The molecular pathology of *BRCA1* and *BRCA2* in breast cancer patients from the West of Scotland

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Declaration

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

Guity Ghaffari

This thesis is dedicated to my beloved father and mother,
who were my first teachers.

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ABBREVIATIONS

A	Adenine
APS	Ammonium persulphate
ARMS	Amplification refractory mutation system
ASO	Allele specific oligonucleotide hybridisation
Bic	Breast cancer information core
bp	Base pair
C	Cytosine
^{0}C	Degree Celsius
CCM	Chemical cleavage analysis
cDNA	Complementary Deoxyribonucleic acid
cm	Centimetre
ddATP	Dideoxy adenosine triphosphate
ddCTP	Dideoxy cytosine triphosphate
ddGTP	Dideoxy guanosine triphosphate
ddTTP	Dideoxy thymidine triphosphate
DGGE	Denaturing gradiant gel electrophoresis
ddNTPs	Dideoxyribonucleotide triphosphate
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	2'-deoxyribonucleoside triphosphate
DTT	Dithiothreitol
E.coli	*Escherichia coli*
EDTA	Ethylenediamine tetraacetic acid
G	Guanine
g	gram
GDB	Genome data base
HA	Heteroduplex analysis
l	Litre
kb	Kilo base
kd	Kilo dalton
LCR	Locus Control Region
M	Molar
µg	Microgram
µl	Microlitre
mg	Milligram
ml	Millilitre
µM	Micromolar
mM	Millimolar
mm	Millimetre
MMLV	Moloney Murine Leukaemia Virus
MOPS	3-(N-morpholino) propanesulfonic acid
mRNA	Messenger RNA
ng	Nanogram

OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
MOPS	3-(N-morpholino) propanesulfonic acid
mRNA	Messenger RNA
ng	Nanogram
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pmol	Picomole
PTT	Protein truncation test
RNA	Ribonucleic acid
r.p.m.	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyle sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SSCP	Single strand conformation polymorphism
T	Thymine
TBE	Tris-acetate ethylenediaminetetra acetic acid
TE	Tris- ethylenediaminetetra acetic acid
TEMED	N,N,N,N-tetramethylenthylenediamine
Tris	Tris(hydroxymethyl) aminomethane
UTR	Untranslated Region
UV	Ultraviolet
V	Vol
W	Watt

Summary

Breast cancer is one of the most common female malignancies in the Western world, affecting one in 12 women during their life time. Five to ten percent of breast cancer may have a genetic component and can be attributed to dominant susceptibility genes. Inherited breast cancer genes, *BRCA1* and *BRCA2* are almost equally responsible and are believed to account for 85-90% of inherited breast cancers.

The overall aims of the present study were to search for and characterise mutations in the *BRCA1* and *BRCA2* genes in the breast cancer patients from the West of Scotland. In this study 40 patients with a family history of breast and/or ovarian cancer were screened for germline mutation in *BRCA1* and *BRCA2* genes.

The strategy for germline mutation analysis involved the initial amplification of the *BRCA1* and *BRCA2* genes by the polymerase chain reaction (PCR) using both genomic DNA and cDNA as templates. After initial amplification by PCR, the product was electrophoresed in an agarose gel in order to check for any abnormal alteration in size due to exon skipping or large gene deletion. If no alteration was identified, the screening for any small alterations was carried out using the protein truncation test (PTT), chemical cleavage mismatch analysis (CCM) and single strand conformational polymorphisms analysis (SSCP). Any differences in the band patterns, when compared to the negative controls in any of the techniques, were characterised by direct sequencing.

Using the above strategy, 16 different positive screening results were detected on analysis of *BRCA1* and *BRCA2* coding sequences. Of these, four are novel and 12 have been described in other studies.

The novel *BRCA2* 5445del7bp results from deletion of TTTAAGT of codons 5445-5451. This deletion results in a translational frame shift leading to a predicted termination at codon 1749. This recurrent mutation was initially identified in an index case from a family with a history of breast cancer who developed breast cancer at age 47 years.

The *BRCA2* 5573delAA results from the deletion of two of seven consecutive As of the normal sequence TCAAAAAAAC involving codons 1773-4. This deletion produces a translational frame-shift with a predicted termination at codon 1792. This mutation was identified in an index case who developed breast cancer at age 56 years.

The *BRCA1* 2800delAA results from the deletion of two of three consecutive As of the normal sequence GAAAC of codons 899-900. This deletion results in a translational frame-shift leading to a predicted termination at codon 901. This recurrent mutation was first identified in an index case who developed breast cancer at age 32 years. The identification of a shared *BRCA1* haplotype constructed with the polymorphic probes D17S855, D17S1322, D17S1323 and D17S1327 in the affected members who are carriers of the defective allele suggests that the prevalence of the 2800delAA frameshift may be associated with a founder effect.

The *BRCA1* 5382insC which is one of the known frequent mutations in *BRCA1* was observed only in one index case with a family history of breast and ovarian cancer who developed breast cancer at age 40 years. Further haplotype analysis of the four markers D17S855, D17S1322, D17S1323 and D17S1327 revealed that affected members of the family share the same haplotype as the British family (Gayther et al. 1995) with the same mutation, which, however, is different from the common haplotype reported for this mutation (Neuhausen et al. 1996).

The novel *BRCA1* skipping of exons 5-7 in *BRCA1* was identified in two unrelated breast cancer patients who developed breast cancer at ages of 23 and 40 years. No RNA from the other member of the family was available to study the presence of this exon skipping in the other member of these two families. No sequence alteration in the splice sites of exons 5, 6 and seven was identified. The absence of these three exons was not observed in the cDNA from 38 index cases from the other families with breast and/or ovarian cancer or in twenty healthy individuals. Taken together, the absence of exons 5-7 in these two families appears to be

directly the cause of predisposition to breast cancer. This is similar to the mutation lacking exon 3 in the cDNA of carriers reported in one of the Berkelies families, where no genomic changes was identified (Friedman et al 1994).

Using RT-PCR and DNA sequencing, an exon 5 deletion variant was found in almost all individuals. The exon 5 deletion transcript was only present in lymphocyte RNA, no abnormalities were detected in genomic DNA flanking exon 5. These findings indicate that caution should be exercised in providing genetic risk assessment on the basis of currently used germline analysis. We also found alternative splicing with the loss of exons 9-10 and 2-10, also reported by Miki *et al.* (1994) and recently by Xu *et al.* (1997). Awareness of the alternatively spliced mRNAs characterised in this report should facilitate analysis of *BRCA1* expression and function.

Seven different polymorphisms were detected in *BRCA1*, six in the coding sequence and one in intron 8 which is reported here for the first time. The one base deletion, 54bp downstream of the exon intron boundary of exon 9, possibly increases the risk of alternative splicing which results in the loss of exons 9-10. In order to assess whether *BRCA1* variants have any effect on the predisposition to breast and ovarian cancer we examined the frequency of five polymorphisms in breast cancer patients and in the normal population. Our data suggests that for each of the polymorphisms there is no genotype difference between the breast cancer patients and the control group.

The present study of 40 breast cancer families from the West of Scotland provides evidence for the involvement of the *BRCA1* and *BRCA2* genes in approximately 22.5% of the cases.

