays consistently yielded fragments of the expected size when was performed on a range of genomic DNA samples. For downstream analysis by PTT the secondary Meta-PCR products were analysed on a 0.7% agarose gel confirming the integrity of PCR products. In spite of huge efforts that I have performed for PCR optimisation, the reaction yields appear to be still inefficient (Fig. 5.16).

Figure 5.16: The secondary Meta-PCR products assembled from BRCA1 and BRCA2 exon 11 (5' and 3'ends) and BRCA1 exons 20 and 2



Lanes 1-6 show the assembled fragments analysed on 0.7% agarose gel using normal and mutant samples. DNA step-ladder alongside the samples shows an approximate of assembled products size.
Meta-PCR products sizes (Internal amplification)=2858bp (2823bp+35bp T_7 promoter)

The size of assembled fragments is smaller than when those amplified individually (2858bp versus 2977bp respectively), because the PCR products of each specific DNA fragment includes hanging linker specific segment either at the both tail or at the one side (See section 5.6.2 and Table 5.4).

5.6.3.2 Recovery of secondary Meta-PCR products following agarose gel electrophoresis

The secondary Meta-PCR products were recovered from agarose gel slices using a Jetsorb™ gel extraction kit (Genomed Inc.) in accordance with manufacturer's protocols. 10µl of purified Meta-PCR products were amplified subsequently, using nested (internal) primers and the same PCR programme, to increase yields for downstream analysis by PTT.

5.6.3.3 PTT analysis

A total of 50ng of reamplified Meta-PCR products from three previously ascertained heterozygotes for BRCA1 and BRCA2 mutations 185delAG and 181insT, in BRCA1 exon 2, and 26261-6262 insGT in BRCA2 exon11 were used as a template for PTT following conventional procedures (see chapter 3 and 4 for more detail). The proteins were revealed by SDS-PAGE (6% acrylamide), treated in solution 3% glycerol, 20% methanol for an hour, dry down for one hour at 80°C by auto dryer and visualized by autoradiography for 24 hours.

Three distinct trials revealed poor de novo protein production (Figure 5.17, a-c).

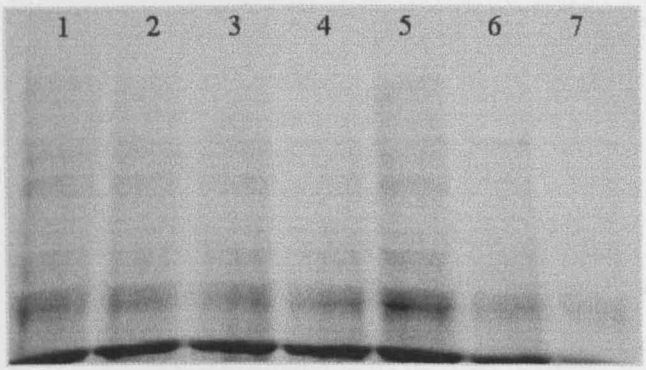
Figure 5.17,a: Lanes 1-7 depict insufficient de novo protein products that lead to unclear interpretation.

Figure 5.17,b: Templates in lanes 1,6 are wild type translated protein; Templates in lanes 2-5 are mutant samples, 26261-6262 insGT, 26261-6262 insGT, 185delAG and 181insT respectively. However no absolute interpretation can be made.

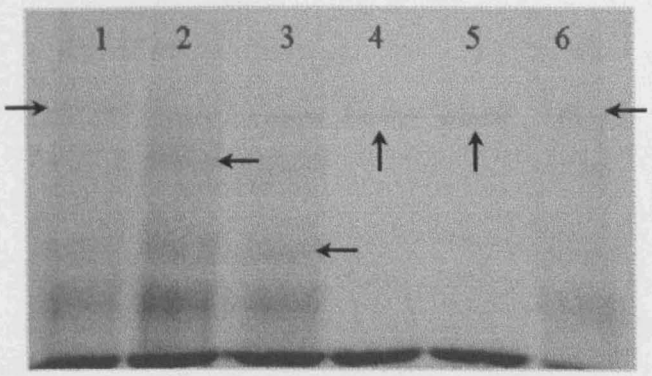
Figure 5.17,c: Lanes 2-9 show insufficient de novo protein products that lead to unclear interpretation. A wild type of Luciferase protein (T_7 Control DNA) was used to exclude failure of PTT procedure (Lane 1).

Figure 5.17: SDS-PAGE images of assembled from BRCA1 and BRCA2 exon 11 (5' and 3' ends) and BRCA1 exons 20 and 2

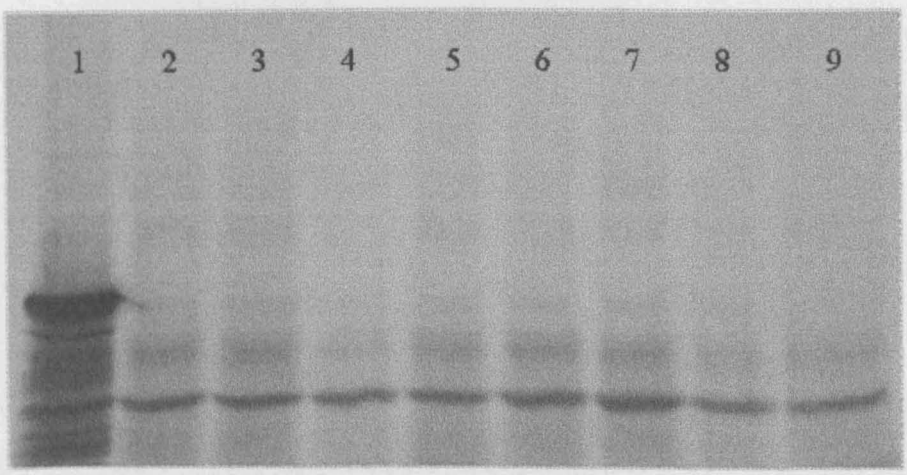
a)



b)



c)



5.7 Discussion

Many disease susceptibility genes are large and consist of many exons throughout which point mutations are scattered almost randomly. BRCA genes fall into this category. The large numbers of exons necessitate large numbers of individual tests, slowing down the screening process. It is in facilitating the screening of large multi-exons genes that Meta-PCR is likely to be most useful. The advantages to diagnostic laboratories already using techniques like CSGE or PTT of coupling Meta-PCR to their mutation scanning method are clearly demonstrated in this chapter.

Meta-PCR could be used to permit simultaneous scanning of disparate mutational hot spots (in the case of BRCA1 gene exons 2 and 20 are notable) thereby allowing a rapid, targeted mutation scanning strategy.

Breast cancers display heterogeneity that can be caused by mutation in the known cancer predisposing BRCA1 and/or BRCA2 genes. Meta-PCR could be used to combine exons from the different genes for simultaneous screening. This could be particularly useful for diseases with mutational hot spots in more than one gene such as BRCA genes.

I have applied Meta-PCR method to improve the overall efficiency of scanning these genes by assembling the more "significant" coding regions and analysing them in a single reaction either by PTT and/or by direct sequencing. These methods were chosen because of their high sensitivity and specificity (each method possesses advantages and disadvantages).

For the PTT approach it was possible to assemble exons 18 of the BRCA2 gene and the exons 2 and 20 of the BRA1 gene successfully. Primer design is the most important criterion for success in this approach. It is critical to ensure the assembled sequences are in frame at the end of the assembly stage. The greatest advantage of this approach compared with direct sequencing is that the length of sequence can be increased up to 5kb for downstream analysis such as PTT.

With the direct sequencing approach, using shorter linker specific primer with higher GC content caused a further improvement in product yield. Primer design is much easier than for the PTT approach, but the length of sequence can be maximized up to 700bp – 800bp with approximate to the length of readily obtained nucleotide sequence.

Both these approaches worked successfully, suggesting that it may be possible to assemble the entire of coding regions of BRCA1 and BRCA2 genes in a multi-step procedure.

By these experiments it become clear that Meta-PCR is a simple, versatile and powerful method for creating chimaeric DNA molecules for the analysis of multi exon genes using a combination of PTT and/or direct sequencing.

In the latest trial (assembling of six segments) insufficient Meta-PCR products lead to poor de novo protein production that might be resulted from several reasons: first, increasing the number of segments; second, differences in fragments sizes; third, a large size of assembled fragment; and fourth, induction a site direct mutagenesis.

However, assembling of more segments may require further PCR optimisation. Therefore, purification of Meta-PCR products by dHPLC may produce better results to compare with agarose gel.

Finally, success in the product is critically depend on, designing the linker specific primer with ~ 50% GC content, suitable position for primer (Gene selection area) with no internal secondary structure, adding adenine residue between genomic and 5' linker segment, making closely equivalent T_m values in both linker segments and genomic specific segments, checking all primers for homology to Alu repeat sequences and the purity of synthesized primers are the most significant parameters, which should not be overlooked.

5.8 References

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Chapter 6

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Chapter 6

"We know that there are keys we can turn to lower risk if we have advanced notice," Jeffrey Weitzel Oct 2001.

6 Genetic counselling and breast cancer prevention

6.1 Genetic counselling

Genetic counseling is the process of providing individuals and families with information on the nature, inheritance, and implications of genetic disorders to help them make informed medical and personal decisions.

Achieving a family history is used in estimating breast cancer risk; it is appropriate to obtain a history of all cancers in biological relatives, especially breast and ovarian cancers. For each cancer, the age of onset, laterality, mode of treatment, and any possible related prior environmental exposures should be noted, if possible. Medical records, including pathology reports, are useful to confirm the history of cancer in the patient and relatives. The following aspects of the family history can be used to identify average-risk, moderate-risk, and high-risk individuals:

- Number of affected relatives
- Ratio of affected to unaffected relatives
- Age at cancer diagnosis
- Presence of bilateral/ multifocal breast cancer
- Presence of ovarian cancer

- Case(s) of male breast cancer
- The ethnicity of the family

The initial pedigree might provide invaluable information about hereditary breast cancer in a specific family. However, updating data on each follow up, may add relevant information. Accurate pedigree can play a valuable role in risk assessment for all probands in a family [1].

6.1.1 Pedigree analysis pitfalls

A clinician may be faced with certain problem in pedigree analysing and accurate risk assessment that cause delays or at least interfere with counselling of the consultand properly. These difficulties are:

1. Incomplete penetrance and variable expressivity of the gene
2. Small families and limited number of women at risk
3. Paternal transmission of disease
4. Death of key relatives before the age of development of breast cancer
5. Adopted individual with breast or ovarian cancer
6. Lack of entire family history due to immigration or other non communication reasons within the families
7. Inconsistency in the family history in following up and poor recall
8. Establishment of false family history of autosomal dominant transmission of breast cancer predisposing gene

6.1.2 Problems in identifying patients at risk of hereditary breast cancer

Identification of women at high risk of hereditary breast cancer begins by taking an accurate family history [2]. At particular risk are women with a family history of early-onset breast cancer, a family history of bilateral breast cancer, those with several affected family members and a family history of other malignancies at an early age. However, a family history of breast cancer may not be a reliable indicator of those who are likely to carry a germline mutation of a breast cancer susceptibility gene.

Approximately 6%-19% of individuals with breast cancer have an affected relative, but this does not reliably identify women who are likely to be carriers of germline alterations [3]. In large families a positive family history may reflect the high incidence of sporadic disease, and in small families the absence of family history does not rule out the presence of a genetic mutation [3]. BRCA1 mutations may also be transmitted through male relatives in a family with no male or female breast cancers being manifest [4]. Other individuals may exhibit de novo germline mutations and consequently have no family history of breast cancer [4]. Identification of BRCA1 carriers by family history may also be obscured by incomplete penetrance of this gene. Lifetime disease penetrance of BRCA1 is believed to be 88 per cent [5]. Carriers of BRCA1 may not be identified in the remaining cases, as they will not develop disease, thereby obscuring the pattern of inheritance.

6.1.3 Genetic risk assessment

Mutations in the BRCA1 and BRCA2 genes are inherited in an autosomal dominant manner. It is necessary to identify the specific cancer-predisposing mutation in an affected family member before molecular genetic testing of either the BRCA1 or BRCA2 gene can be used in genetic counselling and testing of a symptomatic at risk family members. Offspring of individuals with a BRCA1 or BRCA2 mutation have a 50% chance of inheriting the mutant gene [6]. Prenatal testing is possible for foetuses at 50% risk; however, requests for prenatal diagnosis of adult-onset diseases require careful genetic counselling.

Women who are at increased risk of breast cancer should preferentially attend a 'high-risk' clinic where facilities for accurate risk estimation, genetic counselling and testing exist.

6.1.4 Stratification of families at the risk of breast cancer

Pharaoh et al (1998) have classified families at risk of breast cancer into three categories [7]. Members of families in the high risk group are postulated to have a 20% or more chance for development of breast cancer due to highly penetrant BRCA1 gene. Moderate risk group has chance to develop breast cancer less than three times to the general population.

Table 6.1: The criteria for breast cancer risk stratification
(Pharoah et al. 1998)

High risk group
1. Families with 4 or more breast /ovarian cancer relatives on the same side of the family affected at any age.
2. Families with three affected breast cancer (only) relatives average age of diagnosis <40.
3. Families with three breast /ovarian cancer relatives, average age of breast cancer <60.
4. Families with a member affected of both breast and ovarian cancer.
Moderate risk group
1. Families with a first degree female relative with breast cancer diagnosed <40 years or first degree male relative with breast cancer diagnosed at any age, or a first degree paternal female relative with breast cancer diagnosed <60 years, or
2. With two first or second degree relative with breast cancer diagnosed <60 years on the same side of the family, or
3. With three first degree relatives with breast cancer at any age on the same side of the families, or
4. With a first degree relative with bilateral breast cancer diagnosed <60 years.
Low risk group
1. Women with a family history of breast cancer not fulfilling the above two sets of criteria.

6.1.5 Estimation of an individual's risk of developing breast cancer

The risk of an individual to develop breast cancer depends on the individual's family history and non-genetic risk factors. If the family history suggests autosomal dominant inheritance of breast or ovarian cancer risk, consideration is given to the use of BRCA1 and/or BRCA2 mutation analysis to evaluate risk status [8, 9, 10]. Such testing should only be considered after pre-test education and obtaining informed consent.

Methods to estimate an individual's risk for developing breast cancer have been developed, and studies to estimate the probability that an individual has a BRCA1/BRCA2 cancer-predisposing mutation have been performed. Both the risk of developing breast cancer and the probability of having a BRCA1/BRCA2 cancer-predisposing mutation depends to some extent on the family history of breast and/or ovarian cancer and by the age of onset [11, 12, 13].

For instance, the risk of breast cancer for a woman with one first degree relative affected with ovarian cancer is about 50% versus for a woman without family history of breast or ovarian cancer, whilst the risk of female breast cancer with a relative of a male breast cancer patient may increase approximately by twofold. Mutation screening test may be offered to the families with small number of breast or ovarian cancer, with at least one case of ovarian cancer, bilateral breast cancer or a case of breast cancer diagnosed before age of 40 [14].

Two approaches, one based on mathematical modelling and the second on genetic testing, are widely used to estimate an individual's risk of developing breast cancer in order to provide information for affected women at risk.

6.1.6 Mathematical models

6.1.6.1 The Clause model

The **Claus model** uses empiric data from the Cancer and Steroid Hormone Study and assumes that inherited risk is attributable to an autosomal dominant gene mutation with high penetrance [11, 13]. The risk estimate is based on a woman's current age and the number of first and second-degree relatives with breast cancer and their age of diagnosis. The Claus model provides the best available estimate of risk based on family history of breast cancer. The Claus model does not take into consideration any other factors known to increase breast cancer risk.

Risk assessment models are not suitable for families with distinct major gene mutation such as mutations in BRCA1 or BRCA2 genes. Members of such families need comprehensive genetic counselling and pedigree analysis. The risk assessment should be base on their relationship within the family and the prior probability of carrying the same mutant gene.

6.1.6.2 The Gail model

The **Gail model** [15] projects individualized probabilities of developing breast cancer using some of the known non-genetic risk factors as well as limited family history information. It is based on the major predictors of risk identified in the Breast Cancer Detection Demonstration Project study: current age, age at menarche, age at first live birth, number of previous breast biopsies, presence of atypical hyperplasia, and number of first degree relatives (mother or sister) with breast cancer [15]. The Gail model does not consider second-degree relatives, paternal relatives, or ages of diagnosis of breast cancer. It provides a useful estimate of risk for women without a family history of breast cancer, but would be expected to overestimate risk in women whose mothers or sisters had breast cancer diagnosed at an elderly age and underestimate risk for women who have second and third degree relatives with early breast cancer.

The Gail model is the basis for the Breast Cancer Risk Assessment Tool, a computer program that is available from the National Cancer Institute (NCI).

6.1.7 Probability of having a BRCA1/BRCA2 mutation

6.1.7.1 Probability based on personal history of breast cancer

Several studies have looked at the probability of detecting a BRCA1 mutation among women with a personal history of breast cancer, unselected for family history. In a population-based sample of women in western Washington State diagnosed with early-onset breast cancer, 6% of women diagnosed with breast cancer before age 35 years had a BRCA1 mutation, and 7% of women diagnosed before age 45 years and with at least one first-degree relative with breast cancer had a BRCA1 mutation [16].

6.1.7.2 Probability based on family history

Initial studies estimated that 50% of families with inherited breast cancer [17] and approximately 75% of families with inherited breast and ovarian cancer had a BRCA1 mutation [18]. From initial studies, BRCA2 mutations were estimated to occur in approximately 15-30% of families with inherited breast cancer [17] and an unknown proportion of families with inherited breast and ovarian cancer [18]. Subsequent clinical studies have identified cancer-predisposing mutations less frequently. These differences may reflect the criteria used to define a high-risk family and the sensitivity of molecular methods currently available, i.e., even families linked to BRCA1 or BRCA2 may not be found to have an identifiable mutation.

Subsequent studies have further attempted to determine the probability of a cancer-predisposing mutation among families with multiple cases of breast and/or ovarian cancer. In a multi-centre study, women were referred for BRCA1 and BRCA2 mutation analysis if they had been diagnosed with

breast cancer before the age of 50 years or ovarian cancer at any age and also had at least one first or second-degree relative with either diagnosis. Among this selected group, the likelihood of the proband having a BRCA1 or BRCA2 cancer-predisposing mutation was over 50% if she had breast cancer diagnosed before age 50 years and three or more of the following: bilateral breast cancer or ovarian cancer, a diagnosis of breast cancer before age 40 years, a relative with breast cancer diagnosed before age 50 years, or a relative with ovarian cancer [19].

6.1.8 Probability of developing breast cancer by age for the several present participants

In this study, due to insufficient participants' pedigree information, risk calculation of developing breast cancer using different models was not applicable. However, CancerGene (BRCApro) software version 3.3.2 was used to calculate the probability of developing breast cancer (BC) for the several unaffected participants who have strong family history of breast or ovarian cancer (see pedigree 6.1-6.5).

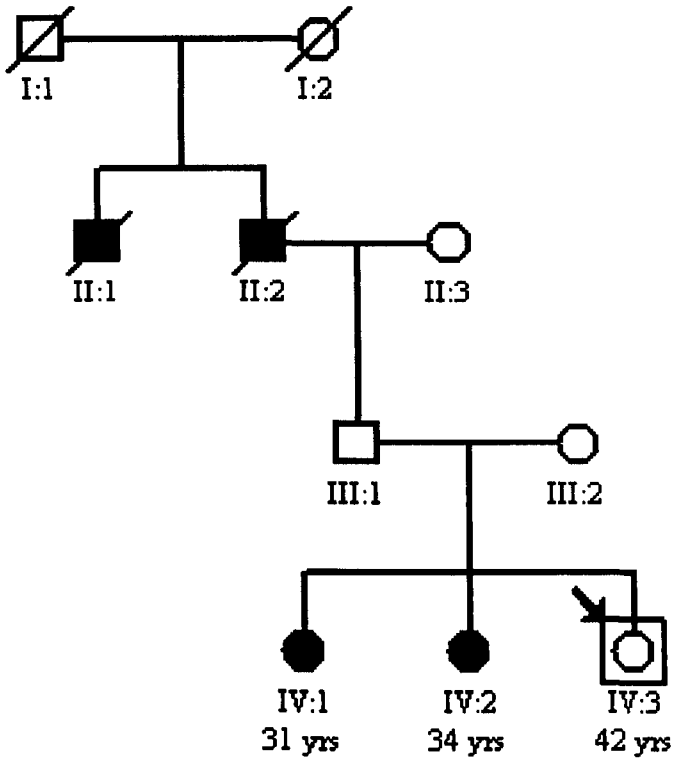
The CancerGene web address is:
http://www.swmes.edu/home_pages/cancergene/

Pedigree 6.1: File record-104UBF

The pedigree shows a proband IV:3 who is seeking advice.