CHAPTER ONE

INTRODUCTION

1.1 Cancer

1.1.1 Definition

For the first half of this century, much of cancer research was focused on identifying agents that can induce cancers in experimental animals. The first agents that were found were viruses. Eventually certain chemicals and also radiation were shown to induce tumours in animals and transform cells in tissue culture. With a deeper understanding of the mechanism of action of viruses and chemicals and radiation as a mutagens, it became apparent that the majority of agents that can induce cancer act at the level of DNA. Today, it is widely accepted that cancer is a disease of malfunctioning cellular genes or unwanted viral gene expression (Watson *et al.* 1988).

During the past 30 years it has become increasingly clear that cancer is a genetic disease that results when multiple mutations accumulate within the DNA of a single somatic cell causing the cell to lose growth control .

Speculation that chromosome rearrangement may play a role in origin of some cancers date back to the early years of the twentieth century. Modern cytogenetic and molecular studies have provided overwhelming verification of this hypothesis . Recent advances in molecular genetics have demonstrated that: **cancer is a "genetic disease" sometimes in the sense of being inherited from generation to generation, and always in the sense that somatic genetic changes are responsible for the growth of tumours** (Yamamota, 1993).

Two general mechanisms account for most of the genetic contributions to cancer: activation and suppression of gene activity. Genes that when activated contribute to growth of a malignancy are referred to as **oncogenes**; the nonactivated native form of such genes are known as **proto-oncogenes**. Activated oncogenes exert a dominant effect on cell function. In contrast, genes whose inactivation contributes to malignancy act

in a recessive manner at the cellular level. These are sometimes referred to as **tumour suppressor genes.**

1.1.2 Oncogenes

As long ago as 1911, Peyton Rous showed that a connective tissue cancer in the chicken was caused by an infectious agent. This agent was subsequently shown to be a virus and was named Rous Sarcoma virus (RSV) after its discoverer. This cancer-causing, tumorgenic virus is a member of the retrovirus class of viruses, whose genome consists of RNA rather than DNA as in most other organisms.

The most exciting aspect of oncogenes was provided by the discovery that these cancer-causing genes are derived genes present in normal cellular DNA. Thus, using Southern blotting techniques Takeya and Hanafusa (1985) detected a cellular equivalent of the viral *src* gene in the DNA of both normal and cancer cells. Many of these cellular equivalents of the viral oncogenes have now been cloned and shown to encode proteins identical or closely related to those present in the retroviruses. To avoid confusion, the viral oncogenes are given the prefix v, as in v-*src,* while their cellular equivalent are designated proto-oncogenes and given the prefix c, as in c-*src.* Proto-oncogenes have been found to encode a variety of growth factors, growth factor receptors, signal transducers and DNA binding proteins.

Since cellular oncogenes occur normally, the question arises as to how they become activated to produce overt cancer. There are three main ways by which proto-oncogenes are activated:

The first mechanism is production of an abnormal product which can occur by (i) point mutations, such as amino acid substitutions or deletion. (ii) Chromosomal translocation resulting in the production of a fusion protein. One example of this is Chronic Myeloid Leukaemia (CML) where chromosome 9 is translocated to chromosome 22. This places ABL oncogene on chromosome 9 next to the break point cluster region (BCR)

gene on chromosome 22 resulting in a fusion gene which in turn produces an abnormal fusion protein.

The second mechanism of activation is over-production of the normal protein by amplification of the proto-oncogene.

The third mechanism of activation occurs through loss of appropriate control mechanisms. This may be due to either insertional mutagenesis that causes transcriptional activation or chromosomal translocation typified by 8:14 translocation in Burkitt's lymphoma that results in juxtaposition of the MYC oncogene on chromosome 8 to one of the immunoglobulin loci on chromosome 14, 2, or 22 resulting in constitutive expression of the transposed MYC gene (Latchman, D. 1995).

1.1.3 Tumour suppressor genes

Following the discovery of cellular oncogenes, however, it became clear that cancer could also result from the deletion or mutational inactivation of another group of genes. This indicated that these genes encoded products which normally restrained cellular growth so that their inactivation would result in abnormal unregulated growth. This idea came after this finding that almost every human cancer can occur in genetically predisposed individuals. The most striking form of genetic susceptibility involves Mendelian dominant inheritance with high penetrance and appearance of cancer at an earlier than usual age, as shown in colon cancer in persons with familial adenomatous polyposis (APC). In this example, the heterozygous states of the germ-line mutation imparts predominantly a high risk of APC although they may be prone to other kind of cancer such as intestin. while in other examples, such as the Li-Fraumeni syndrome (LFS), it predisposes to several kinds of cancer, although never to all forms. The most frequent cancer in LFS is carcinoma of breast, although it does not afflict all female carriers. This incomplete penetrance of a gene for a particular cancer typifies the heterozygous state for a familial cancer gene; it's presence is not a sufficient condition for cancer. A simple explanation for

this incomplete penetrance is that oncogenes require a somatic mutation in some target cells, an event that may never occur in some heterozygous carriers; two mutations one germinal and one somatic would be needed. This hypothesis also relates to the hereditary and nonhereditary forms of cancer by a common mechanism; the same mutations will operate in both, the first mutation being germinal in the former and somatic in the latter. In the hereditary case all of the somatic cells would carry a first "hit" whereas in the nonhereditary case only a clone of somatic cells would do so. What might the targets of these two hits be? The simplest answer is the two copies of some autosomal gene; oncogenesis would be recessive at the cellular level in both hereditary and nonhereditary cases. The presence of one normal (wild type) copy of the gene would interfere with oncogenesis, and the normal allele could therefore be considered as an anti-oncogenes, or tumour supressor gene (Knudson, 1993; Weinberg, 1993).

Thus far a number of tumour supressor genes have been cloned, and several putative genes of this class have been mapped to specific chromosomal bands. *BRCA1* and *BRCA2* which are involved in breast and ovarian cancer are now known to be tumour supressor genes. The evidence that *BRCA1* and *BRCA2* are tumour supressor genes comes primarily from loss of heterozygosity (LOH) studies. In breast tumours, heterozygous loss of chromosomal regions including *BRCA1* and *BRCA2* are frequently observed. In tumours from individuals that carry *BRCA1* and *BRCA2* susceptibility alleles, the region that is deleted invariably corresponds to the wild type homologue. This fits with the classical model of tumour supressor genes in which the susceptibility is inherited as a dominant Mendelian trait. The predisposing allele generally behaves in a recessive manner in somatic cells, thus a single copy of the mutation causes predisposition to malignancy and the loss or inactivation of the wild type allele propels the cell toward malignancy (Stratton, 1996).

1.2 Breast cancer

1.2.1 Epidemiology:

Breast cancer is one of the most common and important diseases of women, affecting one in 12 women in northern Europe and southern America during their life- time. Ovarian cancer, less frequent than breast cancer, is often rapidly fatal and is the fourth most common cause of cancer mortality in American women (Parkin *et al.* 1992). By the year 2000 it is estimated that breast cancer will account for 500,000 deaths annually (Pisani *et al.* 1993).

The large majority of breast cancer cases are thought to be sporadic; that is, they are not attributed to inherited traits, and their incidence is predominantly influenced by environmental or life style risk factors such as diet, reproductive behaviour and radiation exposure (Newman *et al.* 1988a). Approximately 5% of breast cancers, however, are believed to arise from inheritance of genes that confer an elevated risk of the disease. In such cases breast cancer usually appears at an early age, is often bilateral, and tends to cluster in families along with other cancer types, notably ovarian cancer (Claus *et al.* 1991). Several genes have been identified that play a role in the occurrence of hereditary breast cancer. *BRCA1* and *BRCA2* genes appear to account for the majority of hereditary breast cancers in the US and European populations. In families with breast cancer consistent with hereditary breast cancer, it has been reported that about 50% may be attributed to *BRCA1* (Easton *et al.* 1993) and 35% to *BRCA2* (Wooster *et al.* 1994). The remaining 15% of hereditary breast cancer could be attributed to a gene or genes which have not yet been identified (Rebbeck *et al.* 1996).

1.2.2 Risk factors in breast cancer:

Researchers have found certain factors that increase a woman's chance of developing breast cancer.

Family history: A family history of breast cancer increases the likelihood of breast cancer development. This may be attributed to genetic and

environmental similarities among family members. The risk of breast cancer is two or three times higher for women with an affected first-degree relative, and less than twice as high with an affected second-degree relative. Risks are further increased if two first relatives have bilateral breast cancer or if the relative's breast cancer was diagnosed before 40-45 years of age. Early age at onset of breast cancer is the strongest indicator of genetic susceptibility (Slattery and Kerber, 1993). Overall only 10-15% of breast cancer is attributed to family history, and about half of this is attributed to dominantly inherited susceptibility genes (Weber and Garber, 1993).

Age: The risk of breast cancer increases as a woman gets older. Most breast cancers occur in women over the age of 50. The risk is especially high for women over 60. This disease is uncommon in women under the age of 35, except in some families with hereditary form of the disease.

Personal history; The risk of breast cancer is greater than average in women who have had a previous breast cancer.

Other risk factors include: Starting to menstruate at an early age (before 12) or having a late menopause (after 55). First pregnancy after 30 increases the risk compared to an early age (less than 20) (Kelsey *et al.* 1993). Obese women have a higher risk for breast cancer over age 50. These factors may all be related to woman's natural hormones. Alcohol consumption and use of hormonal medications have been associated with increased risk. Scientists are also studying whether diet, exercise, pesticides, electromagnetic fields , contaminants in food and water, abortion and miscarriage are related to breast cancer risk . Some studies suggested that eating plenty of vegetables, fruits, and whole grains or other lifestyle modifications may reduce the risk (Hulka and Stark, 1995).

1.2.3 Inheritance :

Cady *et al.* (1970) described a family in which 3 sisters had bilateral breast cancer. Together with the reports in the literature, this suggested to him the existence of a family with a particular tendency to early onset,

bilateral breast cancer. Anderson (1974) concluded that the sister of the woman with breast cancer whose mother also had breast cancer has an age specific risk that is at a 47 to 51 times higher risk than control women (from ages 30-39); a revised estimate was 39 times (Anderson, 1976). The disease in these women usually developed before the menopause, was often bilateral, and seemed to be associated with ovarian function. About 30% of daughters with early-onset of bilateral breast cancer inherited the susceptibility. The risk of breast cancer for women with affected relatives is higher when the diagnosis is made at an early age and when the disease is bilateral. Ottaman *et al.* (1983) provided a table that gives the cumulative risk of breast cancer to mother and sister at various ages. The highest risk group is sisters of pre-menstrual probands with bilateral disease. Among the sisters of women with breast cancer, Anderson and Badzioch (1985) found the highest lifetime risks when the proband had bilateral disease, an affected mother (25+/-7.2%), or an affected sister (28+/-11%). The risks were reduced to (18+/- 3.3%) and (14+/- 2.6%) respectively, with unilateral disease.

Petrakis (1977) listed the evidence for a genetic role in breast cancer as follows:

1) family history of breast cancer, especially bilateral breast cancer;

2) marked difference in rates between certain racial groups.

3) lack of major change in incidence over many years despite dramatic decline in other cancers;

4) concordance in monozygotic twins;

5) concordance of laterality in closely related persons.

Lynch *et al.* (1984) found evidence consistent with hereditary breast cancer syndrome in 5% of 225 consecutively ascertained patients with verified breast cancer. From a maximum-likelihood Mendelian model, the frequency of susceptibility allele was 0.0006 in the general population, and the lifetime risk of breast cancer was 0.82 among susceptible women and 0.08 among women without the susceptible allele. They concluded that

inherited susceptibility affected only 4% of the families in the samples; multiple cases of this relatively common disease occurred in other families by chance. From complex segregation analysis of 200 Danish breast cancer pedigrees, William and Anderson (1984) concluded that the distribution of cases was compatible with transmission of an autosomal dominant gene. Newman *et al.* (1988) used complex segregation analysis to investigate patterns of breast cancer occurrence in 1,579 nuclear families. They concluded that an autosomal dominant model with a highly penetrant susceptibility allele fully explained disease clustering. Segregation analysis of a population-based study series of families (unselected for family history) indicated that highly penetrant autosomal dominant susceptibility genes were responsible for a 5-10% of all breast cancers (Claus *et al.* 1991).

1.3 Mapping of the *BRCA1* and *BRCA2* Genes

1.3.1 Mapping and cloning the candidate BRCA1 gene:

Hall *et al.* (1990) studied 23 extended families with 146 cases of breast cancer. The families shared the epidemiological features of familial, versus sporadic, breast cancer: younger age at diagnosis, frequent bilateral disease, and frequent occurrence of disease among men. They were tested simultaneously for genetic linkage and heterogeneity of breast cancer in families. They suggested both the presence of a gene for early-onset breast cancer linked to D17S74, which is located in band 17q21 and genetic linkage heterogeneity of the disease. Negative LOD scores were found in families with late-onset disease. They estimated that 15 out of 23, all of early onset cases are linked to D17S74 locus. This finding was further supported by Narod *et al.* (1991). who demonstrated linkage to the same region in breast and breast-ovarian cancer families. Using meiotic breakpoints, a consortium of investigators subsequently narrowed the region containing *BRCA1* to a ~12 centi-morgan (cM) interval flanked proximally by D17S250 and distally by D17S588 (Feunteun *et al.* 1993; Chambrian and *et al.* 1993).

The candidate genes in this region of chromosome 17q12-21 include the estradiol-17-beta-dehydrogenase (EDH17B1), retinoic acid receptor alpha (RARA1), thyroid hormone receptor (THRA1), ERBB2. Gene mapping in extended families with inherited, early-onset breast and ovarian cancer using polymorphic markers at the THRA1 and D17S78 excluded THRA1, this study localised *BRCA1* to the interval between THRA1 and D17S78. Haplotype analysis using markers flanking RARA1 and D17S78 in two affected sisters who developed breast cancer at ages 32 and 35, and carrying two different haplotypes excluded RARA1, no recombination was observed between EDH17B2 and *BRCA1*, however, direct sequencing of overlapping polymerase chain reaction product (PCR) containing the EDH17B2 gene in 4 unrelated affected women did not uncover any sequence variant in EDH17B2 (Simard *et al.* 1993).

A subsequent physical map comprised of overlapping Yeast artificial chromosomes (YACs), P1, bacterial artificial chromosomes (BACs) and cosmid clones allowed localisation of *BRCA1* to minimal region of 1-2 cM and subsequently to a region of about 600 kilobases (Neuhausen *et al.* 1994; Miki *et al.* 1994a).

Miki *et al.* (1994b) developed a detailed map of transcripts for the 600kb region of 17q-21 between D17S1321-and D17S1325. Sixty-five candidate expressed sequences were characterised by DNA sequencing, database comparison, transcript size, expression pattern, genomic structure and DNA sequence analysis in individuals from kindreds that segregate 17q-linked breast cancer susceptibility. Three expressed sequences eventually were merged into a single transcription unit whose characteristics strongly suggested that it was *BRCA1* . This transcription unit is located in the centre of a 600kb region spanning D17855 and was referred as the *BRCA1* gene. Conceptual translation of cDNA revealed a single long open reading frame with a presumption initiation codon flanked by sequences resembling the Kozak consensus sequence. This reading frame encodes a protein of 1836 amino acids. Probable predisposing mutations were detected in 5 out of 6

kindreds which were thought to have breast cancer allele due to *BRCA1* (Miki *et al.* 1994b).