Her two sisters IV: 1 and IV: 2 were diagnosed with breast cancer at the age of 31 and 34 respectively.

Her paternal grand father and her cousin II: 1 and II:2 died from colon cancer. The figure below depicts the probability of development breast cancer by ages for probands IV: 3.



Claus Family History Model

P104UBF
104

The Claus table used in this calculation is: Two first-degree relatives

29	39	49	59	69	79
1.8	6.2	14.8	26.5	37.1	43.7

Remaining Risk

Age	%
46	4.67
51	10.01
56	16.37
61	22.48
66	28.24
71	33.12
76	36.71

Probability of Developing Breast Cancer by Age

To Age 79: 38.86 %

As above diagram shows this proband (IV:3) has about 39% per cent risk of developing breast cancer by age of 79.

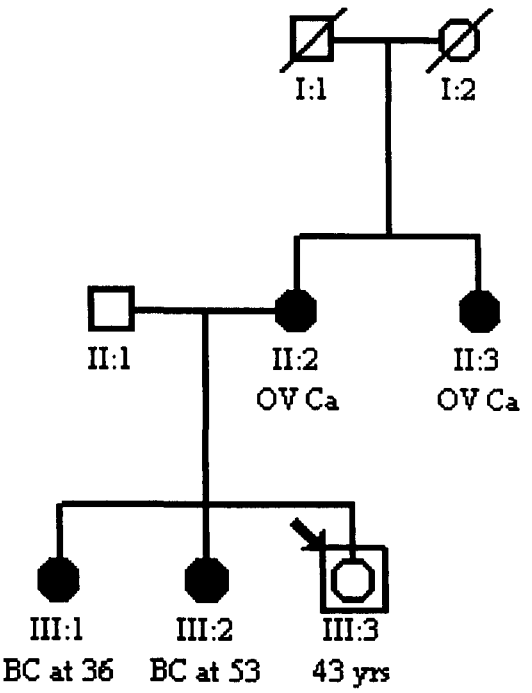
Pedigree 6.2: File record-120UBF

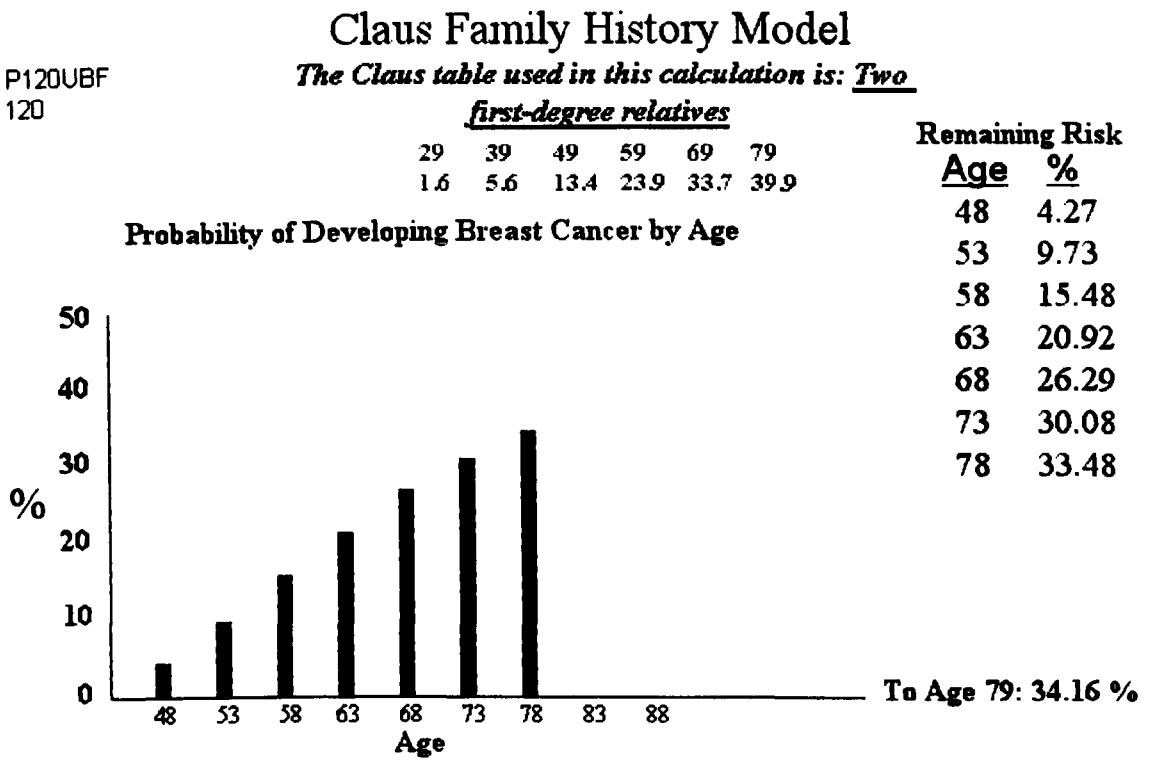
The pedigree shows a proband III:3 who is seeking advice.

Her mother II:2 and aunt II:3 both have been diagnosed with ovarian cancer.

Her two sisters III:1 and III:2 was diagnosed with breast cancer at the age of 36 and 53 respectively.

The figure below depicts the probability of development breast cancer by ages for probands III:3.





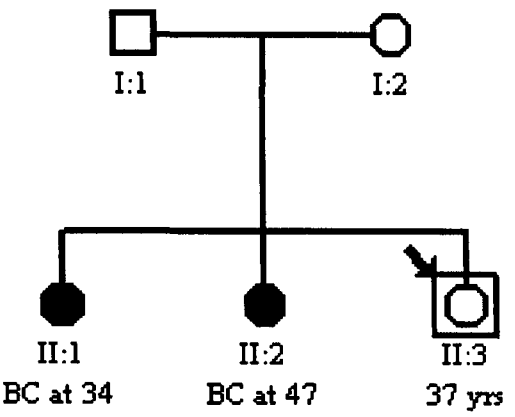
As above diagram shows this proband (III:3) has about 34% per cent risk of developing breast cancer by age of 79.

Pedigree 6.3: File record-134UBF

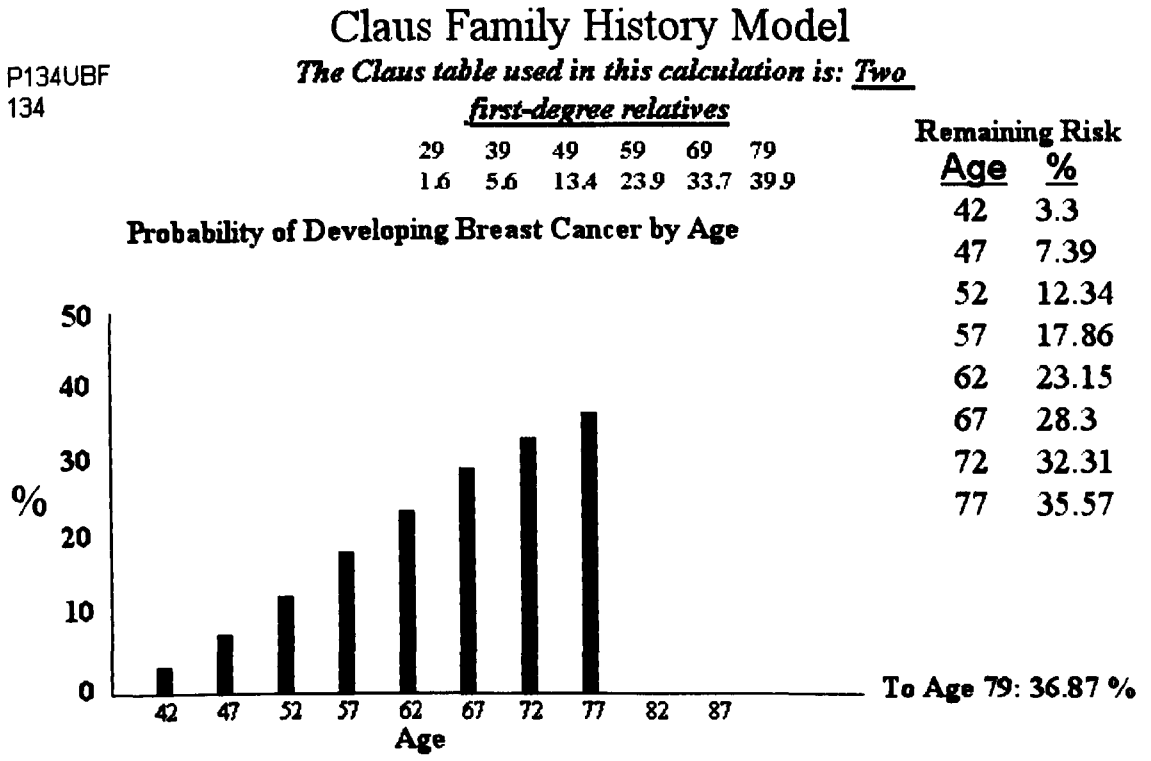
The pedigree shows a proband II:3 who is seeking advice.

Her two sisters II:1 and II:2 were diagnosed with breast cancer at the age of 34 and 47 respectively.

No more information was available to make pedigree analysis informative.



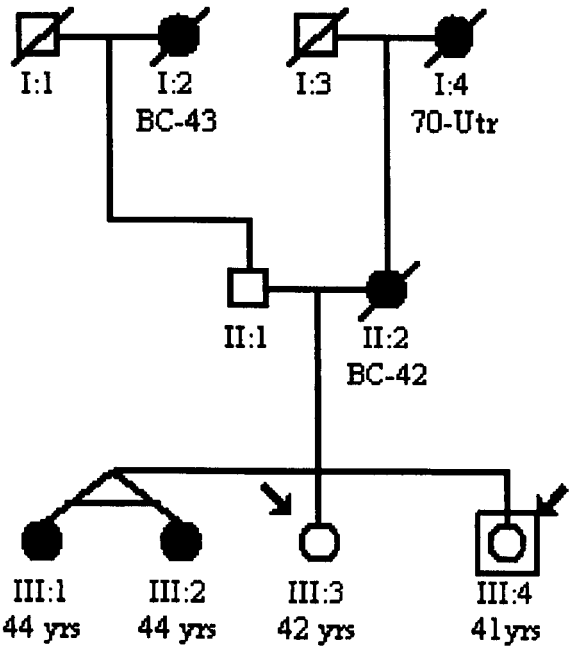
The figure below depicts the probability of development breast cancer by ages for probands II:3.

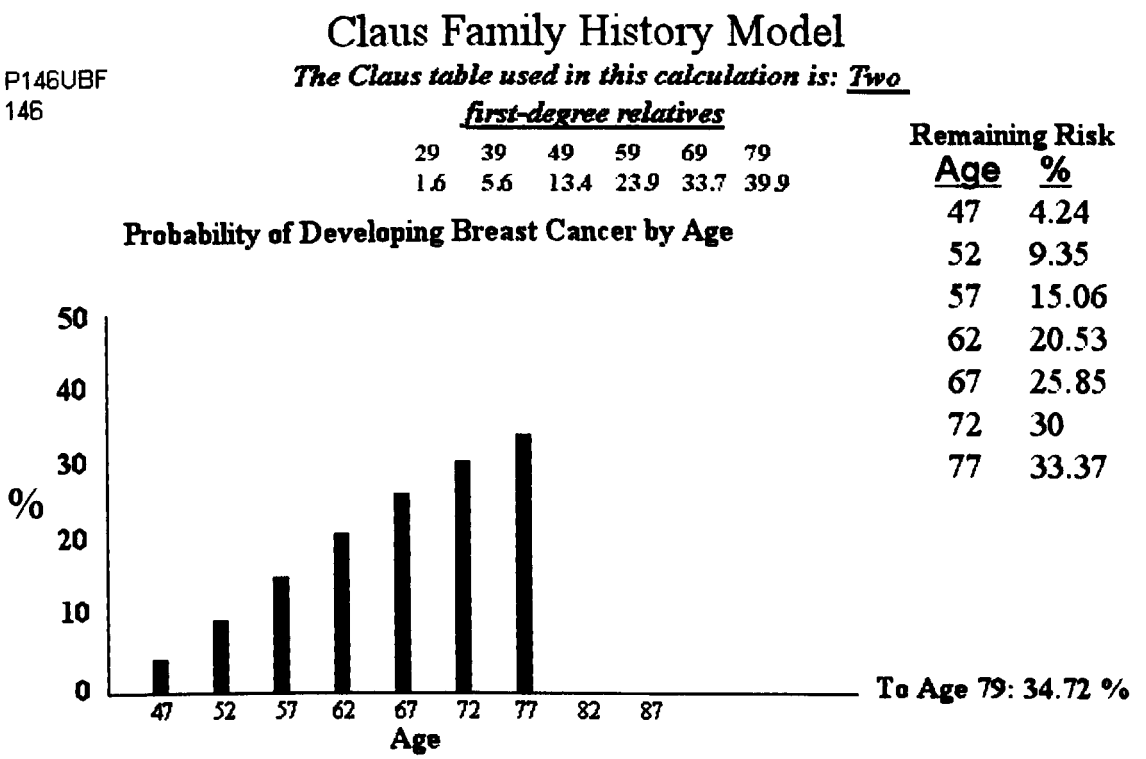


As above diagram shows this proband (II:3) has about 37% per cent risk of developing breast cancer by age of 79.

Pedigree 6.4: File record-146UBF

The pedigree shows a family with affected twins. Probands III: 3 and III: 4 are seeking a genetic test. Their sisters (twin) probands III: 1 and III: 2 who has been diagnosed with breast cancer at the age of <40. Their mother proband II: 2 died with breast cancer at the age of 42 years, maternal grandmother I: 4 died from uterine cancer at the age of 70 and paternal grandmother I: 2 died with breast cancer at the age of 43 years.





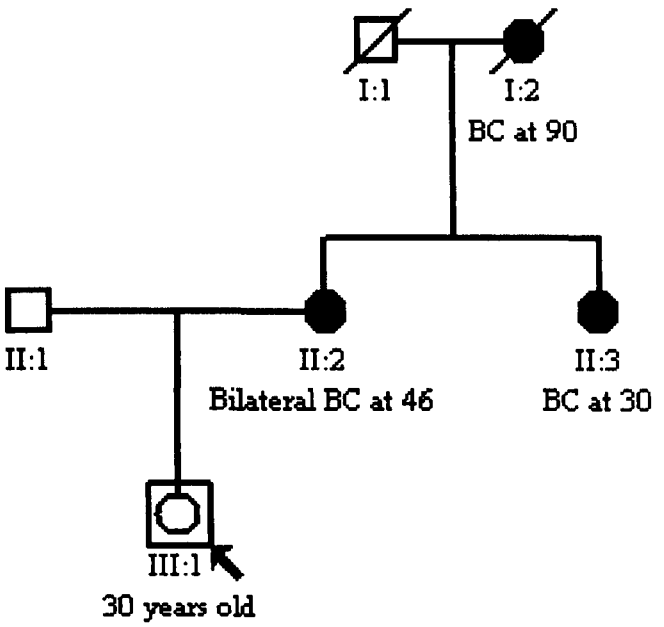
As above diagram shows these probands (III:3 or III:4) has about 35% per cent risk of developing breast cancer by age of 79.

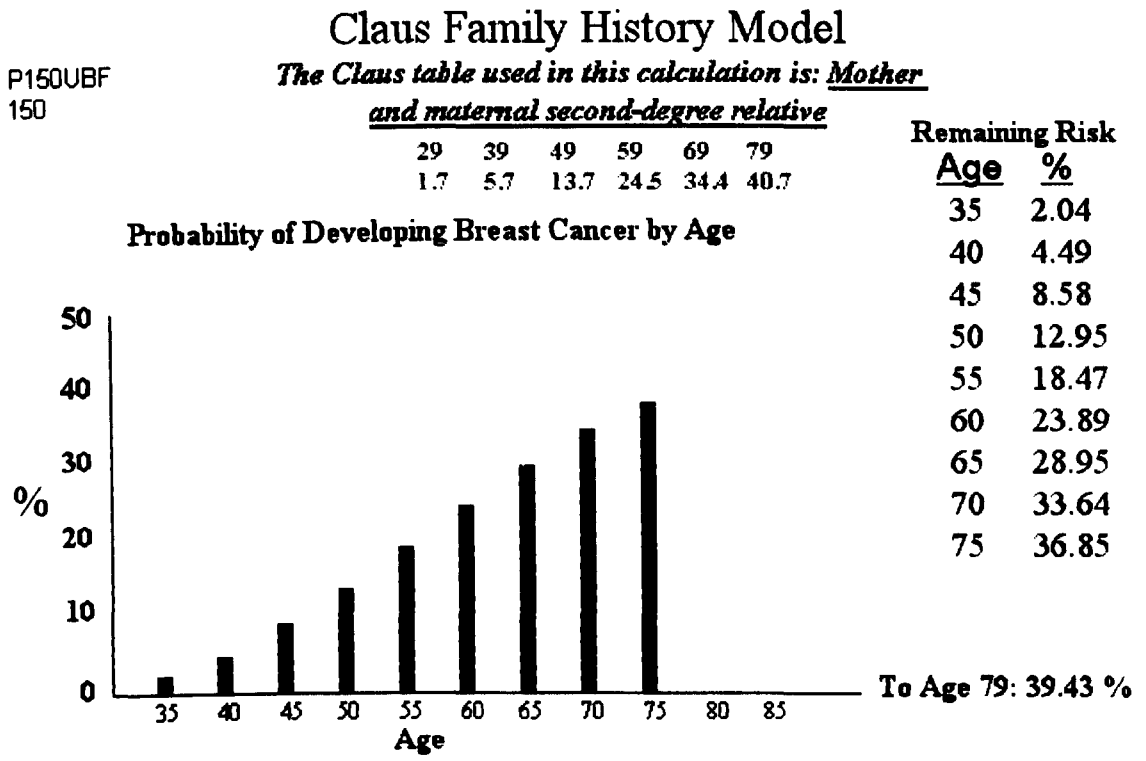
Pedigree 6.5: File record-150UBF

The pedigree shows a proband III:1 who is seeking advice.

Her mother II:2 and her aunt both were diagnosed with breast cancer at the age of 46 and 30 respectively. Her maternal grand mother I:2 died from breast cancer at age of 90.

The figure below depicts the probability of development breast cancer by ages for probands III:3.





As above diagram shows this proband (III:1) has about 40% per cent risk of developing breast cancer by age of 79.

6.1.9 Cancer risks associated with mutations in BRCA1 and BRCA2 genes

The penetrance of BRCA1 or BRCA2 mutations- or likelihood of cancer when a cancer-predisposing mutation is present - is the most significant clinical aspect of BRCA1 and BRCA2 mutations. The penetrance is uncertain and probably variable depending on the population used for analysis [20, 21, 22]. The reasons for differences in penetrance observed between studies could be methodological (pedigree-based studies by definition contain many affected individuals), stochastic (due to the play of chance within families and populations), biological (due to modifying genes in families with many cases of breast and/or ovarian cancer), or environmental (due to diet, smoking, and lifestyle).

The accumulated evidence indicates that some individuals with cancer-predisposing mutations survive to an elderly age without developing cancer. Among those who develop cancer, the age of onset, as well as the type of cancer, varies. No clear explanation exists for the observation that some individuals with a cancer-predisposing mutation may have multiple primary cancers before age 50 years, while others with the same cancer-predisposing mutation may not develop cancer until after age 70 years [23, 24]. These uncertainties are important to consider in pre-test counselling.