1.3.2 Mapping the BRCA2 gene:

Evidence for the second major breast cancer susceptibility gene initially emerged from the analysis of 214 families that followed the location of *BRCA1* to chromosome 17 (Hulka and Stark, 1995). This showed that only about half of families with breast cancer could be attributed to *BRCA1* although most families with breast and ovarian cancer were due to this gene. Additional evidence supporting the existence of another gene came from the study of families with at least one case of male breast cancer (and usually several of female breast cancer and/or ovarian cancer) in which strong evidence against linkage to *BRCA1* was obtained (Wooster *et al.* 1994).

Sobol *et al.* (1992) pointed to genetic heterogeneity of early-onset familial breast cancer, in an extensively affected family they found no evidence of linkage to markers on 17q. Wooster *et al.* (1994) performed a genetic linkage search in 15 high- risk breast cancer families that were unlinked to the *BRCA1* locus on 17q21. This analysis uncovered a second breast cancer susceptibility locus, *BRCA2,* located in a 6-cM between D13S289 and D13S269 on chromosome 13q12-13 that was defined on the basis of meiotic recombination in early onset breast cancer cases. It appeared that *BRCA2,* while conferring a high risk of breast cancer (the same as *BRCA1),* does not confer an important elevated risk of ovarian cancer as in the case with *BRCA1.*

1.4 The *BRCA1* gene

1.4.1 Molecular genetics of the BRCA1 gene:

The *BRCA1* gene is composed of 22 coding exons and two noncoding exons distributed over roughly 100 kb of genomic DNA (figure 4.3). The size of the exons varies from 100 bp to 3700 bp. Exon 11 is the largest exon which contains 3726 bp. *BRCA1* encodes a 7.8 kb transcripts and produces a protein of 1863 amino acids. Much of the *BRCA1* shows no homology to

other known genes, with the exception of a 126 nucleotide sequence at the amino terminus, which encodes a RING finger motif. The transcript is most abundant in testis and thymus, but is also present in breast and ovary. Analysis of the 5′ flanking region of the *BRCA1* gene shows two distinct transcripts differing by the first exons. These transcripts were found and shown to be generated by alternative use of dual promoters and alternative splicing, suggesting the presence of exons 1a and 1b. The 5′ flanking regions of both *BRCA1* transcripts were analysed, and neither contained TATA box initiator elements, which have been proposed to mediate transcription. The *BRCA1* gene shows a complex pattern of alternative splicing downstream of the start codon. There is also considerable heterogeneity in splice junction usage near the 5′ end of *BRCA1* gene (Miki *et al.* 1994b)

1.4.2 The epidemiology of breast cancer susceptibility due to BRCA1

BRCA1 is associated with a high risk of early onset familial breast cancer. Preliminary observation suggested that mutation in *BRCA1* confers a higher risk of ovarian cancer than *BRCA2. BRCA1* is also associated with less risk of breast cancer in males than *BRCA2.* Mutation in *BRCA1* is thought to account for approximately 45% of families with significantly high breast cancer incidence and at least 80% of families with increased risk of both early-onset breast and ovarian cancer (Easton *et al.* 1993). 13% of women diagnosed with breast cancer under the age of 30 and 7% diagnosed under the age of 35 have been found to carry *BRCA1* mutations (Ford *et al.* 1995; Narod *et al.* 1995). However as will be discussed later, these high frequencies apply to the exceptional families with numerous numbers affected by breast cancer.

1.4.3 Risk of developing breast cancer in BRCA1 mutations carriers

The estimated lifetime risk of breast cancer in *BRCA1* mutation carriers who belong to highly selected families is about 50% by the age of 50 and 85% by the age of 70. The corresponding estimates of risk for ovarian

cancer are 23% by the age 50 and 63% by the age 70 (Narod *et al.* 1995). In the high-risk families evaluated for linkage, women who inherited a *BRCA1* mutation had 80% lifetime risk of breast cancer and were at increased risk for ovarian cancer (Newman *et al.* 1988b). Women who carry the *BRCA1* mutations have a lifetime risk of 80-90% for breast cancer and 40-50% of ovarian cancer by the age 70 (Ford *et al.* 1994). Genetic and environmental or lifestyle factors that may modify the risk of breast cancer in *BRCA1* mutation carriers are now being studied. Low parity, which is a risk factor for sporadic breast cancer, appears to have a similar effect in carrier of *BRCA1* mutations (Narod *et al.* 1995). It has also been proposed that a rare allele of the H-ras VNTR which may regulate expression of HRAS, may be associated with an elevated risk of ovarian cancer in *BRCA1* mutation carriers (Phelan *et al.* 1996b).

1.4.4 Mutation in the BRCA1 Gene

Most of the mutations that have been reported so far disrupt the normal function of the protein either by introducing a premature stop codon, by inserting or deleting short sequences to result in a frameshift mutation, or by reducing the amount of *BRCA1* transcript produced. Occasional missense mutations which may have more limited effects on protein structure as a whole have also been identified. It has been reported that around 10% of the mutations in *BRCA1* are nonsense and more than 70% are frameshifts which would be predicted to result in a truncated protein (Shattuck-Eidens *et al.* 1995).The mutations are scattered widely over the coding sequence of the gene showing no hot spots. The only possible evidence of clustering is in the zinc finger motif. There is no significant evidence between the position of the mutations within the gene, and the ratio of breast to ovarian cancer incidence in the family. However, there is some data which suggests a transition in risk such that mutations in the 3′ third of the gene are associated with a lower proportion of ovarian cancer. However, mutations in the 3′ portion of *BRCA1* are less likely to lead to ovarian cancer than mutations in the 5′ portion of the gene (Gayther *et al.* 1995). However, phenotypically

severe mutations have been found in the extreme 5′ end of *BRCA1* and in the 3′ portion of the gene.

1.5 The *BRCA2* Gene

1.5.1 Structure of BRCA2

The *BRCA2* gene is composed of 27 exons distributed over roughly 70 kb of genomic DNA (Tavtigian *et al.* 1996). The *BRCA2* cDNA consists of 11,385 bp which does not include the polyadenylation signal or poly A tail. A CpG-rich at the 5′ end of *BRCA2* extending upstream suggests the presence of regulatory signals often associated with CpG 'islands'. Unlike most human genes, the coding sequence is AT rich (>60%). The *BRCA2* cDNA contains an open reading frame (ORF) beginning at nt position 229 and encoding a protein of 3,418 amino acids which has no similarity to other proteins. There is no obvious signal at the N-terminus, and no obvious membrane spanning region. The highest levels of expression of *BRCA2* are in breast, thymus, lung. Ovary and spleen have slightly lower levels of expression (Wooster *et al.* 1995; Tavtigian *et al.* 1996).

1.5.2 The epidemiology of breast cancer susceptibility due to BRCA2

BRCA2 is associated with a high risk of early onset familial breast cancer, similar to that seen in families with *BRCA1* mutations. Preliminary observations suggested that mutations in *BRCA2* confer a lower risk of ovarian cancer than defects in *BRCA1,* but the risk is still higher than in the normal population. *BRCA2* is also associated with greater risk of breast cancer in males than *BRCA1.* However, since the absolute risk of males in these families is still small (approximately 5% by the age 70), it is likely that the *BRCA2* mutation in many families will be of a type restricted to expression in female breast cancer.