Germline mutations in BRCA1 have been identified in 15–20% of women with a family history of breast cancer and 60–80% of women with a family history of both breast and ovarian cancer [25, 26]. Female mutation carriers have a lifetime breast cancer risk of 60–80% [20, 21], although penetrance estimates as low as 36% have been reported in a series of Jewish breast cancer cases selected regardless to family history [22].

The median age of diagnosis in mutation carriers is 42 years, more than 20 years earlier than the median for unselected women in the US and

Western Europe [27]. Lifetime ovarian cancer risks are estimated at 20–40%, but, unlike breast cancer, age-specific penetrance is not heavily skewed toward early onset disease [28, 29].

The lifetime breast cancer risk for carriers of BRCA2 mutations also is estimated to be 60–85%, and the lifetime ovarian cancer risk is estimated to be 10–20%. However, unlike the situation with BRCA1, men with germline mutations in BRCA2 have an estimated 6% lifetime risk of breast cancer, representing a 100-fold increase over the male population risk.

6.1.9.1 The cumulative risks of breast cancer conferred by BRCA1 mutations

The initial studies of the penetrance of mutations in the BRCA1 gene involved BRCA1 mutation-positive families ascertained by the presence of multiple individuals (usually four or more) affected with breast cancer or ovarian cancer at any age. The cancer risks seen in these families are high and may over-estimate the risk within all families with BRCA1 mutations. The estimates of the cumulative risk of breast cancer for women with a BRCA1 mutation from these high-risk families are summarized in Table 6.2 [30].

Table 6.2: Cumulative risk by age of breast cancer in women from families with BRCA1 mutations

Age (Years)	Cumulative Risk
30 yrs	3.2%
40 yrs	19.1%
50 yrs	50.8%
60 yrs	54.2%
70 yrs	85%

Male breast cancer is rarely associated with a BRCA1 mutation; however, several families with male breast cancer have shown linkage to the BRCA1 locus [31].

6.1.9.2 The cumulative risks of breast cancer conferred by BRCA2 mutations

Women with BRCA2 mutations appear to have a breast cancer risk similar to that of women with BRCA1 mutations [31, 32, 33]. Current risk estimates are based on observations from high-risk families participating in research studies. The average age at which cancer occurs in women with BRCA2 mutations may be later than for women with BRCA1 mutations [31, 34]. The estimates of the cumulative risk of breast cancer for women with a BRCA2 mutation from high-risk families are summarized in Table 6.3.

Male breast cancer has been observed in families with BRCA2 mutations, including some families with multiple cases of male breast cancer and no cases of female breast cancer [35, 36, 37].

Table 6.3: Cumulative risk by age of breast cancer in women from families with BRCA2 mutations

Age (Years)	Cumulative Risk
30 yrs	4.6%
40 yrs	12%
50 yrs	46%
60 yrs	61%
70 yrs	86%

6.1.9.3 The risks of ovarian cancer conferred by BRCA1 and BRCA2 mutations

Although ovarian cancer represents only 4% of women's cancers, it causes more deaths than any other cancer of the female reproductive system. [38].

Overall, women have a 1/55 (almost 2%) chance of developing ovarian cancer by age 70 [39]. However, if a woman carries a BRCA1 mutation, the risk of ovarian cancer is substantially increased: by age 70, the reported risk of the disease in BRCA1-mutation carriers ranges from 16% to 44% [40, 41].

For BRCA2, family-based studies have estimated a risk of 27% by age 70, which is lower than risks obtained by comparable analyses for BRCA1 [31]. Although a lower ovarian cancer risk for BRCA2 is not supported by the population-based studies of Ashkenazim, there is evidence that the prevalent BRCA2 6174delT mutation in this population is associated with a higher risk of ovarian cancer than other BRCA2 mutations [42].

6.1.9.4 The risks of second cancers conferred by BRCA1 and BRCA2 mutations

Even for women who have already had breast cancer, genetic susceptibility testing may be of value. Women with breast cancer who carry BRCA mutations are at high risk of second cancers, which may arise in the ovary or as second breast tumours. For example, if a woman carries a BRCA1 mutation and has had breast cancer, her risk of developing a second breast cancer has been estimated to be as high as 64% by age 70 [40].

Similar figures are not available for BRCA2, but mutations in this gene are presumed to similarly increase the risk of a second breast cancer.

Women with breast cancer and germline BRCA mutations also are at increased risk of developing ovarian cancer [42]. The risk of these second cancers may be a reason for considering BRCA1 and BRCA2 testing in women who are diagnosed with breast cancer and whose family history indicates hereditary breast and ovarian cancer.

6.1.9.5 The risks of other cancers conferred by BRCA1 and BRCA2 mutations

Several other cancers are believed to form part of the BRCA1 and BRCA2 phenotype. Because so few cases are observed, risk estimates are imprecise but are clearly much lower than for female breast cancer and ovarian cancer. Increased risk for prostate and colon cancer have also been reported, with relative risks of 4.1 and 3.3, respectively [32, 43, 44]. An increased risk of prostate cancer was reported among carriers who harbouring 185delAG or 5382insC in BRCA1 gene [45].

Mutations in BRCA2 confer an elevated risk of male breast cancer, prostate cancer, pancreatic cancer, ocular melanoma, thyroid cancer, leukemia, and gall-bladder cancer [46].

6.1.10 Genetic testing

Following consultation (family history and pedigree analysis), genetic testing might be offered to the high-risk individuals who seeking advice individually and to their relatives at risk. In general, families may offered DNA testing if their breast and/or ovarian cancer incidence suggested a >10% prior probability of being due to BRCA1 mutation [47].

Molecular genetic testing for BRCA1 and BRCA2 cancer-predisposing mutations is now offered both as a clinical service and within research protocols. The specific DNA-based testing techniques performed by various laboratories may include: Protein Truncation Testing (PTT), Single Stranded Conformational Polymorphism (SSCP), complete direct sequencing, Conformation Sensitive Gel Electrophoresis (CSGE) [48], or a combination of these and/or other techniques. It is important to note that no currently available technique can guarantee the identification of all cancer-predisposing mutations in the BRCA1 or BRCA2 genes [31].

6.1.10.1 Benefits of genetic testing

Since early detection of breast cancer is one of the most significant factors in determining survival, it becomes extremely important to identify those women who are at greater risk [13, 49]. Genetic testing can help a pre-symptomatic individual from high-risk families to reveal her/his carrier status and relieve stress in an uncertain condition. In carriers it may help to choose an early preventive strategy prior to development of any related cancer. In affected individual it may effect on treatment strategy.

6.1.10.2 Testing strategy in a family

It is strongly recommended that the genetic test be offered to an affected individual prior to offering it to at-risk unaffected family members. Testing an affected individual is the most effective way of determining if a BRCA1 or BRCA2 mutation is causative of breast cancer within a family. After a cancer-predisposing mutation has been identified in an affected family member, BRCA1 or BRCA2 mutational analysis is more informative for unaffected relatives.

6.1.10.3 Pre-test education and counselling

The importance of providing education and obtaining informed consent prior to performing testing for cancer-predisposing gene mutations has been emphasized by several expert groups [50, 51, 52].

Pre-test education for patients includes discussion of the following suggested components:

- The patient's motivation for requesting testing and preconceived beliefs about the test
- The patient's perceptions of his/her risk of developing cancer
- The patient's readiness for testing and optimal timing for testing
- Alternatives to testing, such as DNA banking
- Inability of genetic testing to detect the presence or absence of cancer

- The patient's and family's support systems, and possible need for additional psychological support
- The patient's need for privacy and autonomy
- The possible effects of positive, negative, or uninformative test

6.1.10.4 Sensitivity of the test

The sensitivity of tests for detecting BRCA1 or BRCA2 mutations is dependent on the method used for DNA analysis and a prior risk of the person tested to have a mutation in either gene based on the person's cancer history, family history, and ethnic background.

6.1.10.5 Interpretation of positive test results for mutations of uncertain clinical significance

When full gene sequencing is the method used for testing, mutations of uncertain clinical significance may be identified, such as previously undescribed missense mutations that are not predicted to result in a loss of protein function. Such alterations present a dilemma for physicians who are making patient management decisions. Three methods are available to determine the clinical significance of these mutations: 1) family studies to determine whether the mutation segregates with cancer in family members (which is the most practical and clinically useful method); 2) allele frequency analysis to determine whether the allele has a higher frequency in cancer patients than in the general population; and 3) protein function assays to measure the effect of the mutation on the protein [53].

6.1.10.6 Interpretation of negative test results

When a BRCA1 or BRCA2 mutation cannot be identified in an affected individual from a family with an increased risk of an inherited predisposition to breast cancer, negative results are uninformative, and the possibility of a false negative test must be considered [50]; thus, the affected individual may still have an inherited cancer-predisposing mutation in the

BRCA1 or BRCA2 gene. She may also have a mutation in some other gene that predisposes to breast cancer.

If the affected family member has no identifiable BRCA1 or BRCA2 mutation or is unavailable for testing, all negative BRCA1 and BRCA2 test results in other family members must be considered uninformative.

An at-risk relative who does not have the BRCA1 or BRCA2 mutation identified in an affected family member is considered to have a true negative result. It is appropriate to advise the individual that a negative result does not reduce her cancer risk below that of the general population. Furthermore, if that person is from a high-risk ethnic group, e.g., of Ashkenazi Jewish descent, it may be prudent to test for all the cancer-predisposing mutations known to be common in that population, even if a single cancer-predisposing mutation has already been identified in an affected family member.

6.1.11 Patient decision making

Patient decision making about genetic testing and how to use such information is not a simple process. Even when a positive result (i.e., a deleterious mutation) is identified, the associated cancer risks cannot be precisely quantified and the efficacy of management options is still very uncertain. When a negative result is obtained, it could be good news or completely ambiguous and raise more questions than it answers. Addressing this issue is especially important because a significant proportion of high-risk families do not harbour deleterious mutations in BRCA1 or BRCA2 [31]. Thus even though interpretation of BRCA1/2 results is relatively straightforward in many circumstances, complex cases are not infrequently encountered in the clinical setting. In such instances, alternative explanations for test results need to be considered, additional

family members may need to be tested, or participation in research studies may be indicated.

The process of genetic counselling is critical both before and after testing to ensure that patients understand the potential medical and psychosocial implications of testing and are aware of available options and resources. A multidisciplinary approach to service delivery, which includes clinicians in genetics and oncology, can facilitate patients' decision making and provide continued access to information and support.

6.2 Breast cancer prevention

The best approach for reducing the high incidence and mortality associated with breast and/or ovarian cancer is the early detection of women at high risk. These women, once identified, can be targeted for more aggressive prevention. To date, certain preventional strategies are offered to the high-risk women. Prophylactic mastectomy and/or oophorectomy, chemoprevention and lifestyle modification are part of these strategies. However, advising carriers is complicated because some of them will not develop cancer. Many scientists now have agreed that there are other factors, both hereditary and environmental, that cause variation in risk among carriers; however, no factor had been identified definitely.

6.2.1 Breast self-examination

Breast self-examination (BSE), clinical examinations every six months, regular mammography and MRI are now suggested as a preventional policy worldwide.

Approximately 10% of breast cancers may be detected by only clinical examination. Breast self-examination could detect breast nodules up to 66% cases in some urban areas [54]. Therefore, BSE is quite cost effective; it reduces the false reassurance

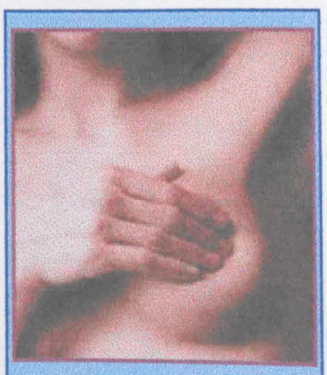


Figure 6.1: Schematic breast self examination

between regular scheduled mammogram and is more beneficial than other methods for women regardless their age.

Typical advice schedule for BSE:
"To check for breast changes, do a self-exam once a month. Use your eyes and hands to search for lumps, thickened areas, swelling and skin changes. Move your fingertips in a circular motion, going around each breast in a spiral pattern, then up under your armpit. Check both lying down and standing. Look in a mirror, arms at your sides and elevated, for changes in breast symmetry. Also check for any unusual nipple discharge."

6.2.2 Mammography

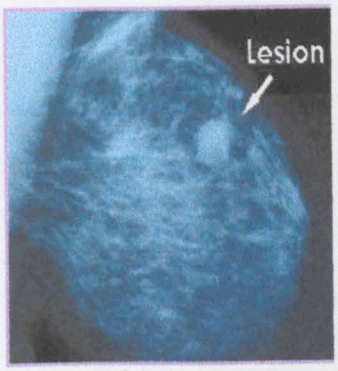


Figure 6.2:Mammogram with a lesion that might be breast cancer.

Since women may not be able to alter their personal risk factors, the best opportunity at present for reducing mortality is through early detection. The National Cancer Institute (NCI) recommends that women in their forties or older obtain screening mammograms on a regular basis; typically every one to two years [55].

Research has shown mammography to have a sensitivity (the ability to find cancer when the woman being screened has the disease) of up to 94%, and a specificity (the ability to find an absence of cancer when the woman being screened does not have the disease) of greater than 90%.

Digital images potentially could enhance the quality of the image and even magnify the view of specific areas of the breast. This technology is expected to improve the sensitivity of mammography, especially in radiographically "dense" breast tissue, which makes visualisation of cancer difficult, and to decrease the dose of radiation used per mammogram. Digital mammography also will allow computer-aided diagnosis and teleradiology.

- Mammograms can detect cancer up to two years before a lump can be felt by a manual exam.
- Mammography and breast self-exam guidelines could reduce deaths from breast cancer in women over age 50 by as much as 35 per cent, and in women in their 40s by 17 per cent.
- Regular screening mammograms every one to two years if you are age 40 or over.
- Education for monthly breast self-examination.

Mammography in younger women is less accurate. It usually reveals only dense breast tissue that may mask an underlying tumour.

Mammography is more accurate in women over 50 whose glandular breast tissue has become atrophic. As women age, glandular tissue disappears, becomes atrophic and is replaced by fat.

In UK, the national breast-screening programme offers mammographic screening every three years for women between the ages 50 and 64. The compound data analysis proved that regular mammography might decline mortality rate of breast cancer by 30%-40% result in early detection [49, 56, 57]. However, Fletcher et al (1993) reported that there was no benefit of mammography for women under the age 50 years. In contrast, the American Cancer society, recommended mammography for women at the age of 40-49 years [58]. Extending screening to cover women aged 40-49 years remains controversial, since the cost effectiveness of this plan is almost five times that in older women [59]. As about 5% of women who screened reveal abnormal mammogram, 10%-20% of which proved to had cancer. In total, it is not agreeable to offer mammography to all women with positive family history of breast cancer.

6.2.3 Prophylactic surgery

Inherited mutations in the BRCA1 and BRCA2 genes predispose women to both breast and ovarian cancers, often at young ages. Furthermore, women with breast cancer who carry a BRCA1 or BRCA2 mutation develop second contralateral breast cancers and ovarian cancers at higher rates than do women with breast cancer who lack a germline BRCA1/2 mutation. For young women with breast cancer and high-penetrance BRCA1/2 mutations, the lifetime probability of developing a contralateral breast cancer may be as high as 65%, and the probability of developing ovarian cancer may exceed 40% [28, 31]. In comparison, young women with breast cancer who lack these mutations face approximate lifetime risks of 10% for contralateral breast cancer and 2% for ovarian cancer [60, 61, 62].

While intensified screening may identify cancers at a favourable stage, it cannot prevent them. Therefore, women with BRCA1/2 mutation-associated breast cancer may consider prevention strategies such as tamoxifen, prophylactic contralateral mastectomy (PCM), or prophylactic oophorectomy (PO) to reduce their risk for second cancers. Although the efficacy of these approaches for BRCA1/2 mutation carriers is not certain, interim estimates can be extrapolated from studies of women at high risk for breast and ovarian malignancy. These data suggest that PCM may reduce breast cancer risk by 90% [63], PO may substantially reduce ovarian cancer risk [64], and 5 years of tamoxifen use may decrease contralateral breast cancer risk by 47% [65].

However, complete prevention of breast cancer seems to be a difficult task, as development of carcinoma in the residual breast tissue after prophylactic mastectomy has been widely reported.

There is insufficient evidence for or against prophylactic oophorectomy as a measure for reducing ovarian cancer risk among BRCA mutation carriers. Women with BRCA1 mutations should be counselled that this is an option

available to them. The uncertainty of current methods for detecting early ovarian cancer, and the high mortality rate associated with this cancer, has been cited as reasons to consider prophylactic oophorectomy [66].

In conclusion, neither prophylactic mastectomy nor prophylactic oophorectomy provides complete protection against the subsequent development of malignant disease and so ongoing surveillance is still required. Obviously, women should be extensively counselled about the matters before any prophylactic surgery [67].

6.2.4 Chemoprevention

The option of chemoprevention of breast cancer in gene carriers has been proposed as an alternative to prophylactic surgery. Potential primary prevention medications, such as tamoxifen [68] and raloxifene [69], reduce breast cancer risk. For women who have already had one primary breast cancer, the risk of a second primary tumour in the contralateral breast is reduced by as much as 40-47 per cent by adjuvant tamoxifen [65, 70].

6.2.5 Lifestyle factors

Numerous studies have identified potentially modifiable non-genetic risk factors for breast and ovarian cancer, including reproductive factors, high levels of dietary fat, high alcohol consumption, low antioxidant vitamin intake, excessive radiation exposure and decreased physical activity [71].

6.3 References

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Chapter 7

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7 District Health System in I.R. Iran

7.1 Introduction

The Islamic Republic of Iran with an area of 1,648,000 sq. km has located in the Middle East area. Tajikistan, Turkmenistan, Azerbaijan and Armenia border it in the north, Afghanistan and Pakistan in the east, and Turkey and Iraq in the west. It has access to the Caspian Sea in the north and the Persian Gulf and the Oman Sea in the south, place Iran in very important geopolitical area among other Asian countries (Figure 7.1).

More than half of the country is covered by mountains, one-quarter by deserts and less than one-quarter in under cultivation. The country is divided into 30 provinces, 260 districts and more than 66000 villages. Tehran is a growing capital city with a population of 7.5 million.

According to the latest census, conducted in 1996, the population of Iran is about 62 million of which 62% are settled in urban areas and 38% in rural communities. 42% of the population are under 15 years old and about 4% are above 65 years old or more [1].

Figure 7.1: Iran geographical boundaries and the location of main universities (Marked with red star) across the country

7.2 Medical Sciences Education and Health Care System

In over past twenty years Iranian government established an exemplary Primary Health Care (PHC) system that covers all of the country [2] which is recently developed to the District Health System (DHS) [3]. At least one University of Medical Sciences and Health Care services in each province is responsible for training medical and paramedical personnel, providing Health Care Services and conducting research activities.

Diagram 7.1 shows the organisation of DHS in Iran in an assumed province. On this diagram, PHC out patient services have only been shown.

"The Health House" is the most peripheral **rural** facility in the "Health Network", covering an average of 1500 population. Usually a male and a female named **Behvarz** are selected from among the young and interested natives and are trained in a **"Behvarz Training Centre"**, which is supervised by the **"District Health Centre"**. The training course in this boarding school lasts two years. The main duty of Behvarz is the provision of PHC services to a well-defined rural population. Behvarzes, in collaboration with the Community and the other Public Sectors, identify the target population including mothers, children, students, workers, and eligible females for Family Planning Programmes and then provide them the PHC services. In case of necessity, Behvarzes meet the first needs of the patients and refer them to the **"Rural Health Centre"**. The "Rural Health Centre" is a village-based facility, covering approximately six "Health Houses". It is staffed by a physician, several health technicians and administrative personnel, who work under the physician's leadership. The "Rural Health Centre" accepts referral patients from "Health Houses" and provide them with more specified services. At present nearly 16000 "Health Houses" and 2600 "Rural Health Centres" provide about 90% of rural inhabitants with PHC services. The private sector is not active in the rural areas.

"Health Post" and **"Urban Health Centre"** provide the **urban** population with the PHC and out patient services. There are about 360 "Health Posts" and 1800 "Urban Health Centres". Each centre covers a population of 12500. "Women Health Volunteers" (WHVs) by providing the urban population with PHC services activate the role of "Health Posts" and "Urban Health Centres". The private sector is also very active in providing the urban community with the out patient services. Therefore 100% of urban inhabitants have access to PHC services.

"District Health Centre" and **"Provincial Health Centre"** are responsible for the management of the related units in district and provincial level respectively.

Education and research departments in the university use the Health Unit and community for the purposes of medical education and health researchers.

7.2.1 Primary Health Care and "Community Participation"

By initiating the **"Women Health Volunteers"** (WHVs) project in Tehran in 1990, the most significant form of "Urban Community Participation" in PHC activities has been manifested. It was attempted to motivate the women from the target communities to activate the role of the Health Centres in providing PHC services to the population of those urban communities.

Each WHV is in charge of 50 households and has regular contacted with the "Urban Health Post" or "Urban Health Centre". The training modules for WHVs, prepared by the Government with support of UNICEF, provide them with proper knowledge to present identified services to their under coverage households.

At the present time, there are 40000 WHVs in the urban areas covering 9 million inhabitants (25% of urban population) [3].

7.2.2 Primary Health Care and "University"

In 1986, the management of the "University of Medical Sciences" integrated with those of "Health Providing Units". This integration presents the knowledge and the experiences of health system managers, health personnel and academician to the other health sectors.

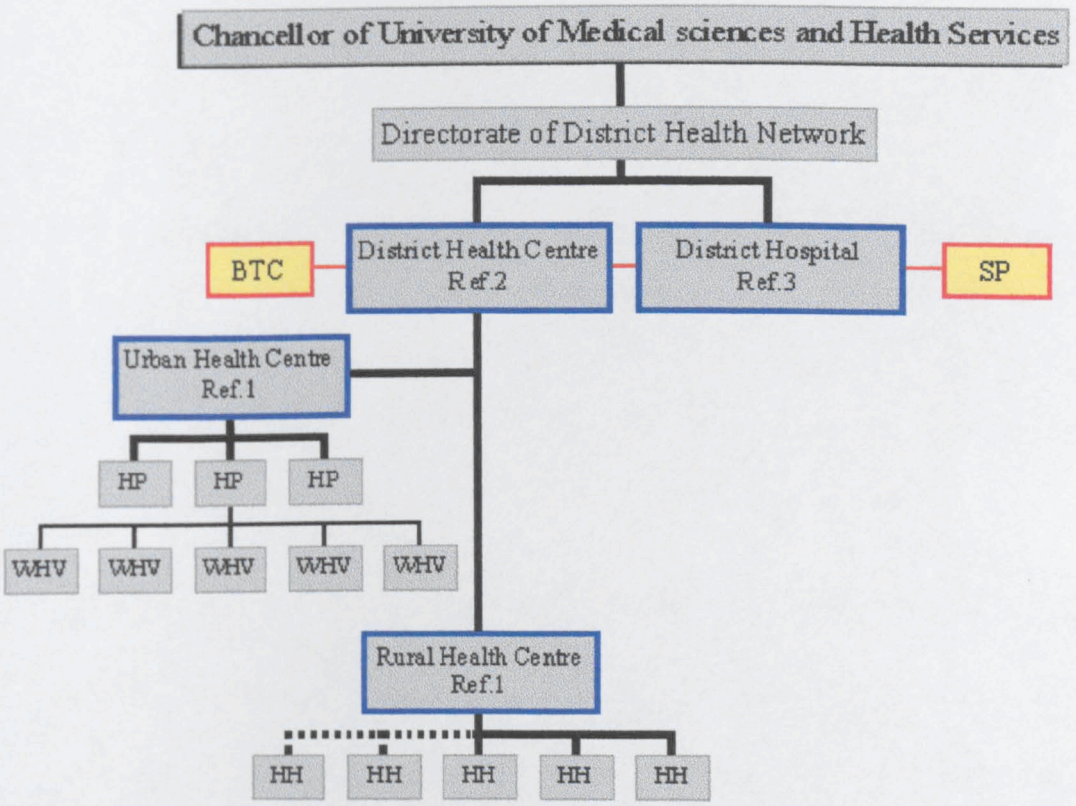
Diagram 7.1 shows a referral pathway system within DHS governs by the University of Medical Sciences.

Diagram 7.1: Organisation of District Health System in Islamic Republic of Iran

The diagram shows the referral pathway of public health system in Iran that is under the management of "Chancellor of University of Medical Sciences and Health Services".

Health services may be offered at three different referral levels, in urban and rural areas, which begin by health staff called Behvarz and Women Health Volunteers at Health House and Health Post respectively. The last referral centre, the District Hospital is linked to Specialised Polyclinic. The diagram depicts only Primary Health Care (PHC) system including ambulatory services and does not include the other activities such as education, hospitalisation services, research, logistic and cultural activities.

Diagram 1: District Health System in I.R. Iran



BTC: Behvarz Training Centre
HP: Health Posts
HH: Health Houses
SP: Specialised Polyclinic
WHV: Women Health Volunteers
Ref. : Referral centre

7.3 History of cancer registration in Iran

Cancer registration activity dates back to 1968 when the first cancer registration program was launched as a collaborative research agreement between the International Agency on Cancer Research (IARC) and the Institute for Health Studies affiliated to the Tehran University School of Public Health Tehran-Iran, in order to study the high incidence rate of oesophageal cancer in the province of Mazandaran-Iran [4]. Activity ceased in early 1980 and then in 1991, the registry started again to collect data on cancer cases referred to local cancer treatment facilities. In 1993, the Cancer Institute affiliated to Tehran University of Medical Sciences with a grant from Ministry of Health and medical Education initiated a cancer registry program, to assist public health authorities in different regions of the country to establish regional population based cancer registries [5].

At the present time three registries cover defined populations; 1) Tehran metropolitan area cancer registry which covers a population of 6,000,000 people residing in 22 municipal districts of Tehran municipality, 2) Mazanderan registry which covers a population of 2,600,000 residing in Mazanderan province, and 3) Fars registry that covers a population of 3,100,000 residing in Fars province [6].

7.4 Current knowledge of the prevalence and incidence rate of breast cancer in Iran

While breast cancer is one of the biggest health problems worldwide, the prevalence and the incidence may differ from country to country. However, the incidence of breast cancer is much higher in developed countries than in less developed countries (Table 7.2) [7]. Oesophageal cancer is the most common cancer in Iran whereas breast cancer is the most common cancer in the world [6] (Tables 7.2-7.6). One of the most important factors, which might have influence on this, is the number of unregistered cases of breast

cancer in the less developed countries. However, breast cancer is a multifactorial disease and results from genetic and environmental influences.

The prevalence and incidence rate of breast cancer in Iran have been reported by different sources, Centre for Disease Control (CDC)-Iranian Ministry of Health [8], the Cancer Institute affiliated to Tehran University of Medical Sciences, Tehran-Iran) [6] and the IARC [9] but it does not necessarily reflect the current picture of the breast cancer in Iran. Indeed, there are some differences between these reports (Table 7.1).

The IARC data for cancer in Iran were estimated using the approximate incidence of cancer at all sites, from cancer registries in the same region, together with some old data - 20 years old - on the frequency of cancers at different sites, from hospitals in Tehran and elsewhere.

Recent relative frequency data show that breast cancer constitutes 20% of cancers among Iranian women [5, 10, 11]. Breast cancer has a uniform geographic distribution in Iran, a higher incidence is seen where urbanization has grown faster [6]. A preliminary study has shown no significant differences in trend of age-specific incidences among Iranian women with breast cancer in compare with UK [6, 12] (Figure 7.2 and Table 7.2).

Infiltrating ductal carcinoma is the most frequent (more than 60%) histological type in Iran [5, 10, 11]. A preliminary analysis of survival data 1023 cases of primary breast cancer referred to the Tehran Cancer Institute from 1990 has shown a median survival time of 75 months and five years survival rate of more than 60% [13].

Table 7.1 The new cases and incidence rate of female breast cancer in Iran from different sources of data

Source	New cases	Incidence rate *	
		Crude	ASR (W)
Iranian Ministry of Health (CDC)- 1996	928	3.14	---
Local researcher –2000	2943	8.0	12.3
IARC-2001	3491	10.46	14.76

1) The column marked with * show the incidence rate (per 10^5 persons) of female breast cancer whilst another depicts the number of new cases.
2) Adapted from GLOBACAN 2000: Cancer Incidence, Mortality and Prevalence Worldwide, Ver. 1.0. IARC CancerBase No.5. Lyon, IARC Press. 2001.

Table 7.1 shows the different figures for the incidence rate of breast cancer from different sources. CDC has been noted (1996); breast cancer incidence is achieved among 11025 new cases of cancer, which were estimated from 1/5 of expected data. However, these numbers have been estimated in different periods. It seems more studies are required to produce accurate estimates.

Figure 7.2: A distribution of estimated age-specific incidence rates of female breast cancer compared in Iran and UK

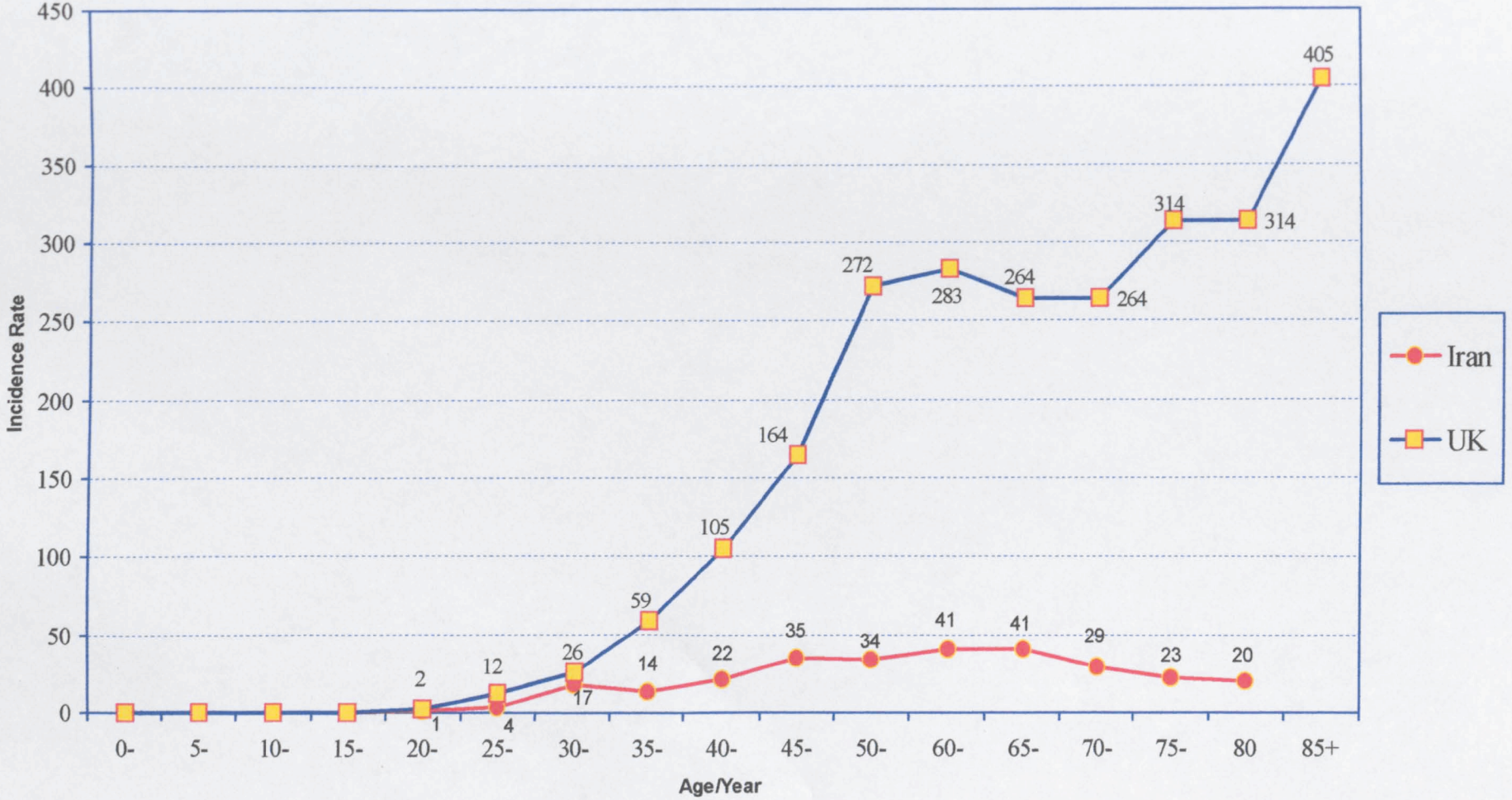


Table 7.2 A distribution of estimated age-specific incidence rates of female breast cancer compared in Iran and UK

Country	Age-specific distribution /Year															
	0-	5-	10-	15-	20-	25-	30-	35-	40-	45-	50-	60-	65-	70-	75-	80-	+80
	Incidence rate ✞																
Iran*	0	0	0	0	1	4	17	14	22	35	34	41	41	29	23	✹	✹
UK✠	0	0	0	0	2	12	26	59	105	164	272	283	264	264	314	314	405

✞ For cancer, the incidence rate is usually expressed as an annual rate per 100,000 persons at risk.

* Age-specific distribution incidence rate from ref. 7

✹There are no data available for this period of age.

✠ Adapted from UK National Statistics web site at http://www.statistics.gov.uk/ (based on data from 1999, reviewed in March 2002)

Table 7.3: The new cases and incidence rate of four common female cancers in Iran compared to other countries

Region	New cases	Incidence rate *		New cases	Incidence rate *		New cases	Incidence rate *		New cases	Incidence rate *	
	Oesophagus	Crude	ASR (W)	Breast	Crude	ASR (W)	Cervix uteri	Crude	ASR (W)	Stomach	Crude	ASR (W)
Islamic R. of Iran	4451	13.34	21.19	3491	10.46	14.76	2059	6.17	8.98	1312	3.94	6.27
United Kingdom	2800	9.35	4.25	34815	116.27	74.93	3537	11.81	9.34	3579	11.9	65.5
Less developed countries	117092	4.89	6.16	471063	19.66	23.07	379153	15.83	18.73	192850	8.05	9.97
More developed countries	16253	2.66	1.34	579285	94.93	63.22	91451	14.99	11.35	125029	20.49	10.96
World	133342	4.44	4.45	1050346	34.94	35.66	470606	15.66	16.12	317883	10.58	10.38

1) The columns marked with * show the incidence or mortality rate (per 10^5 persons) of different types of cancers whilst others depict the number of new cases.

2) Adapted from GLOBACAN 2000: Cancer Incidence, Mortality and Prevalence Worldwide, Ver. 1.0. , IARC Cancer Base No. 5. , Lyon, IARC Press. 2001.

Table 7.3: Shows the incidence rate of four common female cancers in order of incidence preferences in Iran and other countries. The oesophageal cancer is the most common female malignancy in Iran (it is even much higher if it is account for both sexes) whilst in UK the incidence of breast cancer is twelve fold higher than oesophageal cancer (crude rate=116.27 vs 9.35 respectively).

However, in general, breast cancer is the most common malignancy worldwide (See also chapter 1 Figure 1.11).

Table 7.4: The new cases/deaths and incidence/mortality rate of six common female cancers in Iran

Cancer type	Cases	Incidence rate*		Deaths	Incidence rate*	
		Crude	ASR (W)		Crude	ASR (W)
Oesophagus	4451	**13.34**	21.19	3940	**11.81**	18.64
Breast	3491	**10.46**	14.76	1563	**4.69**	6.71
Stomach	1312	3.94	6.27	1122	3.36	5.38
Cervix uteri	2059	6.17	8.98	1097	3.29	4.96
Colon/Rectum	1314	**3.94**	5.69	849	**2.55**	3.67
Lung	289	0.87	1.41	266	0.8	1.3

1) The columns marked with * show the incidence/mortality rate (per 10^5 persons) of different types of cancers whilst others depict the number of new cases/deaths.

2) Adapted from GLOBACAN 2000: Cancer Incidence, Mortality and Prevalence Worldwide, Ver. 1.0. , IARC Cancer Base No.5, Lyon, IARC Press. 2001.

Table 7.5:The new cases/deaths and incidence/mortality rate of six common female cancers in the UK

Cancer type	Cases	Incidence rate*		Deaths	Incidence rate*	
		Crude	ASR (W)		Crude	ASR (W)
Breast	34815	**116.27**	74.93	14415	**48.14**	26.81
Colon/Rectum	15924	**53.18**	25.28	9047	**30.21**	13.76
Lung	13423	44.83	21.76	13231	44.19	21.06
Stomach	3579	11.96	5.5	3199	10.69	4.76
Cervix uteri	3537	11.81	9.34	1906	6.37	3.91
Oesophagus	2800	**9.35**	4.25	2640	**8.81**	3.95

1) The columns marked with * show the incidence/mortality rate (per 10^5 persons) of different types of cancers whilst others depict the number of new cases/deaths.

2) Adapted from GLOBACAN 2000: Cancer Incidence, Mortality and Prevalence Worldwide, Ver.1.0. , IARC Cancer Base No.5. , Lyon, IARC Press. 2001.

Table 7.6: The new cases/deaths and incidence/mortality rate of six common female cancers in the world

Cancer type	Cases	Incidence rate*		Deaths	Incidence rate*	
		Crude	ASR (W)		Crude	ASR (W)
Breast	1050346	**34.94**	35.66	372969	**12.41**	12.51
Cervix uteri	470606	15.66	16.12	233372	7.76	7.99
Colon/Rectum	445963	**14.84**	14.44	237595	**7.9**	7.58
Lung	337115	11.22	11.05	292700	9.74	9.53
Stomach	317883	10.58	10.38	241352	8.03	7.81
Oesophagus	133342	4.44	4.45	110600	3.68	3.65

1) The columns marked with * show the incidence/mortality rate (per 10^5 persons) of different types of cancers whilst others depict the number of new cases/deaths.
2) Adapted from GLOBACAN 2000: Cancer Incidence, Mortality and Prevalence Worldwide, Ver. 1.0. , IARC Cancer Base No.5. , Lyon, IARC Press. 2001.

Tables 7.4 - 7.6 compare the incidence and the mortality rate of six common female cancers in Iran, UK and world.

The oesophageal cancer is the most common female malignancy in Iran; the incidence and mortality rates reach to 1.3 and 2.5 fold respectively than breast cancer.

In UK the incidence rate of breast cancer 12 fold higher than oesophageal cancer (crude rate=116.27 vs 9.35) and its mortality rate rise to 5.5 fold, whilst colorectal cancer is the second common cancer in British female.

The incidence rate of breast cancer is 2.4 fold higher than colorectal cancer and their mortality rates are relatively equal worldwide. Therefore, oesophageal cancer is not a common malignancy.

Table 7.7: Comparison of the new cases/deaths and incidence/mortality rate of female breast cancer in Iran and other countries

Region	Cases	Incidence rate*		Deaths	Incidence rate*	
		Crude	ASR (W)		Crude	ASR (W)
Islamic Republic of Iran	3491	10.46	14.76	1563	4.69	6.71
United Kingdom	34815	116.27	74.93	14415	48.14	26.81
Less developed countries	471063	19.66	23.07	183768	7.67	9.12
More developed countries	579285	94.93	63.22	189203	31.01	18.61
World	1050346	34.94	35.66	372969	12.41	12.51

1) The columns marked with * show the incidence/mortality rate (per 10^5 persons) of different types of cancers whilst others depict the number of new cases/deaths.

2) Adapted from GLOBACAN 2000: Cancer Incidence, Mortality and Prevalence Worldwide, Ver. 1.0. , IARC Cancer Base No. 5. , Lyon, IARC Press. 2001.