1.6 The spectrum of mutations in genetic disease

1.6.1. Definitions

Disease-causing mutations can be classified into two broad groups; those causing a significant change in gene structure (large deletions, insertions, inversions, or duplications) and those that produce only a minimal change in DNA structure (small deletions, insertions, inversions, duplications, and base substitution).

1.6.1.1 Base substitutions

Base substitutions involve replacement of usually a single base but in rare cases several clustered bases may be replaced simultaneously as a result of a form of gene conversion (Weatherall, 1991). Base substitutions are among the most common mutations and can be grouped into two classes: **i) Transitions** are substitutions of a pyrimidine (C or T) by a pyrimidine, or of a purine (A or G) by a purine. **ii) Transversions** are substitutions of pyrimidine by a purine or of a purine by pyrimidine. Both coding and noncoding DNA transitions are more common than transversions due to the high frequency of C to T transition, resulting from instability of cytosine residues occurring in the CpG dinucleotides; a hot spot for mutations (Cooper, 1983). In such nucleotides the cytosines are often methylated at the $5'$-C atom such $5'$-methylcytosines are susceptible to spontaneous deamination to give thymine (Cooper and Krawczak, 1993). Base substitution may affect the transcription of the gene into mRNA or the processing of mRNA into protein (Krawczak *et al.* 1992).

On the basis of the amino acid or codon changes, base substitutions have been classified into four groups; **1- A silent substitution** results in no change in an amino acid. This class of mutations is the most frequent observed in coding DNA because they are neutral mutations.

2- A missense mutation, results in the substitution of one amino acid for another amino acid due to a single nucleotide alteration. Missense mutations can be classified into two subgroups: **i) a conservative substitution** results

in replacement of an amino acid by another that is chemically similar to it. Often the effect of such substitutions on protein function is minimal because the side chain of new amino acid may be functionally similar to that of the amino acid it replaces . **ii) a nonconservative mutation** is a mutation that results in replacement of one amino acid by another, which has a dissimilar side chain. Sometimes a chain difference is introduced. Other changes may involve replacement of polar side chains by nonpolar ones and vice versa. Base substitution at the first and second positions can often result in nonconservative mutation.

3-Nonsense mutations are the substitutions of a single nucleotide resulting in a premature termination codon and the formation of a truncated protein product. Nonsense mutations are usually associated with severe phenotypes and can have at least three different consequences on the protein: **i) Truncated protein;** a premature termination codon can result in a product that lacks some of the normal C-terminal sequence , **ii) Unstable mRNA;** sometimes the mRNA which contains a premature termination codon is unstable and difficult to detect. **iii) Exon skipping;** some nonsense mutations appear to induce skipping of constitutive exons . If exon skipping does not result in a frameshift and the deleted amino acids are not essential for the function of the protein the phenotypic consequences of the mutation may be less severe than expected. Exon skipping is also known to be induced by other mutations which occur within exons.

4- Termination mutations which change a termination codon to one that codes for amino acids and result in the translation of a protein beyond the normal termination codon (Weatherall, 1991).

1.6.1.2 Insertions

An insertion is the addition of nucleotides in part of a DNA sequence. An insertion can involve one, a few or several hundred bases. In rare cases this involves transposition from another locus. **Copy** or **duplicative transposition** involves a sequence from one locus being replicated and the

copy inserted to another locus. **Noncopy transition** involves simple transposition of a DNA sequence from one locus to another. Insertional mutation, involving the introduction of <10bp of DNA sequence into a gene coding region, is not a random process and appears to be highly dependent on the local DNA sequence context. The majority of insertion type mutations can be explained by: i) symmetric elements; ii) slipped mispairing; ii) inverted repeats (Krawczak and Cooper, 1991).

1.6.1.3 Deletions

Deletions are the absence of part of the DNA sequence and can be classified according to the length of deleted DNA as follows:

1- **Large deletions** involve a large part of the DNA. Large deletions may be generated by one of these mechanisms: **i)** some large scale deletions and insertions may be generated by pairing of nonallelic interspersed repeats followed by breakage and rejoining of chromatid fragments, **ii)** unequal crossing over between nonallelic sequences on either nonsister chromatids of pairs of homologs or sister chromatids which is called **unequal sister chromatid exchange.** Since the crossing over occurs between mispaired chromatids the exchange results in a deletion of one of the chromatids and an insertion on the other one. Both mechanisms occur predominantly at locations where the tandemly repeated units are moderate to large in size. In such cases, the very high degree of sequence homology between the different repeats can facilitate pairing of nonallelic repeats on nonsister chromatids or sister chromatids.

2- Small deletions eliminate one or more nucleotides (<20bp) from a sequence. Direct repeats (a feature of a number of recombinations), palindromes (which potentiate the looping out of single stranded DNA), symmetrical sequence elements (found at the sites of single base deletion) have been found to be the cause of small deletions (Krawczak and Cooper, 1991). Deletion and insertion can result in a **frameshift mutation.** If the number of bases inserted or deleted is not divisible by three, a change in the

reading frame **(out of frame mutation)** alters the reminder of the translation of the protein and most often a stop signal is encountered prematurely. If the number of bases inserted or deleted is divisible by three, the reading frame will not change (**in-frame mutation**) resulting in an insertion or deletion of one or more amino acids. Sometimes **in-frame** mutations do not have a profound effect on gene expression or translation, however, sometimes the loss or gain of the extra amino acids may result in an unstable RNA or a non-functional polypeptide (Strachan and Read, 1996).

1.6.1.4 Inversions

The term inversion signifies that a region of DNA is back-to-front in respect of its normal orientation in the genome. Pathogenic inversion can be produced by intrachromatid recombination between inverted repeats. The high degree of sequence similarities between inverted repeats facilitates pairing of the repeats by a mechanism that involves a chromatid folding back upon itself. Subsequent chromatid breakage at the mispaired repeats and rejoining can then result in an inversion. The classic example of a pathogenic inversion caused by such mechanism occurs in more than 40% of patients with severe haemophilia A (Lakich et al. 1993).

1.6.2 Pathogenic mutations

Pathogenic mutations can occur at three types of DNA sequences at a gene locus:

1-The coding sequence of the gene: The great majority of recorded pathogenic mutations have been identified in the coding sequence. Those due to nucleotide substitution are, in the vast majority of cases, missense substitutions and mostly occur at first and second base positions of codons. However, rarely a silent codon substitution is not neutral as expected, but may cause a disease by activating a cryptic splice site. The CpG dinucleotides and tandem repeats within coding sequence have been reported to be hotspots for pathogenic mutations within coding DNA (Cooper and Yousoufian, 1988).

2-Intragenic noncoding sequences: This is restricted to sequences which are necessary for correct expression of the gene, such as important intronic elements, notably the highly conserved GT and AG dinucleotides at the ends of introns, however, there are other conserved sequence elements which, if mutated, can also cause aberrant splicing. Mutations which alter such sequences can have different phenotypic consequences, depending on whether there is a failure of splicing, or the use of an alternative illegitimate (cryptic) or natural splice site. The use of an intronic cryptic splice site will introduce new amino acids, while using an exonic cryptic splice site will result in a deletion of coding DNA. Some apparently innocuous synonymous mutations in coding DNA may also not be neutral as expected because they activate a cryptic splice site and can be pathogenic (Cooper et al. 1995). Mutation of a splice donor site may also result in skipping of a whole upstream exon while mutation of the splice acceptor sequence results in skipping of the downstream exon. Often, the exclusion of an exon has a profound effect on gene expression: it may result in a frameshift, unstable RNA transcripts or a non-functional polypeptide because of the loss of a critical group of amino acids. Often such mutations represent a small component (about 10-15%) of total pathogenic mutations at a gene locus (Cooper et al. 1995). However, in some disorders pathogenic splicing mutations may be common. In the case of the collagen disorder, osteogenesis imperfecta, they constitute very common pathogenic mutations leading to the replacement of the highly conserved, structurally important glycine residues.

3-**Regulatory sequences outside exons**: Many genes naturally undergo alternative forms of RNA splicing. In addition, mutations sometimes produce an aberrant form of RNA splicing which is pathogenic. Sometimes this results in the sequence of exons being excluded from the mature RNA (exon skipping) and on other occasions the abnormal splicing pattern may exclude part of a normal exon or result in new exonic sequences. Point mutations located in regulatory sequences have been identified to be pathogenic. Most

mutations located in regulatory sequences have been identified in conserved elements which are located just upstream of the first exon, notably the promoter (these conserved elements are normally required for RNA splicing). Occasionally, however, aberrant splicing of a gene can be induced by mutation of other sequence elements which are more distantly located from regulatory elements. For example, deletions which eliminate LCR but leave the β-globin gene and promoter intact result in almost abolishment of β-globin gene expression and contribute to β -thalassemia.

1.6.3 Factors governing the expression of pathogenic mutations.

The degree to which a pathogenic mutation results in an aberrant phenotype depends on several factors:

1- The mutation class and the way in which the expression of the mutant gene is altered. This may depend on the location of the mutation within the gene (Table 1.1). Most mutations abolish or substantially reduce the gene expression, but some lead to inappropriate gene expression (e.g. overexpression or ectopic expression, that is expression in tissues where the gene is not normally expressed).

2- The degree to which aspects of the aberrant phenotype are expressed in the heterozygote. The presence of a single normal allele may be sufficient to maintain a clinically normal phenotype, or a milder phenotype when compared with that of mutant heterozygotes, as in dominantly inherited disorders where the mutation is simple loss of function of the gene.

3- The proportion or the nature of the cell in which the mutant gene is present. Generally, mutations which are present in all the cells of an individual (inherited mutations) or in many of them (somatic mutations acquired very early in development) are likely to have a more profound effect than those present in a few cells (somatic mutations which arise at much later stages) or in cell types where the relevant gene is not expressed. Cancers, however, arise from unregulated division of the cells produced from a single original mutant cell.

Location and nature of mutation	Effect on gene function	Comments
Extragenic mutation	Normally none	Rare mutation may result in inactivation of distant regulatory elements required for normal gene expression
Multigene deletion	Abolish	Associated with contiguous gene syndrome
Whole gene deletion	Abolish	
Whole gene duplication	Can have effect due to altered gene dosage	
Whole exon deletion	Abolish or modification	May cause shift in reading frame; protein is often unstable
Within exon	Abolish	If loss/change of key amino acids, shift of the reading frame or introduction of premature stop codon
	modification	If nonconservative /substitutions, small in-frame insertions or other mutations at some locations
	None	If conservative/ silent substitution or mutation at nonessential sites
Whole intron deletion	None	
Splice site mutation	Abolish or modification of expression	Conserved GT and AT signals are critically important for normal gene expression. Mutation may induce exon skipping
Promoter mutation	Abolish or modulation of gene expression	Deletion, insertion or substitution within promoter may alter expression. Complete deletion abolish function
Mutation of termination codon	Modification	Additional amino acids are included at the end of the protein until another stop codon is reached.
Mutation of poly(A) signal	Abolish or modulation of expression	Deletion, insertion or substitution of nucleotides within poly(A) site may alter expression. Complete deletion abolishes function
Elsewhere in intron/UTS	Usually none	

Table 1.1 Effect of the location and class of mutation on gene function (Strachan and Read, 1996).

1.7 Methods for mutation detection

1.7.1 Direct mutation testing methods

Direct mutation testing methods can be classified as:

1- <u>Mutation detection</u> methods test the DNA samples for the presence or absence of one specific mutation.

<u>2- Mutation screening</u> methods screen a sample for any deviation from the standard sequence.

1.7.1.1 Mutation detection methods

Mutation detection methods are involved in the detection of known pathogenic mutations in DNA or RNA samples. Generally, these techniques require prior knowledge of the types of mutations commonly found and involve use of specialised approaches for efficient, accurate, and cost effective testing. Methods of direct analysis differ with specific disorders. Detection of deletions in the dystrophin gene in males with Duchenne/Becker muscular dystrophy is based on multiplex PCR and can be generalised to other X-linked disorders in which deletion is a common mechanism of mutation. Simultaneous detection of multiple point mutations using allele-specific oligonucleotides is another example of mutation detection methods. This technique has been used to identify point mutations responsible for cystic fibrosis gene, but is also applicable for any disease in which point mutations commonly occur. Amplification-refractory mutation system (ARMS) presented another protocol for detection of point mutations. This is based on the use of PCR primers that specifically amplify mutant or wild type allele. It should also be widely applicable to the task of efficient analysis of clinical specimens for known mutations. Several disorders have recently been identified in which variation in the size of triplet repeat region is the basis for gene mutation. Such mutations have been found to be involved in fragile X syndrome, for instance. In some cases, a mutation will create or destroy a restriction enzyme recognition site. In this case the mutation is detected by southern analysis, following digestion with the

enzyme and hybridisation with a specific probe. The size of the hybridisation band (or bands) will indicate the presence or absence of mutation. This can be done by PCR, the amplification product is digested with the enzyme and analysed on an ethidium bromide-stained agarose gel.

Mutations may also be detected by hybridising an oligonucleotide homologous to either the mutant or wild type sequence to a Southern blot or to PCR products bound to a membrane.

Point mutation screening by ARMS: PCR amplification lends itself to another mechanism of mutation detection based on the sequence specificity of PCR primers. A primer whose 3′ end coincides with a site of point mutation will work most efficiently only if the 3′ base is correctly matched to the target sequence. Amplification can be carried out with one common primer and another designed with either the wild-type or mutant 3′ base. Under the condition where the annealing temperature is relatively high, only the correctly matched base will produce a PCR product. A homozygote will react with only one primer, and a heterozygote will react with both (Newton *et al.* 1989).

Mutation screening by RT-PCR: Mutations that affect splicing sites can be inferred from the mRNA analysis of a gene. This is mostly done by reverse transcription PCR (RT-PCR). A cDNA copy is made by reverse transcription and primed with oligo-dT, a set of random hexamers, or with primer specific to a gene. This cDNA then serves as substrate for PCR. The products are analysed by a gel electrophoresis. Products of abnormal size indicate deletion of exon material or inclusion of intron as a result of splicing mutations.

Allele specific oligonucleotide hybridisation (ASO) : This method is based on the hybridisation of labelled oligonucleotide probes which have complementary sequence to either normal or mutant DNA sequence at the point of mutation to dot blot containing polymerase chain reaction (PCR)-amplified DNA products generated from the sequence of interest. Under very stringent conditions, such probes will only hybridise to their prefect

homologous sequences and not to those that vary by even a single nucleotide residue. Therefore, a normal allele can be detected using the wild type probe and the mutant allele with the probe containing the same mutation. Multiple mutations can be detected in a single reaction by mixing several hybridisation probes (Saiki *et al.* 1986).