Figure 7.3: The incidence and the mortality rate (Crude rate) of female breast cancer in Iran and other countries

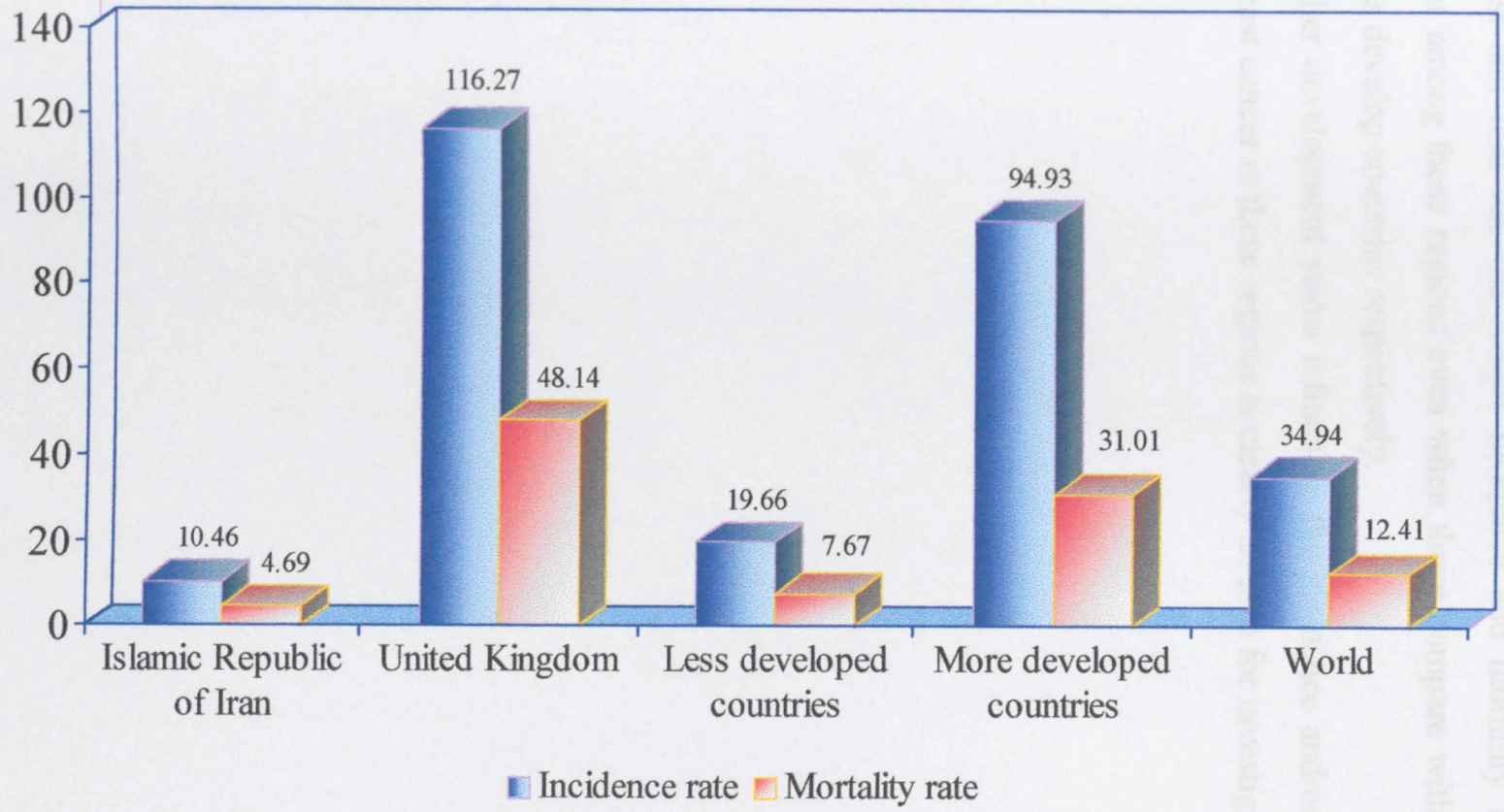


Table 7.7 and figure 7.3 reveal the incidence and the mortality rate of female breast cancer in Iran and other countries compared. UK has the highest and Iran has the lowest incidence and mortality rates of breast cancer among these regions even when those compare with more develop or less develop countries respectively.

Whether development status influences the incidence and/or mortality rate of breast cancer in these regions is clearly an area for investigation.

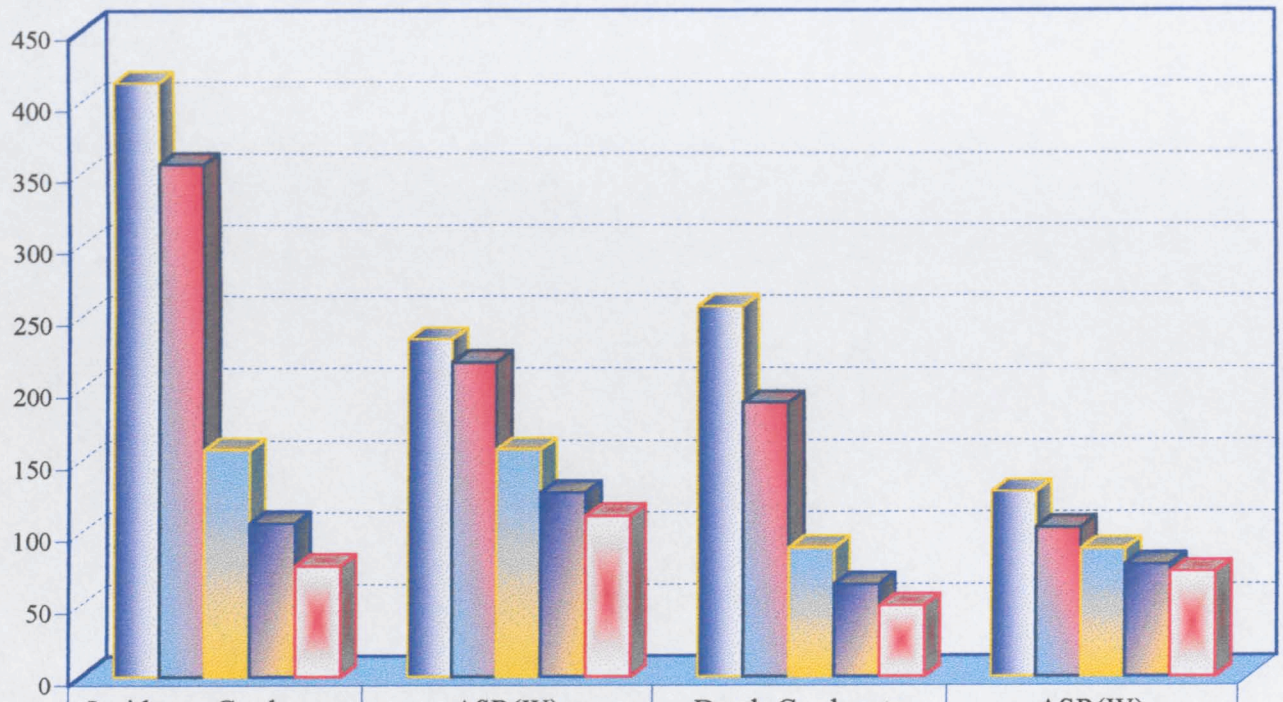
Table 7.8: Comparison of the new cases/deaths and incidence/mortality rate (Crude rate) of all types of female cancer (but not skin) in Iran and other countries

Region	Cases	Incidence rate*		Deaths	Incidence rate*	
		Crude	ASR (W)		Crude	ASR (W)
United Kingdom	123876	**413.69**	234.25	76923	**256.89**	128.05
More developed countries	2175974	356.59	218.31	1157634	189.71	103.08
World	4737646	**157.62**	157.84	2686313	**89.37**	88.3
Less developed countries	2561666	106.93	127.9	1528670	63.81	77.48
Islamic Republic of Iran	25799	**77.34**	111.77	16362	**49.05**	72.57

1) The columns marked with * show the incidence/mortality rate (per 10^5 persons) of different types of cancers whilst others depict the number of new cases/deaths.

2) Adapted from GLOBACAN 2000: Cancer Incidence, Mortality and Prevalence Worldwide, Ver. 1.0. , IARC Cancer Base No. 5. , Lyon, IARC Press. 2001.

Figure 7.4: Comparison of the incidence and the mortality rate of all types of female cancer (but not skin) in the UK, World and Iran



	Incidence-Crude rate	ASR(W)	Death-Crude rate	ASR(W)
United Kingdom	413.69	234.25	256.89	128.05
More developed countries	356.59	218.31	189.71	103.08
World	157.62	157.84	89.37	88.3
Less developed countries	106.93	127.9	63.81	77.48
Islamic Republic of Iran	77.34	111.77	49.05	72.57

As can be seen in Table 7.8 and Figure 7.4, UK has the highest incidence and the mortality rate (crude rates) of female malignancy in the regions compared. Since in most other countries of the developing world, Iran possess a conspicuously "young" population, with over 42% of the population under the age of 15 with about 4% above 65 years old or more, these rates have been estimated to be lower in Iran than in other regions, although these figures seem to be dominated by age distribution (Age pyramid) in these regions.

7.5 Breast cancer policy

Taking all these figures together, what is the most appropriate health policy for breast cancer screening in Iran?

In the first step, any given country should make a decision individually based on their own criteria such as female age distribution, socio-economic circumstances, culture and religion, priority of public health services including common cancers and common diseases, geographical situation, resources (personnel, finance, logistics, knowledge, etc), availability of technology and finally political conditions.

The government plays a basic leadership role in such national programmes and their support is an absolute necessity. Community leaders/organisations, media (TV, newspapers), schools and universities should also be encouraged to participate.

An understanding of the prevalence and incidence rate of breast cancer is a critical point. In order to obtain an accurate picture of the prevalence and incidence rate of breast cancer in Iran, cancer registration as part of a national health programme should be carry out more vigorously.

It would be necessary for three main groups to participate in such a cancer registry: Behvarz (in rural areas), Women Health Volunteers (in urban areas) and physicians (General Practitioner, Gynaecologist, General

Surgeon, Oncologist and Pathologist) throughout the country. Other people such as health technicians and nurse-specialists might also participate indirectly. I believe that registration data could lead to the establishment of an appropriate strategy for cancer screening at the lower cost and lead to a reduction in the mortality and the morbidity of the breast cancer in the long term.

At the present time, Iran possesses numbers of knowledgeable and dedicated physicians and excellent District Health System. Based on the latest official information, about 10732 General Practitioner (GP), 1060 Gynaecologist, 967 General Surgeon are working in the public health services (Ministry of Health) throughout the Iran those might participate in a breast cancer screening programme, services and treatment by any means [14]. Equal numbers of these groups of physicians work for the private sector; however, the private sector might not be interested to participate in cancer studies. With such this picture the Ministry of Health should govern all cancer studies particularly a breast cancer screening programme. It seems with close collaboration of twelve main universities (Three in Capital, Mashhad, Esfahan, Shiraz, Tabriz, Ahvaz, Yazd, Kermanshah, Bandar-e-Abbas, Zahedan) located across the Iran (See figure 7.1) a cancer campaign, initially by cancer registry, can be deployed effectively in Iran.

It should be noted that, the population of Iran (62 million in 1996) is expected to increase rapidly, 42% of the population are less than 15 years old and only 4% are older than 65 years old. The Annual Growth Rate (AGR) was 1.4 in 1996 [1], which will result in around 17 million Iranian women being placed at risk of breast cancer. If one assumes that a woman's lifetime risk for breast cancer is 10 per cent on average, then around one and half (1.5) million Iranian women will develop breast

cancer in their lifetime. Although Oesophageal cancer is the most common cancer in Iran, breast cancer is sufficiently common to be considered as a main health problem for women.

The distribution of estimated age-specific incidence rates of breast cancer among Iranian women is almost similar to UK, up to the age of 70 years, [6, 12] however, it is increased markedly over the age of 70 for UK and decreased sharply among Iranian women. This might be dominated by pyramidal age distribution and/or unregistered cases of Iranian women. Unregistered cases could be resulted in non-attending of elderly patients in clinic due to social, physical and cultural difficulties particularly in rural areas.

7.6 Glossary:

European and World standard population by age:
An age-standardised rate (ASR) is a summary measure of a rate that a population would have if it had a standard age structure. Standardisation is necessary when comparing several populations that differ with respect to age, because age has such a powerful influence on the risk of cancer. The most frequently used standard populations are the world and the European standard populations. The calculated incidence or mortality rate is then called the World age standardised or European age standardised incidence or mortality rate. They are expressed as a rate per 100000 (Table 7.9).

Cumulative Rate:
Cumulative incidence is the probability or risk of individuals getting the disease during a specified period. For cancer, it is expressed as the number of newborn children (out of 100, or 1000) who would be expected to develop a particular cancer before the age of 65 (or 70, or 75) if they had the rates of cancer currently observed. Like the age-standardized rate, it permits comparisons between populations of different age structures.

Population at risk:
The part of a population that is susceptible to have a specific cancer. It is defined on the basis of demographic data, such as place of residence, sex, age group, etc.

Crude Rate:
Data on incidence or mortality are often presented as rates. For a specific tumour and population, a crude rate is calculated simply by dividing the number of new cancers or cancer deaths observed during a given time period by the corresponding number of people in the population at risk. For cancer, the result is usually expressed as an annual rate per 100,000 persons at risk.

Prevalence:
The prevalence of cancer is the number of cancer cases in a given population at a specified point in time. It depends on the incidence and on the duration of the disease, that is, on survival.

Incidence:
The incidence is the number of new cancer cases arising in a given period in a specified population. Cancer registries collect this information routinely.

Mortality:
The mortality is the number of cancer deaths occurring in a given period in a specified population.

Table 7.9: European and World standard population by age

(After Doll et al., 1966)

Age group	European standard population	World standard population
0-4	8000	12000
5-9	7000	10000
10-14	7000	9000
15-19	7000	9000
20-24	7000	8000
25-29	7000	8000
30-34	7000	6000
35-39	7000	6000
40-44	7000	6000
45-49	7000	6000
50-54	7000	5000
55-59	6000	4000
60-64	5000	4000
65-69	4000	3000
70-74	3000	2000
75-79	2000	1000
80-84	1000	500
85+	1000	500
Total	**100000**	**100000**

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Chapter 8

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8 Discussion and Conclusion

8.1 General Discussion

Breast cancer results from genetic and environmental factors leading to the accumulation of mutations in essential genes. Genetic predisposition may have a strong, almost singular effect, as with BRCA1 and BRCA2, or may represent the cumulative effects of multiple low-penetrance susceptibility genes.

During the last decade has seen many important advances in understanding genetic susceptibility to breast cancer, there remains much to learn [1, 2].

Unanswered questions include the number and nature of genetic variants that predispose women to breast cancer, the interplay between those variants and environmental factors, and the optimal use of that information to reduce both the personal and social costs of breast cancer.

The ultimate aim of all these efforts is to diminish the mortality and morbidity of breast cancer. In achieving to this aim, most of the studies have been focused on specific aspects of the breast cancer such as epidemiological studies (to investigate factors those might involve in developing of breast cancer), molecular studies (to understand functions of cancer predisposing genes with a view deeply that lead to designing

suitable therapeutic drugs), methods and technical development (to investigate high risk individuals at early stages of breast cancer development by developing mutation detection methods and making improvements in medical imaging instruments and techniques) and finally studies on cost-effectiveness of breast cancer screening in public services.

8.1.1 Clinical point of view

The identification of BRCA1 (Miki et al, 1994) and BRCA2 (Wooster et al, 1995) were landmarks in the investigation of the genetics of breast cancer. Even before the genes were identified, it was clear that management issues following on from gene identification would need to be addressed quickly if the impetus gained from these discoveries was not to be lost. So what are the keys questions facing clinicians who care for women with BRCA mutations? One is that of penetrance-that is, what is the likelihood that a carrier of a mutated allele of a breast-cancer-susceptibility gene will develop breast cancer by a given age? The next important question is, when advising individuals at risk, whether counsellors should give more weight to the mutation itself (the gene affected and the type of mutation), the position of the mutation along the gene, or the family history? A third question is whether the prognosis of BRCA1-related breast cancer differs from that of BRCA1-negative breast cancer. Finally, and most importantly, there is the question of the choice of medical and surgical preventive measures.

8.1.2 Penetrance

(Which mutations have a higher penetrance?)

Several population-based studies have demonstrated differences in the penetrance of BRCA genes [3, 4, 5]. Thus, efforts are underway to identify additional high- and low-penetrance genes. Although, it is unlikely that the

final list of breast-cancer-susceptibility alleles will be divided into high- and low-penetrance genes, and will more likely represent a spectrum of penetrance with each modified by multiple gene-gene and gene-environment interactions. It is now apparent that most familial breast cancer risk is not accounted for by mutations in the high-penetrance susceptibility genes BRCA1 and BRCA2.

8.1.3 Prevalence and incidence

Breast cancer is the most common cancer in UK and is the most common female malignancy worldwide. However, according to the latest data from Iran, oesophageal cancer obtaining the highest incidence rate of cancer among Iranian population (see chapter seven in great detail). One should consider a priority of cancer research within a given population or country. Attempts are now making to address why the prevalence and incidence rate of breast cancer is much higher in developed countries than undeveloped countries? Is it due to social and cultural, diet, geographical condition, life style effects, and efficient cancer registry system and/or resulted in unknown modifier genes?

8.1.4 Screening pitfalls

- **Clinical**: Self Breast-Examination (SBE) is the best method for large scale screening programme either individually or by an expert physician (every six months) and is highly cost effective but it has yet to be accepted widely by the general public. The key step here to overcome such attitudes is education.
- **Radiological**: Advanced technology such as MRI, which is much more sensitive than mammography, is too expensive to be affordable in public services. Of the women screened by mammography, about 10% of the x-

ray images record something suspicious, which requires further investigation. However only a small number of those cases will be cancerous. Mammography therefore seems not to be sufficiently sensitive and informative to be used in large-scale screening.

- **Molecular**: At the present time, despite of the considerable efforts to develop more sensitive new methods for screening BRCA genes, there is still no currently available technique that can guarantee the identification of all cancer-predisposing mutations. All routine diagnostic methods such as Direct Nucleotide Sequencing, PTT, SSCP, HA, DGGE, DHPLC, etc suffer two limitations:

1) They are quite laborious and expensive for use in a diagnostic services, which needs to produce answers quickly and within a modest budget.
2) They detect differences between the patient's sequence and the published normal sequence but they do not generally distinguish between pathogenic and non-pathogenic changes.

On the other hand it is nevertheless clear that molecular methods should form part of any population-screening program. How this is integrated with conventional methods of clinical investigation requires open debate.

8.1.5 Interpretation of test result

(What distinguishes those who develop cancer from those who do not?)

Since the cloning of BRCA1 and BRCA2, genetic testing for breast and ovarian cancer susceptibility has become more widespread. However, interpretation of test results is not always straightforward. In many high-risk families, a deleterious mutation in BRCA1 or BRCA2 is not identified in an affected proband. Even when a positive result (i.e., a deleterious mutation) is identified, the associated cancer risks cannot be precisely quantified and the efficacy of management options is still very uncertain.

When a negative result is obtained, it could be good news (in the case of a true negative for a familial mutation), or a negative result could be completely ambiguous and raise more questions than it answers. There are several potential explanations for such a finding, namely that an undetected mutation in BRCA1 or BRCA2 may exist, or there could be a mutation in a rare or undiscovered gene. In addition, the possibility that women with breast cancer represent sporadic cases within hereditary cancer families must also be considered. The occurrence of BRCA1/2 variants of uncertain significance, often missense mutations, further complicates the risk assessment. Addressing this issue is especially important because a significant proportion of high-risk families do not harbour deleterious mutations in BRCA1 or BRCA2.

In some of these instances, extending testing to relatives can be helpful to clarify results. When hereditary breast cancer cannot be ruled out, individuals may still be at increased risk for cancer and therefore need to obtain appropriate surveillance.

The management of known carriers of breast cancer susceptibility genes remains controversial. Options include prophylaxis against the development of breast and/or ovarian cancer, by prophylactic mastectomy, prophylactic oophorectomy and/or using Tamoxifen for five years, or careful follow-up with screening for overt disease. Offering any preventional strategies should absolutely consider age, religion, social and culture of carries as well as benefit of that. Therefore, balanced information that includes estimates of the magnitude of survival benefit from these interventions as well as their potentially adverse effects on quality of life should be discussed with patients, especially those who are young and have good prognoses. **It must be considered "what the preventional strategies should be to cover both breast and ovarian cancer?"**

8.1.6 When does cancer develop in breast cancer predisposing gene carriers?

Many recent studies have shown that the penetrance of BRCA1 or BRCA2 mutations is uncertain and probably variable and the prevalence of BRCA1 mutations in the general population is estimated to be between 1/500 and 1/1000 [6]. Segregation analyses have estimated that about 1 in 20 women with breast cancer are carriers of a breast cancer predisposing gene that is dominantly inherited and highly penetrant. A carrier with a deleterious mutation in BRCA1 or BRCA2 gene has 80%-90% chance of developing breast cancer [7]. Assessing of all proband information genetic testing should be able to predict, not just whether a woman is likely to develop breast cancer, but when she might get it [8, 9] (See chapter 6 section 6.1.9). This is the most significant question that should be considered before genetic counselling and/or testing.