1.7.1.2 Mutation screening methods

The reason for mutation screening is either to determine whether a candidate gene is causally related to a phenotype or to identify new alleles at a known locus. The latter involves the detection of novel and recurrent mutations in previously characterised genes, while the former is essential to confirm the causative role of a particular gene in the pathogenesis of human disease. Methods for general mutation screening usually examine and determine the difference between the sequence under test and some standard sequence.

In general, the methods for screening of mutations can be subdivided into those that grossly alter gene structure and those that produce only a minimal change in genomic structure. The methods for the screening of large mutations include, pulsed-field gel electrophoresis, southern blot hybridisation and restriction mapping (Southern, 1975). In the case of small mutations the most direct way to discriminate the normal and mutant sequence is DNA sequencing. This offers the advantage that any sequence variant will be detected and the mutation will be fully characterised but this can be time-consuming and expensive for genes like *BRCA1* and *BRCA2* which are relatively large. By using other screening techniques such as; single strand conformational polymorphism (SSCP) analysis, heteroduplex analysis (HA), chemical cleavage mismatch analysis (CCM), denaturing gradient gel electrophoresis (DGGE) and Protein truncation test (PTT) the specific affected region of the gene can first be identified and then the DNA sequencing will define the nucleotide change involved. All those techniques depend on PCR amplification of DNA or mRNA samples prior to analysis (Grompe, 1993). The advantages of using mRNA as the template are that

large non-coding regions are omitted thus permitting the analysis of a potentially smaller target region (with fewer PCR) and the rapid detection of gross deletions or splicing errors.

Single strand conformational polymorphism (SSCP) analysis

SSCP was originally described by Orita (Orita *et al.* 1989). It is based on the fact that single-stranded DNA has a tendency to fold up and form a complex structure stabilised by weak intermolecular bonds, notably base pairing hydrogen bonds. The electrophoresis mobilities of such structures on non-denaturing gels will depend not only on their length but also on their conformations, which are directed by the DNA sequence. SSCP fragments amplified by PCR can be separated into single strands and electrophoresed on non-denaturing polyacrylamide gels. It is a simple and relatively sensitive technique, it has a detection rate of about 70-95% for segments about 200bp in length. The sensitivity of the method is less than 50% when analysed fragments are more than 400bp in length (Grompe, 1993).To increase the sensitivity of the technique, a radioactive label may be necessary, but silver staining of the gel also shows equally good results. However, SSCP analysis detects mutations but does not localise them within a fragment.

Heteroduplex analysis (HA)

The heteroduplex analysis is based on the fact that mutant and wild type samples, when presented simultaneously in a PCR reaction, generate heteroduplex as well as homoduplex during the late cycles in a PCR reaction (Nagamine *et al.* 1989). The electrophoresis mobility of heteroduplex in polyacrylamide gel is less than of the homoduplex, and can then be detected as extra slow moving bands. The technique is simple and detects 80-90% changes in fragments less than 300bp (Groompe, 1993). However, as SSCP, HA can not localise the mutations and for the full characterisation of mutation, DNA sequencing has to be done.

Chemical cleavage mismatch analysis (CCM)

CCM is based on the principle of hybridisation of a radiollabled probe to the tested DNA, in order to create a heteroduplex between radiolabelled wild type and mutant DNA (Cotton *et al.* 1988). Chemical modification at the site of mutation is then carried out using hydroxylamine and osmium tetroxide which is based on the fact that mismatched cytosine and thymine are more reactive with the compounds hydroxylamine and osmium tetroxide, than are Watson-Crick cytosine and thymine bases. This is then subjected to cleavage at the site of modification using piperidine followed by denaturing gel electrophoresis and autoradiography (figure 1.1) . CCM is one of the most sensitive methods of mutation detection with a detection rate of >95% when the wild type DNA is labelled and 100% when both the wild type and mutant are labelled (Forrest *et al.* 1991). It can be applied to kilobase-length samples and it reveals the position of the mismatch (by the size of cleaved fragment) and the type of base change is determined from the cleaved reagent. Its disadvantages are that the chemicals, especially osmium tetroxide, are very toxic, and some practice is required before it works well. This technique has been improved with the use of silver stain instead of [α-^{32}p]dCTP] .

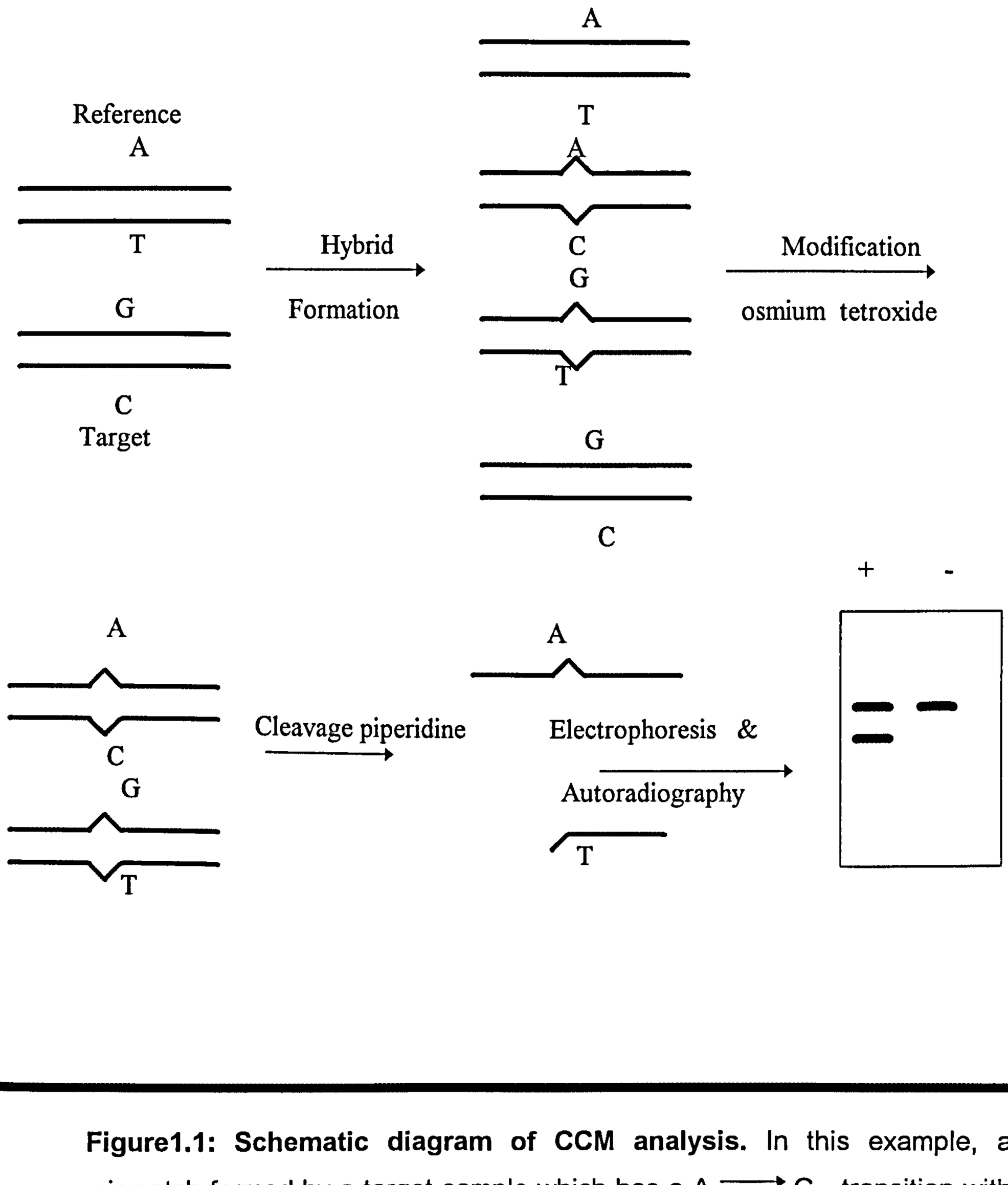


Figure1.1: Schematic diagram of CCM analysis. In this example, a mismatch formed by a target sample which has a A ⟶ G transition with respect to the labelled reference sample is detected using osmium tetroxide modification. The schematic autoradiograph shows bands expected when the target sample is different from (+) sample or from the (-) reference sample.