8.1.7 Uncertainty of prognosis

What is the difference of prognosis in carriers and control cases?

Accurate estimates of breast cancer prognosis in individuals with BRCA1 and/or BRCA2 mutations would require prospective longitudinal studies with large numbers of women. Such studies have yet to be reported.

Most studies on the prognosis of breast cancer have not found a significant difference in survival between individuals with BRCA1 or BRCA2 mutations and controls [10, 11, 12, 13, 14] but conflicting reports of both better [15, 16] and worse [17, 18] prognoses exist.

8.2 Technical Discussion

8.2.1 Data collection

One of the most important steps in any research project is data collection, depending on significance of project's aims. For such a this study, as a PhD course, which was performed for the first time on this selected group, data gathering was not a easy task mostly because of time constraint and modest budget.

Over a period of three months agreements were obtained from two main centres for cancer research and treatment in Tehran-Iran to establish collaboration on this study. This was followed by recruiting relevant three employees for initial jobs, reviewing more than four thousands files to invite appropriate participants for collection of their relevant personal and medical information, informing attendants and obtaining their consent and their blood samples, setting up laboratory equipments and reagents for DNA extraction. Finally the genomic DNA and other data from more than one hundred participants were brought to the UK for further analysis.

Additional attempts have been made to collect formal information from the Iranian Ministry of Health to evaluate the capacity of the Iranian District Health System for integration of breast cancer screening programme.

8.2.2 Mutation detection methods pitfalls

The advantages and disadvantages of each method those used in this study have been discussed in the relevant chapters in detail. What I would like to discuss here is, the cost effectiveness of molecular diagnostic approaches versus clinical and medical imaging diagnostic methods in breast cancer screening programme.

Although new mutation detection techniques have resolved diagnostic problems for some genetic disorders, breast cancer diagnosis remains problematic. The following questions still remain unanswered.

1) How many per cent genetic factors contribute to the development of all types of breast cancer?

2) Would it (detection methods) be readily available (instrumental, technicians, reagents) in most genetic laboratories worldwide?

3) Would these methods detect all sequence variants in known predisposing breast cancer genes accurately?

4) Would it be affordable in the public health services? If not, who will pay for?

Let us assume that there are only two known genes (BRCA1 and BRCA2) that contribute to the development of familial or hereditary breast cancer. Myriad Genetic Laboratories, Inc. offer BRACAnalysis® to international customers for $2580 (BRCA1 only $1290, BRCA2 only $1555, Single Amplicon Testing $295). Now a question arises, at the time of offering BRCA genes analysis by private sectors, how many genetic laboratories are available to scan guaranteed these genes worldwide?

With such above costs, how many per cent of high-risk families do afford to take this test?

Answer to the above questions might help to make an informed decision regarding public breast cancer screening strategies. In my knowledge no currently available molecular scanning methods is cost effective in offering diagnostic information to high-risk individuals in the public health services. However, molecular diagnostic approaches once fully developed might be applied in hereditary breast cancer for prenatal diagnosis.

Alongside the developmental of mutation detection methods in the last decade, significant advances have been seen in medical imaging

technologies. Magnetic resonance imaging (MRI), sonography, digital mammography and etc are now used in breast cancer screening but all have both advantages and disadvantages.

Mammography is not sufficient sensitive to detect cancerous from normal breast tissue in women at age of 20-25 years because of high density pattern of normal breast tissue in this stage. Further investigation of a problem detected at the screening stage has involved the patient being referred for an ultrasound scan to help clarify whether or not their 'lump' is malignant. More recently scientists at The Institute of Cancer Research (UK) have developed a modified ultrasound system known as **freehand elastography** for diagnosis of breast cancer. The new technique is expected to differentiate more accurately between benign and malignant tumours and avoid the need for more invasive procedures such as biopsy.

The new system creates images using information from tissue stiffness, the same property that gives rise to the sensation of a hard lump found within suspicious breast tissue when it is felt with the fingers. It is achieved through a computer process in which images are recorded while the breast is pressed gently by a hand-held ultrasound probe. Freehand elastography in conjunction with conventional ultrasound scanning might improve accuracy of diagnosis and used as a conventional ultrasound equipment thus minimising costs to public hospitals. It is hoped medical imaging will form a fundamental part of the diagnosis and treatment of breast cancer.

No one approach provides comprehensive breast cancer screening; several approaches (Clinical, Technological and Molecular) should be deployed continuously.

8.3 Conclusion

◈To date, germline mutations in the BRCA1 and BRCA2 genes in patients with early-onset breast and/or ovarian cancer have not been identified within the Iranian population, so that there are no published data of the mutation spectrum in this selected group. Although only ten mutations have been detected (See chapter 3 Table 3.4 and chapter 4 Table 4.5) in one hundred and four participants tested, some circumstances may have contributed to this outcome.

Firstly, this number (104 participants) included sixteen unaffected individuals who were screened and no sequence variant has been detected among them.

Secondly, I have only analysed hot spot regions for mutations in both BRCA1 and BRCA2 genes not the entire coding regions. Comparison of data from other studies suggests that, using this strategy (analysing selected coding regions) approximately 14% and 22% of mutations in BRCA1 and BRCA2 gene respectively would be missed [19, 20, 21, 22, 23]. However, there are no data to indicate the presence of population-specific mutations in Iran, and therefore, these data must be considered preliminary.

Thirdly, genomic DNA has only been analysed for coding regions variants, mutations in the promoter regions and those within the introns would have been missed and analysis of cDNA may uncover other alterations.

Finally, SSCP is known to be insufficiently sensitive for the detection of all sequence variants.

◈The 185delAG BRCA1 germline mutation has been reported in Ashkenazi Jewish individuals both in familial breast and ovarian cancer and in the general population most commonly [24, 25, 26]. Moreover, other Jews and non-Jews population studied showed the same mutation [27, 28, 29]. Sporadic cases of the 185delAG BRCA1 germline mutation in Jewish non-

Ashkenazi breast/ovarian cancer families have previously been reported [30].

In this preliminary study 185delAG BRCA1 germline mutation was identified once in an Iranian non-Jewish breast cancer family, none of the participants tested in this study was Jewish in origin. However, this particular mutation has previously been detected in one Iranian Jewish individual diagnosed with breast cancer [27]. Further study such as haplotype analysis may require for originating this particular mutation within Iranian population.

◈It has been shown that the histological characteristics of breast cancers due to BRCA1 and BRCA2 mutations differ from those of sporadic breast cancers [31]. Several studies shown that invasive ductal carcinoma is the most frequent (more than 60%) histological type in Iranian women who diagnosed with breast cancer [32, 33, 34]. In this study, a total of five different breast/ovarian tumour types were histologically reported in BRCA1/2 mutant carriers and non- carriers. One type, invasive ductal carcinoma was identified dominantly (See Tables 8.1 and 8.2).

Table 8.1: Breast/ovarian tumour types observed in Iranian women carrying the BRCA1/2 germline mutation and non-carriers

Tumour type	Abbreviation	Total observed cases
Invasive Ductal Carcinoma	IDC	71
Papillary Serous Cystadenoma (OV)	PSCA	3
Medullary Carcinoma	MC	2
Invasive Lobular Carcinoma	ILC	1
Granulosa Cell Tumour (OV)	GCT	1

OV= Ovarian cancer

Of 104 individuals tested 88 patients were diagnosed with breast or ovarian cancer, sixteen participants tested were unaffected (See chapter two section 2.1.1) obviously no pathologic reports have been made for them.

Finally a total of seventy-seven pathology reports were available in their record reviewed from 88 women diagnosed with breast/ovarian cancer (tumour grading was not requested in questionnaire from).

Table 8.2: Breast/ovarian tumour types in Iranian women carrying the BRCA1 or BRCA2 germline mutation

Gene/exon	Mutation and nucleotide change	Tumour type	Coding effect	Family history	Age at diagnosis
BRCA1-exon 2	185-186 delAG	IDC	Frameshift	2BC-1PS	37
BRCA1-exon 2	181-182 insT	IDC	Frameshift	1-OV	41
BRCA1 exon 11	2335-2336 delAA	IDC, MC	Frameshift	(2BC<40)	42*
BRCA2 exon 11	6261-6262 insGT	IDC	Frameshift	(2BC<40)	27
BRCA2 exon 11	3979-3980 insA	IDC	Frameshift	Negative	40
BRCA2 exon 11	5972 C>T T1915M	n.a.	Missense	Negative	41
BRCA2-exon 17	IVS16-14T>C IVS16- 6T>G	n.a.	Close to splice site	n.a.	n.a.
BRCA2-exon 18	8345A>G (N2706S)	IDC	Missense	Negative	38
BRCA1-exon 20	IVS20+48 dup gtattccactcc	IDC	Polymorphism	2BC<42	27
BRCA2-exon 23	9266C>T T3013I	IDC	Missense	Negative	31

BC= Breast Cancer OV= Ovarian Cancer PS= Prostate Cancer n.a= not applicable
IDC= Invasive Ductal Carcinoma MC= Medullary Carcinoma
*This woman who carrying a deleterious BRCA1 mutation was identified histologically with both IDC and MC type.

Although IDC was dominantly reported in BRCA1/2 carriers and non-carriers breast families, due to inadequate cases of hereditary and sporadic breast/ovarian cancers (control cases) in this study, it is difficult to compare this observation with the other studies [31, 35] and make a correct interpretation. Further study consist of a large number of breast/ovarian families including familial and sporadic breast/ovarian cancer cases (control cases) and tumour type with the sufficient histological features is required to investigate potential use of breast tumour type in identifying carriers with BRCA1/2 mutation in this selected group.

Inclusion of breast-cancer histology in the design of genetic testing policies, particularly for young women without a family history of breast cancer, may be clinically appropriate and cost-effective.

8.3.1 Mutation detection rate

The present study analysed the BRCA genes based on specific criteria, therefore the outcome mainly depends on a prior chance of carrier having a mutation in the BRCA genes as well as on the sensitivity and specificity of methods used. Mutation detection rates within BRCA1 and BRCA2 genes have been reported in cohort studies to vary between 6.2% and 16% [26, 36, 37, 38, 39, 40]. The present cohort studied all had early-onset (under the age of forty-five years) breast cancer; a sub-group had a family history, which was quite limited in comparison to the majority of other studied [37, 38, 39, 41]. These preliminary data suggest that the spectrum of mutations identified differs little from that seen in other studied with no recurrent mutation and with the mutations spread throughout the genes. Among 83 breast cancer families five putative (Frameshift) mutations were detected in the group as a whole (6.02% detection rate) in which 4 mutations was detected among 14 individuals with a family history (28.6% detection rate with 95% binomial confidence interval 0.09-0.58) whilst one mutation was seen in an

patient among 69 individuals without family history that is apparently sporadic early-onset breast cancer (1.5% detection rate with 95% binomial confidence interval 0-0.09) [42].

I could find no sequence variant in some cases that diagnosed with early-onset breast cancer (bilateral) with or without strong family history. However, it is clear that the known genes cannot explain most of the variation in breast cancer risk in the population. The difficulties of identifying further genes by linkage indicate that the remaining genes may be numerous, with relatively common alleles conferring moderate risks. Only by identifying these genes with the true pattern of risk and the mechanism behind them become clear [43].

The detection rate in those individuals with a family history was high especially when compared with other studies where the selection criteria for screening required a much stronger family history. This suggests that the screening, albeit incomplete, was well targeted. The low detection rate in those with an apparently sporadic early-onset breast cancer suggests that early-onset occurrence alone is insufficient to warrant breast cancer screening in the Iranian population.

Identification of these distinct mutations suggests that any given population should develop a mutation database for its programme of breast cancer screening. The pattern of mutations seen in the BRCA genes does not appear to differ from other population studied. Early-onset breast cancer (less than 45 years) and a limited family history is sufficient to warrant mutation screening with a detection rate of over 25% in this group whilst sporadic early-onset breast cancer (detection rate less than 5%) is unlikely to be cost-effective. If these results were confirmed on a large cohort, molecular methods might form a vital part of any screening programme in

Iran; this programme could be readily administered through the Iranian Primary Health Care Network.

8.3.2 GenBank accession numbers

The sequences variants that identified in the current study were submitted to the GenBank with accession numbers:

AF274503, AF284812, AF288936, AF288937, AF288938, AF309413, AF317283, AF348515, AY008850, AY008851.

The current study has been published in Breast Cancer Research Journal. 2002; Vol. 4 No. 4

8.3.3 The future prospects

Breast cancer is not only a health problem to the Western countries so we should consider it globally. Screening programmes can be offered at two levels: 1-public services 2-research. Any given government's policy should consider this issue.

At the level of public services, medical team (Nurse specialist, General Physician, Gynaecologist, Surgeon, Psychologist, Genetic Consultant), and molecular geneticist, both should collaborate very closely. Both should be aware of the limitations and complexity of breast cancer management, and must exploit all possible clinical and technical approaches to provide a much better outcome and benefit to the probands.

At the level of research, international collaboration is critical to understand those significant factors (environmental, ethnic founder effect, life style and etc.) additional to genetic predisposing factors that might contribute to breast cancer development, such global research efforts will also be needed to investigate other high or low penetrance genes and possible other unknown breast cancer pathways.

Previous studies have estimated different penetrances for breast cancer risk in women with germline BRCA1 and BRCA2 mutations among the population used for analysis. In summary the existing breast cancer database that includes epidemiologic factors have been gathered mostly from selected populations, some other countries may uncover other factors either genetic or environmental contributing to the development of breast cancer.

Thus far, present data have revealed that cancer incidence and prevalence in Iran is much lower than the most parts of the world, confirming that more studies are urgently needed. However, it seems that there is a strong correlation between age distribution (Age pyramid) of Iranian population and the incidence rate of breast cancer.

It appears to be timely for uncovering populations where epidemiologic factors that may contribute significantly to the development of breast cancer remain uncharacterised.

The last point is, which I would like to emphasis on that, worldwide breast cancer incidence rate (new cases) approaches around one million per year, if we assume that hereditary breast cancer is accounted for 5-9 per cent of all breast cancers types, the new cases of hereditary breast cancers reach to 100000 so that 900000 new cases remain to be detected at early stage by any means rather than genetic tests. Taking this figures, are we driving in a right way? Do we have a clear plan for early detection of all types of breast cancer? Working together may lead to prevent and cure breast cancer in near future.

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PRESENTATIONS AND PUBLICATION

Presentations:

1) Novel mutations in the BRCA1 and BRCA2 gene in Iranian women with early-onset Breast Cancer. 10th International Congress of Human Genetics, 15-19 May 2001, Vienna, Austria

2) Gene assembling, a new approach in mutation detection techniques: An application for BRCA genes scanning. Human Genome Meeting 2002, 14-17 April 2002, Shanghai, China

Publication:

1) Novel mutations in the BRCA1 and BRCA2 gene in Iranian women with early-onset Breast Cancer. Breast Cancer Research Journal. 2002; Vol 4 No 4

Abstract 1: Novel mutations in the BRCA1 and BRCA2 gene in Iranian women with early-onset Breast Cancer. 10th International Congress of Human Genetics, 15-19 May 2001, Vienna, Austria

Novel germline mutations in the BRCA1 and BRCA2 gene in Iranian women with early-onset Breast Cancer

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To report the initial experience in identifying germline mutations in the *BRCA1 and* BRCA2 breast and ovarian cancer susceptibility genes, to assess the spectrum *of such mutations and* to determine the frequency of recurrent mutations in Iranian women with *early-onset breast cancer, we* analyzed a total of 90 samples derived from Iranian women under the age 40 years. 70 samples derived from patients diagnosed with early-onset breast cancer and 20 samples from unaffected women who have first-degree family history of at least one breast cancer under the age 40.

We distinguished mutations identified by analysing BRCA1 exons 2, 3, 5, 11, 13 and 20 and BRCA2 exons 9,10,11, 17, 18 and 23 using Protein Truncation Test (PTT) and Single Strand Conformation Polymorphism (SSCP) analysis on PCR-amplified genomic DNA. Modified primers containing T7 promoter and a perfect Kozak consensus sequence were used to generate PCR products of the whole of exon 11 of BRCA1 and exon exons 10 and 11 of BRCA2 that were suitable for PTT.

Ten distinct sequence variants were identified. Five frameshifts (putative mutations-four novel); three missense changes of unknown significance and two polymorphisms, one seen commonly in both Iranian and British populations.

Identification of these novel mutations suggests that any given population should develop a mutation database for its programme of Breast Cancer screening. The pattern of mutations seen in the BRCA genes does not appear to differ from other population studied. Further studies should be performed to address the prevalence of these mutations in Iranian population.

It is nevertheless clear that molecular methods should form part of any population-screening program. How this is integrated with conventional methods of clinical investigation needs open discussion.

Novel mutations in the BRCA1 and BRCA2 gene in Iranian women with early-onset Breast Cancer

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Background:

Cancer is a multifactorial disease involving interaction of environmental, hormonal, and dietary risks in addition to genetic predispositions.

Breast cancer is the most common cancer among women, excluding skin cancers, with a lifetime risk of 10 percent in the general population. In spite of earlier detection and better treatment, largely due to recent technological advances it is still the second leading cause of cancer death in women, exceeded only by lung cancer. Hereditary breast cancer is suspected in an individual who has a family history of breast cancer or breast and ovarian cancer consistent with autosomal dominant inheritance.

Breast cancer is not a systemic disease at its inception, but is a progressive disease and surviving can arrest its development, because treatment of advanced breast cancer is often futile and disfiguring. In 1990, the first breast cancer susceptibility gene, BRCA1, was localised using linkage analysis to chromosome 17q21. In 1994, Miki and associates isolated the gene. Wooster and associates identified the new gene, BRCA2, in 1995. *Figure1*

Women who carry BRCA1and BRCA2 mutations have a significantly increased chance of developing breast cancer before the age of 50. Breast cancer is not a systemic disease at its inception, but is a progressive disease and surviving can arrest its development. Because treatment of advanced breast cancer is often futile and disfiguring.

Mutational Spectrum

At the present time over 250 different disease-causing mutations, scattered throughout the BRCA1 gene, have been discovered. These variants are distributed along the entire coding region of the gene and more than 50 per cent of them have been identified only once.

Over 100 mutations have been identified in *BRCA2* that, like *BRCA1*, are not limited to a particular region of the gene. To date, however, only a handful of somatic mutations have been described in sporadic breast and ovarian cancers.

Design and screening methods

With the cooperation of two referral centres for cancer in Tehran-IRAN, Iranian Centre for Breast Cancer (ICBC) and Cancer Institute in Imam Khomeini Hospital, 186 Iranian women' file records who have been under screening for breast or ovarian cancer were studied.

A total of 90 samples derived from Iranian women under the age 40 years for germline mutations in the BRCA1 and BRCA2 genes.70 samples derived from patients diagnosed with early-onset breast cancer and 20 samples from unaffected women who have first-degree family history of at least one breast cancer under the age 40.

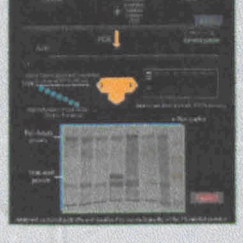
Using Promega DNA Purification Kit and according to the manufacturer's protocols genomic DNA were extracted from peripheral blood lymphocytes at Pasteur Institute in IRAN-Tehran.

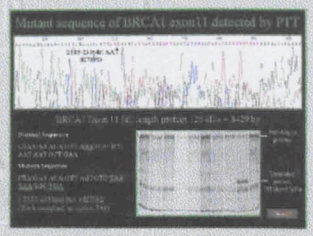
We analyzed BRCA1 exons 2, 3, 5, 11, 13, 20 and BRCA2 exons 9,10,11, 17, 18, 23 using Protein Truncation Test (PTT) by TNT-T7 Coupled Reticulocyte Lysate System (commercial Promega Kit) and according to the manufacturer's protocols, and Single Strand Conformational Polymorphism (SSCP) assay on PCR-amplified genomic DNA. Two sets of primers were designed to screen whole BRCA1 and BRCA2 exon11 in one single reaction using couple transcription – translation system using PTT.

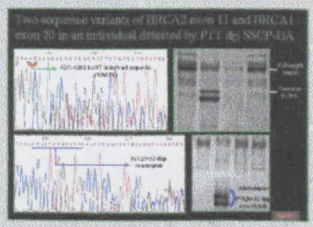
Mutation Detection Methods

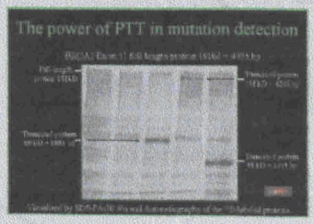
Protein Truncation Test (PTT)

PTT is a specific test for frameshift, splice or nonsense mutation, which truncate a protein product. *Figure 2* PTT analysis detects premature stop codons in a single reaction by amplifying DNA fragments of the coding region using a special primer, which carries at the 3'end a T7 promoter followed by an eukaryotic translation initiator sequence, transcribing the fragments into mRNA, and translating the mRNA into radiolabeled peptides.Products from the transcription/translation reaction can be analysed by SDS-PAGE. The sample to be fractionated is denatured and coated with detergent by heating in the presence of SDS and a reducing agent. The SDS coating gives the protein a high net negative charge that is loaded on a Polyacrylamide gel and high voltage is applied, causing the protein components to migrate toward the positive electrode (anode).Since all of the proteins have a net negative charge that is proportion to their size, the proteins are separated solely on the basis of their molecular mass- a result of the sieving effect of the gel matrix. The molecular mass of a protein can be estimate by comparing the gel mobilities of a band with protein standards. Following gel electrophoresis, gel bands may be visualized by autoradiography. If the product is full length, no truncating mutation is present in the sequence cloned. In samples where premature termination has occurred the protein product will be smaller than that generated from the non-mutant sequence. Assessment of the size difference between these two products allows the position of the putative mutation to be located.







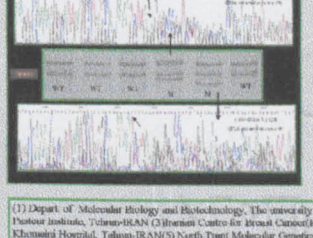


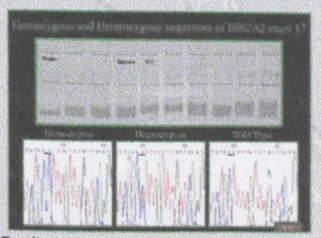
Combined Single Strand Conformation Polymorphism assay (SSCP) and Heteroduplex Analysis (HA)

Single – stranded DNA has a tendency to fold up and form complex structures stabilised by weak intramolecular bonds. The electrophoretic mobilities of such structures on nondenaturing gel will depend not only on their chain lengths but also on their conformations, which are dictated by the DNA sequence. For SSCP, wild type & test samples will amplified by PCR from genomic, denature at 99C° , rapid cooling then loaded on to a non-denaturing PAGE that allows the single – strand molecules to form secondary structure, which affects their rate of migration, products can be detected by silver staining. Some of each product renaturated to give double-stranded DNA. This runs faster in the gel. The single-stranded DNA runs more slowly in the same gel. Since each strand takes up different structure, two bands are seen in wild-type DNA. Even a single base change alters the secondary structure, so a mutant DNA molecule produces two bands in a different position and a heterozygous will produce four bands (two wild type, two mutant). If fragments of less than 150-200 bp (sequence dependent) are tested, insertions, deletions and most single base substitutions are detectable. In SSCP, the precise pattern of bands seen is very dependent on detail of the conditions.

SSCP and heteroduplex analyses reputedly each have sensitivities of between 60 and 80%. The electrophoretic mobility of heteroduplexes in polyacrylamide gels is less that of the homoduplexes, and they can be detected as extra slow moving base.*Figure 6,7*

It is important to note that no currently available technique can guarantee the identification of all cancer-predisposing mutations in the BRCA1 or BRCA2 genes.

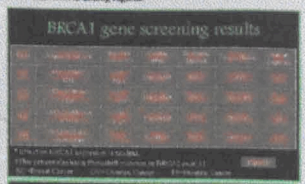


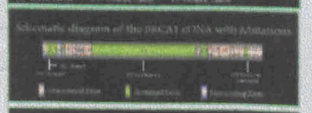


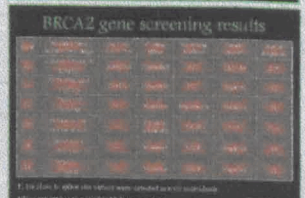
Results: We distinguish mutations identified by analyzing BRCA1 exons 2, 3, 5, 11, 13, 20 and BRCA2 exons 9,10,11, 17, 18, 23 using Protein Truncation Test (PTT) and Single Strand Conformation Polymorphism (SSCP) analysis on PCR-amplified genomic DNA.

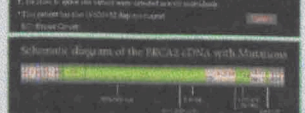
Ten distinct and novel sequence variants were identified. Five of these are frameshifts (four of which are novel), three missense (non-conservative changes), two polymorphisms (one intronic 12bp duplication [IVS20+52] and one close to splice site -BRCA2 [IVS 16-14 T>C]. Sixty similar splice site variants (32 heterozygous and 8 homozygous individuals) were detected in BRCA2 [IVS 16-14 T>C].Except one that has been previously reported all these sequence variants are novel. No sequence variant was detected in unaffected individuals.

It is important to note that no currently available technique can guarantee the identification of all cancer-predisposing mutations in the BRCA1 or BRCA2 genes. The *Figures 5,9* depict the location of each observed mutation within BRCA1 and BRCA2 coding regions.









Conclusion: Although only ten mutations have been detected in 90 probands tested, some circumstances may have contributed to this outcome:(i) This number included 20 unaffected individuals who were screened and no sequence variant has been detected among them. (ii) We have only analysed hot spot regions for mutations in both BRCA1 and BRCA2 genes not entire the coding regions. (iii) Genomic DNA has only studied for coding regions variants. Mutations in the promoter and those deepest within the introns would have been missed. Analysis of cDNA may uncover other alterations. (iv) SSCP is not enough sensitive to detect all sequence variants. (v) PTT may have missed some mutation at 5' and 3' end of exon 11 in both BRCA1 and BRCA2 genes.

The intronic 12bp duplication [IVS20+52] effect on BRCA1 expression is still unclear but may be it is significant region for transposone elements.

The identification of these novel mutations suggests that any given population should develop a mutation database for its Breast Cancer screening program. Further studies should be performed to address the prevalence of these mutations in Iranian population.

Finally it is nevertheless clear that molecular methods should form part of any population-screening program. How this is integrated with conventional methods of clinical investigation needs open discussion.

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Abstract 2: Gene assembling, a new approach in mutation detection techniques: An application for BRCA genes scanning. (Accepted) Human Genome Meeting 2002, 14-17 April 2002, Shanghai, China

Gene assembling, a new approach in mutation detection techniques: An application for BRCA genes scanning

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Many disease susceptibility genes are large and consist of many exons in which point mutations are scattered throughout. There has been an increasing demand for a rapid and accurate method for scanning point mutations in BRCA genes particularly.
Meta-PCR is a new method for creating chimaeric DNA molecules using a modified PCR reaction (Wallace et al 1999) that allows maximising the length of sequence that can be scanned by downstream technique.
Here we present data to demonstrate the assembles of exons 2, 20,23 and 24 of the BRCA1 gene and their subsequently analysis by direct sequencing.
The BRCA1 exons 2 and 20 are hot spot regions that are known to harbour particularly deleterious mutations. In order to avoid missing any mutation in these two exons, the above four exons were assembled in the following order of preferences: 23 20, 2 and 24. However, the order of fragments can be predetermined by primer design.
We verified by direct sequencing that the order and sequence of the component exons in the Meta-PCR products were as predicted. Meta-PCR products from three previously ascertained heterozygotes for BRCA1 mutations were directly sequenced and gave the same sequence patterns.
Scanning each exon of BRCA1 and BRCA2 genes individually represent a tedious task. Meta-PCR technique might circumvent this problem and is likely to be useful for clinical molecular diagnostic laboratories, helping them to fulfil the demand for scanning for complex genetic diseases at a lower cost.

Gene assembling, a new approach in mutation detection techniques: An application for BRCA genes scanning

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NHS Trust

Introduction

Many disease susceptibility genes are large and consist of many exons throughout which point mutations are scattered almost randomly. The large numbers of exons necessitate large numbers of individual tests, slowing down the screening process. A variety of scanning methods are available to identify unknown point mutations. The choice of technique being determined by several competing factors, two of the most important factors is the sensitivity of the method and the complexity of the procedure. Some widely used mutation detection methods such as Single Strand Conformation Polymorphism assay [1] and Heteroduplex Analysis [2] are technically simple but are only capable of analysing small DNA fragments and even then with variable sensitivity. Other mutation-scanning techniques like Protein Truncation Test [3] are capable of locating mutations in DNA fragments much larger than the average exon. There has been an increasing demand for rapid and accurate methods for scanning of point mutations in large multi-exon genes. Meta-PCR [4,5] is a versatile method for generating chimeric DNA molecules that can minimize the length of sequence scanned by downstream techniques. Breast cancer display heterogeneity that can be caused by mutation in the known cancer predisposing BRCA1 and/or BRCA2 genes. Meta-PCR could be used to combine exons from the different genes for simultaneous screening. This could be particularly useful for diseases with mutational hot spots in more than one gene such as BRCA genes. Currently, up to five PCR amplifiable fragments can be combined to form a single linear amplicon.

Method and materials

The Meta-PCR reaction is self-assembling and takes place in two coupled, stages carried out in a single reaction vessel. The main phases of the primary Meta-PCR reaction are illustrated in Figure1A and B. During this phase, genomic DNA is amplified with limiting quantities of primers. In the initial cycles, the reaction behaves like a simple multiplex PCR. However, as the amplimers begin to increase in concentration, there are insufficient amount of each primer to hybridise to and re-extend the entire single-stranded DNA. This "encourages hybridisation to occur between the linker segments of the linker primers" forming two types of intermediate (Fig.1A). One of these intermediates (intermediate 1 in Fig.1A) is capable of being extended by *Taq* polymerase, leading to a fusion product comprising the two fragments. As progressively more extendible intermediates form, longer fragments accumulate, the full-length product arises (Fig.1B). Reamplification of a small aliquot of the primary Meta-PCR in a secondary reaction (secondary Meta-PCR) with an excess of internal nested primers reamplifies the full-length product with high specificity, producing sufficient product for downstream analysis (Fig.1B).

In this study BRCA1 exons 2, 20, 23 and 24 were chosen as the target to be assembled and were analysed by direct sequencing in one reaction. The BRCA1 exons 2 and 20 are hot spot regions that are known to harbour particularly deleterious mutations. In order to avoid missing any mutation in these two exons, the above four exons were assembled in the following order of preference: 23 20, 2 and 24. However, the order of fragments can be predetermined by primer design.

Primer design

The genomic specific segments were selected to have closely equivalent Tm values for the positioning of the primers. The complementary 5' tetramer, or linker segments of the Meta-PCR primers, were designed typically with approximately 50% GC content, avoiding any internal secondary structure, and to have Tm values that exceeded those of the genomic specific segments to favour self-assembly. A supplementary unmatched adenine residue was inserted between the genomic and 5' linker segments of the self-assembling primers to accommodate the 3' adenine overhange added by Taq polymerase to the nascent DNA strand (Table1). All primers were checked to avoid homology to Alu repeat sequences using the BLAST analysis program available at:

http://www.ncbi.nlm.gov/cgi-bin/BLAST/nph-blast.

Meta-PCR amplification

Genomic DNA was used as a template for amplification. Primers were purified by (HPLC). Meta-PCR amplification was carried out in two stages. The primary reactions were performed in 20µl volumes using 100ng of genomic DNA, 1µl of each primer (40nM), 3 µl of mixture of dNTP (2mM), 3µl MgCl2(4mM, 2µl Buffer B(x10) 1 µl *Taq* DNA polymerase (Promega ™ Cat#M1665) according to the manufacturer's recommendations and dH2O to a final volume of 20µl.

For amplification, each sample was denatured at 94°C for 2 minute and followed by 30 cycles of PCR (at 94°C for 1minute, at 60°C for 1minute and extending at 72°C for 2 minutes on a Perkin-Elmer-Cetus DNA thermal cycler) were followed by a final synthesis 72°C for 10 minutes.

The secondary Meta-PCR were carried out in separate 20µl volumes using 3-5µl of the primary PCR product, 2pl of each internal (nested) primer (5pM), 2µl of mixture of dNTP (2.5mM), 2µl MgCl2(4mM, 2µl Buffer B x10 (identical to the above) and 1 µl *Taq* DNA polymerase.

The thermal cycling conditions were identical to those used for the primary amplification. On completion of the secondary PCR, a 3µl portion of the PCR products were checked on a 1% agarose gel to confirm the presence of the expected fragments size (Figures 2 and 3).

Table 1: Details of linkers specific primer segments and genomic specific fragments used for amplification and assembling of BRCA1 exons 23, 20, 2 and 24 by Meta-PCR technique

Name&	Length	Sequence 5'-3'	Genomic T_m	Linker T_m
Ex 23F (ext.)	25	atg atg aag tgt cag ttc cag tag t	59.9	—
Ex 23R-L1F	53	ctg cgc cgc ggc ggc tA cag aag gat ccc ttt tag cac	59.8	65
Ex 20S-L1R	55	agc cgc cgc gca gg tct tct gct cca ct cca ttc	59.3	65
Ex20R-L2F	58	tcg ggc ccg ccg ccg aA cct gtg tca aag tat cta aca ctg	59.7	64
Ex2F-L2R	58	tcc ggc ggc ccg ccg aA tgt gtt aaa gtt cat tgg aac tgg	60.3	64
Ex 2R-L3F	57	agg ccg ggc ggc ggc tA ccc aaa ttc ata cac tct tgt gc	59.6	65
Ex 24F-L3R	58	agc ggc ccg ggc ggc tA cga ttg att aga gac tag tcc a	59.9	65
Ex 24R (ext.)	23	tgt tgc tac gac gac tgt tga tgc	59.7	—
Ex 23F (Int.)	24	TCC TAC TTT GAC ACT TTG AAT GCT	61.1	—
Ex 24R (Int.)	21	GTC CTG TGG CTC TGT ACC TGT	59.8	—

Gene of interest sequence= Black colour — Linker= Blue colour

A = supplementary unmatched adenine located between 3' end of the linker specific primer segment and 5' end of the genomic specific fragment

Gene of interest sequence: Black colour — Internal primer sequence: BLACK UPPER CASE

Internal amplification PCR product size: 850bp

The secondary Meta-PCR products were recovered from agarose gel slices using a Jetsorb™ gel extraction kit (Genomed Inc.) according to the manufacturer's instruction. A total of 80-100ng of each sample was sequenced in both orientation using internal nested primers with BigDye terminator cycle sequencing kits (Perkin-Elmer, Applied Biosystems). The manufacturer's protocols were followed with the exception that the annealing temperature for the cycle sequencing reaction was increased from 50°C to 55°C to reflect the high T_m of internal primers (Figure 4a-f).

Fig. 4: Electropherograms from analysis of full-length forward sequences of wild type Meta-PCR products (850bp)

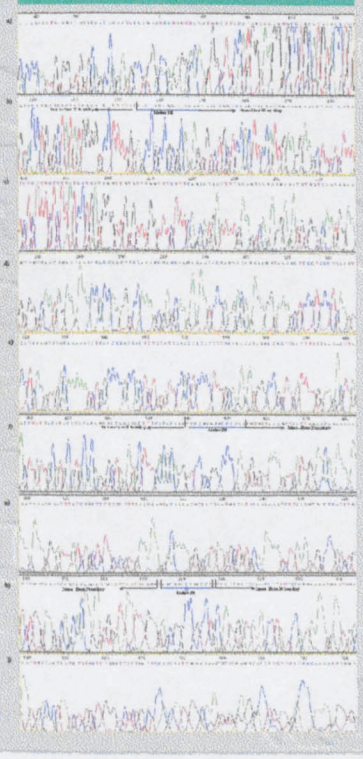
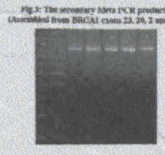


Fig.2: Analysing of PCR products of BRCA1 exon 23, 20, 2 and 24 individually

Monitoring of PCR products on 10% PAGE. PCR amplification of each exon above the primer dimmer, confirming the quality and the quantity of PCR products as expected.
Lane 1: PCR products of BRCA1 exon24 =324 bp
Lane 2, PCR products of BRCA1 exon20 =128 bp
Lane 3, PCR products of BRCA1 exon23= 187 bp
Lane 4, PCR products of BRCA1 exon2 =349 bp

Fig.3: The secondary Meta PCR products (Assembled from BRCA1 exons 23, 20, 2 and 24)

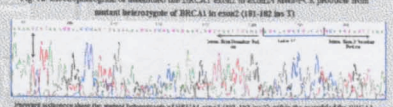


Monitoring of PCR products on 4% agarose gel. Lanes 1-5 show the assembled fragments (PCR products sizes ~ 850bp)

Results

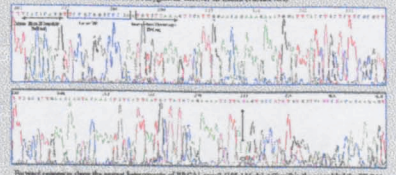
Detection of three previously ascertained heterozygous BRCA1 mutations: To test the suitability of the Meta-PCR products for downstream analysis by direct sequencing, Meta-PCR products from three previously determined heterozygous BRCA1 mutations in exon 2 and exon 20, were sequenced to confirm that the genotype present in genomic DNA was correctly expressed in the Meta-PCR products. The three mutant heterozygotes were BRCA1, 181-182 insT, 185 delAG and a 128bp duplication [IVS20+52]. These were clearly visible in the heterozygotes in figures 5a-c.

Fig. 5a: Electropherogram of assembled the BRCA1 exon2 to exon24 Meta-PCR products from mutant heterozygote of BRCA1 in exon2 (181-182 ins T)



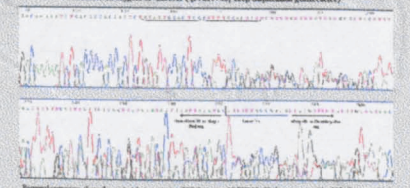
Forward sequences show the mutant heterozygote of BRCA1 exon2 (181-182 ins T) within the assembled the BRCA1 exon2 to exon24 by Meta-PCR technique. Arrow depicts the location of [T] insertion and frameshift sequence consequently.

Fig. 5b: Electropherogram of assembled the BRCA1 exon20 to exon2 Meta-PCR products from mutant heterozygote of BRCA1 in exon2 (185delAG)



The mixed sequences show the mutant heterozygote of BRCA1 exon2 (185-186 del AG) within the assembled the BRCA1 exon20 to exon2 by Meta-PCR technique. Arrow depicts the location of [AG] deletion and frameshift sequence consequently.

Fig. 5c: Electropherogram of assembled the BRCA1 exon20 to exon2 Meta-PCR products from mutant heterozygote of BRCA1 (IVS20+ 48) 128p duplication (gtatttcaccatcc)



Forward sequences show the mutant heterozygote of BRCA1 exon20 [IVS20+52 dup c(atttcaccatcc)] within the assembled the BRCA1 exon20 to exon2 by Meta-PCR technique. Arrow brackets depict the location of 128p duplication (atttcaccatcc) and frameshift sequence consequently occurred.

Conclusion

Breast cancer predisposing genes are large and consist of many exons in which point mutations are scattered throughout almost randomly. Screening each exon of BRCA1 and BRCA2 genes individually represents a tedious task, slowing down the screening process.

Meta-PCR could be used to combine exons from the different genes (BRCA1 and BRCA2 gene) for simultaneous screening of diagnostic mutational hot spots (in the case of BRCA1 gene exons 2 and 20 are notable) thereby allowing a rapid, targeted mutation scanning strategy. Meta-PCR technique is likely to be most useful for clinical molecular diagnostic laboratories, helping them to fulfil the demand for screening for complex genetic diseases at a lower cost.

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Research article

Novel mutations in the *BRCA1* and *BRCA2* genes in Iranian women with early-onset breast cancer

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Abstract

Background: Breast cancer is the most common female malignancy and a major cause of death in middle-aged women. So far, germline mutations in the *BRCA1* and *BRCA2* genes in patients with early-onset breast and/or ovarian cancer have not been identified within the Iranian population.

Methods: With the collaboration of two main centres for cancer in Iran, we obtained clinical information, family history and peripheral blood from 83 women under the age of 45 with early-onset breast cancer for scanning of germline mutations in the *BRCA1* and *BRCA2* genes. We analysed *BRCA1* exons 11 and *BRCA2* exons 10 and 11 by the protein truncation test, and *BRCA1* exons 2, 3, 5, 13 and 20 and *BRCA2* exons 9, 17, 18 and 23 with the single-strand conformation polymorphism assay on genomic DNA amplified by polymerase chain reaction.

Results: Ten sequence variants were identified: five frameshifts (putative mutations – four novel); three missense changes of unknown significance and two polymorphisms, one seen commonly in both Iranian and British populations.

Conclusions: Identification of these novel mutations suggests that any given population should develop a mutation database for its programme of breast cancer screening. The pattern of mutations seen in the *BRCA* genes seems not to differ from other populations studied. Early-onset breast cancer (less than 45 years) and a limited family history is sufficient to justify mutation screening with a detection rate of over 25% in this group, whereas sporadic early-onset breast cancer (detection rate less than 5%) is unlikely to be cost-effective.

Keywords: *BRCA1*, *BRCA2*, breast cancer, Iranian population, mutation detection methods

Introduction

Worldwide, breast cancer is the most common cancer in women, excluding skin cancers [1], with a lifetime risk of 10% in the general population [2]. In spite of earlier detection and better treatment, largely due to recent technological advances, it is still the second leading cause of cancer death in women, exceeded only by lung cancer [3]. The three commonest cancers, excluding skin cancer, in the Iranian population (females, all ages), are cancers of the oesophagus, breast and cervix, in diminishing order of incidence [4]. A preliminary study showed no significant differences in age-specific

PTT = protein truncation test; SSCP = single-strand conformation polymorphism.

incidences among Iranian women with breast cancer, in comparison with other studies [5,6].

Hereditary breast cancer is suspected in an individual who has a family history of breast cancer, or breast and ovarian cancer, that is consistent with autosomal dominant inheritance [7]. Many efforts are now under way to reduce the high incidence and mortality associated with breast and ovarian cancer by the early detection of women at high risk. These women, once identified, can be targeted for more aggressive prevention programs. Breast cancer is not a systemic disease at its inception but is progressive; screening can arrest its development and the treatment of advanced breast cancer is often futile and disfiguring [8].

In 1990, the first breast cancer susceptibility gene, *BRCA1*, was localised by linkage analysis to chromosome 17q21 [9]. Miki *et al.* [10] isolated the *BRCA1* gene in 1994. Subsequently, Wooster *et al.* [11] identified a new gene, *BRCA2*. Both *BRCA1* and *BRCA2* are large genes, consisting of 24 and 27 exons, respectively. Mutations in *BRCA* genes are distributed throughout the coding region. Women who carry *BRCA1* and *BRCA2* mutations have a significantly increased chance of developing breast cancer before the age of 50 years [12–14]. Some studies indicate that 13% of women who are diagnosed with breast cancer under the age of 30 years, and 7% of women who are diagnosed with breast cancer under the age of 35 years, have germline *BRCA1* alterations [12,15]. No molecular genetics study of *BRCA1* and *BRCA2* germline mutations has been reported in the Iranian population and there are few individuals with strong family histories that have as yet been identified. We therefore selected women who were most likely to harbour germline mutations in these genes.

Mutational spectrum

At present, over 878 distinct mutations, polymorphisms and variants throughout the *BRCA1* gene have been discovered [16]. Over 900 distinct alterations have been identified in *BRCA2* [16] and, as in *BRCA1*, they are not limited to a particular region of the gene. Most mutations in both genes are private [16]. *BRCA1* is considered to be responsible for about one-half of all cases of early-onset breast cancer and for the majority of familial breast and ovarian cancers [9,17]. *BRCA1* somatic mutations have never been reported in sporadic breast cancer [18] although they have occasionally been found in sporadic ovarian cancers [19,20].

Materials and methods

Design

We performed the present study to obtain initial experience in identifying germline mutations in the *BRCA1* and *BRCA2* genes in Iranian women diagnosed with early-onset breast cancer.

With the cooperation of two main centres for cancer research and treatment in Tehran, Iran, namely the Iranian Centre for Breast Cancer and the Cancer Institute at Tehran University of Medical Sciences, 83 samples derived from 82 unrelated Iranian families were selected for screening of germline mutations in *BRCA1* and *BRCA2*.

During 3 years since 1997, the medical records of 152 women diagnosed with breast cancer at the Iranian Centre for Breast Cancer were reviewed and 39 patients were selected for screening. In the 12 months after December 1999, a consecutive series of 44 patients from the Cancer Institute who were diagnosed with breast cancer were chosen for screening. All selected patients from these two centres were under the age of 45 years.

All selected women were informed that their DNA samples would be analysed for known mutations in genes associated with susceptibility to breast cancer; they were offered the opportunity to receive the results and were asked to sign a second consent form if they chose to learn the results.

DNA isolation and mutation analysis

Using a Promega DNA purification kit (catalogue no. LA1620) and in accordance with the manufacturer's protocols, genomic DNA was extracted from peripheral blood lymphocytes at the Pasteur Institute in Tehran, Iran.

We analysed *BRCA1* exon 11 and *BRCA2* exons 10 and 11 by the protein truncation test (PTT) [21–23], and *BRCA1* exons 2, 3, 5, 13 and 20 and *BRCA2* exons 9, 17, 18 and 23 by the single-strand conformation polymorphism (SSCP) assay [24] and heteroduplex analysis [25] by amplification from genomic DNA with the polymerase chain reaction (PCR).

Although mutations are scattered throughout both *BRCA1* and *BRCA2*, some coding regions were particularly chosen for several reasons: first, it has been shown that they have a significant role in protein function (exons 2–5, Ring finger domain [26]; exon 11 and exon 20, BRCT domain) [27]; second, exons 10 and 11 cover a large segment of the gene; third, many putative mutations have been reported in these regions; and fourth, the coding regions listed above have been shown in other significant studies [28–32] to be most likely to harbour germline *BRCA1* and *BRCA2* mutations. A comparison of data from other studies suggests that, with this strategy, about 14% and 22% of mutations in *BRCA1* and *BRCA2*, respectively, would be missed. However, there are no data to indicate the presence of population-specific mutations in Iran; these data must therefore be considered preliminary [28–32].

The PTT method

Most mutations reported in *BRCA1* and *BRCA2* cause premature termination of translation, which is readily

detectable by PTT; PTT is a very sensitive and efficient tool for mutation detection [28].

Modified primers containing a T7 promoter and a perfect Kozak consensus sequence were used to generate PCR products of the whole of exon 11 of *BRCA1* and exons 10 and 11 of *BRCA2* that were suitable for PTT. Each primer pair amplified 3446 base pairs (bp) and 4959 bp for *BRCA1* exon 11 and *BRCA2* exon 11, respectively. Regions (the 5′ and 3′ ends) of exon 11 in both genes were screened separately to identify any potential decrease in the sensivity of PTT resulting from the use of such large fragments.

PCR programme

PCRs were performed with genomic DNA containing 50 ng of genomic DNA, 1 μl of each primer at 5 pmol/μl, 2 μl of a mixture of dNTPs (each at 2.5 mM), 2.5 μl of 5 × PCR buffer A [300 mM Tris-SO_4 (pH 9.1 at 25°C), 90 mM $(NH4)_2SO_4$ and 5 mM $MgSO_4$] and 2.5 μl of buffer B [300 mM Tris-SO_4 (pH 9.1 at 25°C), 90 mM $(NH4)_2SO_4$ and 10 mM $MgSO_4$], 1 μl of Elongase® Enzyme Mix (Invitrogen, Paisley, UK) in accordance with manufacturer's recommendations, and distilled water was added to a final volume of 25 μl. For amplification, each sample was denatured at 94°C for 1 min and subjected to 31 cycles of PCR (94°C for 25 s, 56°C for 1 min, and extension at 68°C for 4 min on an Applied Biosystems DNA thermal cycler, Applera Europe BV, Cheshire, UK); this was followed by incubation at 68°C for 10 min. The PCR products were checked on a 0.7% agarose gel and a 1 μl sample was used for a coupled transcription–translation reaction (TNT® T7 Quick for PCR DNA kit from Promega, Southampton, UK; catalogue no. L5540), in accordance with the instructions of the manufacturer. Full-length artificial proteins of BRCA1 and BRCA2 exon 11, 126 and 182 kDa, respectively, labelled with ^{35}S, were detected after SDS-PAGE [33] (see Fig. 2) on a 2 × Mini-Protean system (Bio-Rad, Hertfordshire, UK, catalogue no. 165-3301).

The SSCP assay

BRCA1 exons 2, 3, 5, 13 and 20, *BRCA2* exons 9, 17, 18 and 23 and the 5′ and 3′ ends of exon 11 of *BRCA1* and *BRCA2* were analysed with the SSCP method.

PCR was performed on 50 ng of genomic DNA, 1 μl of 5 pmol/μl forward primer, 1 μl of 5 pmol/μl reverse primer, 1.6 μl of a mixture of dNTPs (each at 2.5 mM), 2 μl of 10 × PCR reaction buffer [750 mM Tris-HCl (pH 8.8 at 25°C), 200 mM $(NH4)_2SO_4$ and 0.1% (v/v) Tween 20], 0.7 unit of Red Hot *Taq* DNA polymerase, made up to a final volume of 20 μl with distilled water. For amplification, each sample was denatured at 94°C for 2 min and subjected to 28–30 cycles of PCR (at 94°C for 30 s, at 55–60°C for 1 min, and extension at 74°C for 1 min on a Perkin-Elmer-Cetus DNA thermal cycler); this was followed by incubation at 72°C for 5 min. Annealing temperatures varied according to the melting temperature, T_m, of the primer template.

SSCP analysis for point mutations was performed under the following conditions: a mixture of 5 μl of a 28-cycle PCR product and 5 μl of loading buffer was denatured for 10 min at 95°C, cooled rapidly on ice and separated on a non-denaturing 14% polyacrylamide gel (57:1 acrylamide:bisacrylamide, 3–10% glycerol) in 0.5 × Tris-borate-EDTA buffer at 12–16°C for 16–20 h at 245 V. Bands were revealed by silver staining.

Direct sequencing

All sequence variants were confirmed by using the PCR products of each sequence variant and Big Dye dideoxy-terminator chemistry (Perkin-Elmer) on an ABI 377 DNA sequencer (Applied Biosystems).

Primer design

For SSCP, splice junctions were included in the analysis in all cases. In addition, four sets of primers were used to screen the 5′ and 3′ ends of exon 11 of *BRCA1* and *BRCA2*. For PTT, two sets of primers were designed to screen the whole of exon 11 of *BRCA1* and *BRCA2*.

Results

We have identified ten sequence variants in this cohort: five frameshifts, four of which were novel (Figs 1 and 2), three missense changes of unknown significance and two polymorphisms.

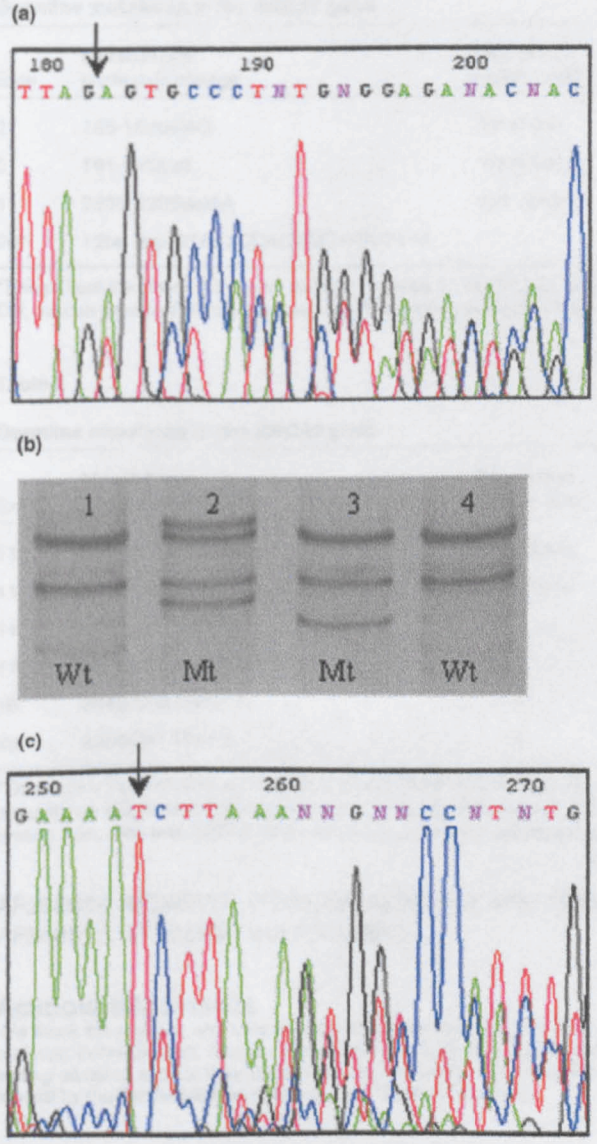
A common polymorphism in *BRCA2* [IVS16-14T>C] was identified in both Iranian and British populations. No sequence variant was detected at the 5′ and 3′ ends of exon 11 in *BRCA1* and *BRCA2* by SSCP analysis, suggesting that there was no loss of sensitivity in analysing such large fragments by PTT.

Tables 1 and 2 describe the distribution of *BRCA1* and *BRCA2* mutations respectively by the type of mutations.

Discussion

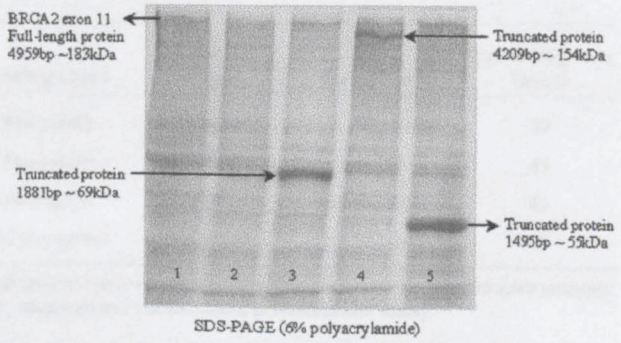
This is the first report to describe mutations in the *BRCA* genes in the Iranian population. The cohort studied all had early-onset (less than 45 years) breast cancer; a subgroup had a family history that was quite limited (Tables 1 and 2) in comparison with most other studies [34–37]. These preliminary data suggest that the spectrum of mutations identified differs little from that seen in other studies, with no recurrent mutation and the mutations spread throughout the genes. Five putative (frameshift) mutations were detected in the group as a whole (6.02% detection rate) in which four mutations were detected among 14 individuals with a family history (28.6% detection rate with 95%

Figure 1



Single-strand conformation polymorphism (SSCP) assay for germline mutation in *BRCA1* exon 2. Lanes 1 and 4 show normal patterns, and lane 2 and 3 depict the abnormal patterns of single-strand DNA mobility seen on a polyacrylamide gel **(b)**. Frameshift mutation in sample (lane 2) confirmed by direct sequencing **(a)** that shows a 2 bp (AG) deletion in *BRCA1* exon 2 at nucleotides 185–186 that leads to the formation of TGA at codon 39. **(c)** A frameshift mutation (lane 3) identified by direct sequencing, which revealed a 1 bp (T) insertion in *BRCA1* exon 2 between nucleotides 181 and 182, leading to the formation of TGA at codon 40. These frameshift mutations are likely to disrupt the function of the *BRCA1* proteins.

Figure 2



SDS-PAGE analysis of the whole of exon 11 of *BRCA2*, revealing the capacity of the PTT technique to detect mutations within 4959 bp coding sequences in a single reaction with the use of a coupled transcription–translation system, TNT® T7 Quick for PCR DNA kit from Promega. Lanes 1 and 2 show the normal pattern of the full-length (181 kDa) protein from exon 11 of *BRCA2*; lanes 3–5 show three different sizes of truncated protein, which were identified on 6% SDS-PAGE. Arrows show the sizes and positions of the normal and truncated proteins. Lane 4 shows a mutation occurring close to the 3′ end of exon 11 and producing a large protein for which the band migrated close to the top of the gel.

binomial confidence interval 0.09–0.58), whereas one mutation was seen in a patient among 69 individuals with early onset breast cancer but without a family history (1.5% detection rate with 95% binomial confidence interval 0–0.09) [38].

In many of the study cohort, we failed to identify a causative mutation, regardless of the presence or absence of a family history and these may simply be sporadic cases. However, it is clear that the known genes cannot explain most of the variation in breast cancer risk in the population. The difficulties of identifying further genes by linkage indicate that the remaining genes might be numerous, with relatively common alleles conferring moderate risks. Only by identifying these genes will the true pattern of risk and the mechanism behind them become clear [39].

Conclusions

The detection rate in those with a family history was high, especially when compared with other studies in which the selection criteria for screening required a much stronger family history. This suggests that the screening, albeit incomplete, was well targeted. The low detection rate in those with apparently sporadic early-onset cancer suggests that early-onset cancer alone is insufficient to justify screening in the Iranian population. If these results were confirmed on a large cohort, molecular methods would form a vital part of any screening programme in Iran.

GenBank accession numbers

These sequence variants have already been submitted to GenBank: accession numbers AF274503, AF284812,

Table 1

Germline mutations in the *BRCA1* gene

Exon	Mutation and nucleotide change	Stop codon at amino acid	Coding effect	Screening method	Family history	Age at diagnosis (years)
2	185-186delAG	39 (TGA)	Frameshift	SSCP/HA	2 BC, 1 PS	37
2	181-182insT	40 (TGA)	Frameshift	SSCP/HA	1 OV	41
11	2335-2336delAA	741 (TAA)	Frameshift	PTT	2 BC <40	42
20*	12bp dup GTATTCCACTCC IVS20+48	–	Polymorphism	SSCP/HA	2 BC <42	27

*This patient also has a frameshift mutation in exon 11 of *BRCA2*. BC, breast cancer (early-onset ages are also shown); HA, heteroduplex analysis; OV, ovarian cancer; PS, prostate cancer; PTT, protein truncation test; SSCP, single-strand conformation polymorphism assay.

Table 2

Germline mutations in the *BRCA2* gene

Exon	Mutation and nucleotide change	Stop codon at amino acid	Coding effect	Screening method	Family history	Age at diagnosis (years)
11*	6261-6262 insGT	2040 (TAA)	Frameshift	PTT	2 BC <40	27
11	3979-3980 insA	1264 (TAA)	Frameshift	PTT	Negative	40
11	5972 C>T T1915M	–	Missense	DS	Negative	41
17†	IVS16-14T>C IVS16-6T>G	–	Close to splice site	SSCP/HA	n.a.	n.a.
18	8345A>G N2706S	–	Missense	SSCP/HA	Negative	38
23	9266C>T T3013I	–	Missense	SSCP/HA	Negative	31

*This patient has also *BRCA1*-IVS20+48 dup GTATTCCACTCC. †This common polymorphism was detected in both Iranian and British populations. BC, breast cancer (early-onset ages are also shown); DS, direct sequencing; HA, heteroduplex analysis; n.a., not applicable; PTT, protein truncation test; SSCP, single-strand conformation polymorphism assay.

AF288936, AF288937, AF288938, AF309413, AF317283, AF348515, AY008850 and AY008851.

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