Denaturing gradient gel electrophoresis (DGGE)

DGGE allows the separation of DNA molecules differing by as little as a single base change (Fischer and Lerman, 1983). In this method, DNA duplex is forced to migrate through an electrophoretic gel in which there is a gradient of increasing amount of a denaturant (usually chemical denaturant, but temperature gradient gel electrophoresis is also used sometimes). Migration continues until the DNA duplex reaches a position in the gel where the strands melt and separate, after which the denatured DNA does not migrate much further. A single base pair difference between normal and mutant duplex is sufficient to cause them to migrate to a different position in the gel. DGGE is potentially highly sensitive, but it requires very careful design of primer, so that the sequence amplified has the right profile of melting domains. Sensitivity is improved by adding a tail "GC clamp" at the end of the primer (Sheffield *et al.* 1989). The location of the sequence difference within the fragment can not be localised and has to be determined by sequencing.

Protein truncation test (PTT)

The PTT is a specific test for frameshifts, splice sites or nonsense mutations which truncate a protein product, and was first described by Roest *et al.* (1993). The procedure is to make cDNA by RT-PCR, using a special primer which carries at the 5′ end a T7 promoter followed by a eukaryotic translation initiator sequence. The cDNA is put into a coupled transcription-translation system, which uses the T7 promoter to make mRNA and the translation initiator to translate it. The protein product is then run out on a gel. If the product is full length, no truncating mutation is present in the sequence cloned. Truncating mutations result in shorter products, the size of which reveals the position of the mutation .

Comparatively large (up to 2.4kb) stretches of coding sequence can be screened. However, the strength and (or) weakness of the PTT is that it detects only certain classes of mutations. In some genes, such as the dystrophin, *APC*, *BRCA1*, and *BRCA2* which most of the mutations result in

truncated protein the PTT has several advantages. It conveniently ignores the non-pathogenic silent mutations or missense base substitution, and it reveals the approximate location of any mutation. Large exons, such as exon 11 of the both *BRCA1* and *BRCA2*, can be tested using genomic DNA rather than by RT-PCR .

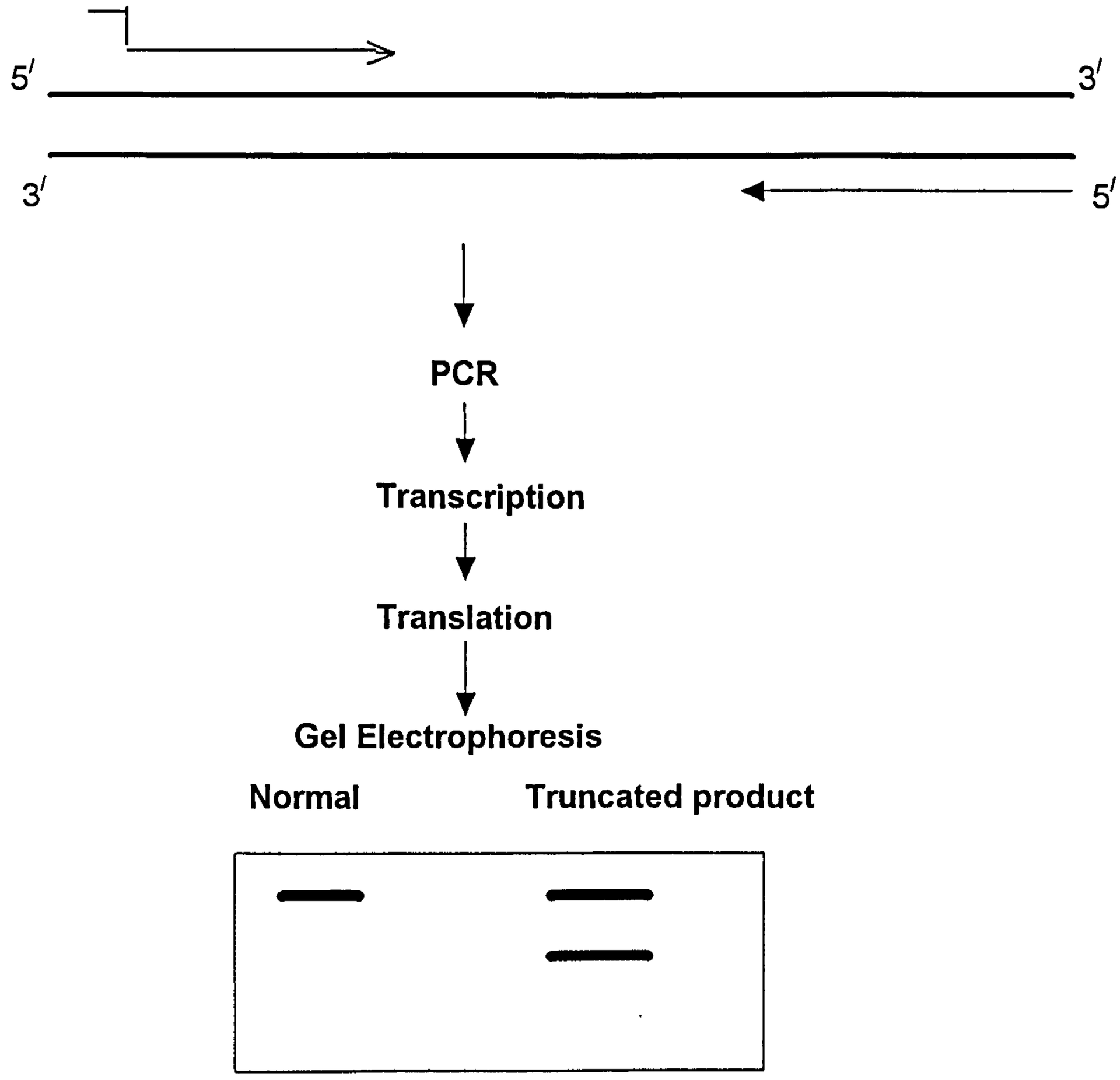


Figure 1.2: Analysis of sequence variation by PTT.

1.8 Direct sequencing

The most direct way to detect any change in DNA sequence is direct sequencing which refers to the direct sequence analysis of a PCR product. There are two sequencing techniques available: Maxam and Gilbert (Maxam and Gilbert, 1977), and Sanger's method (Sanger *et al.* 1977). Maxam and Gilbert's method is the chemical method that breaks a terminally-labelled DNA molecule partially at each repetition of a base. The length of the labelled fragments then identify the positions of that base. Reactions were used that cleave DNA preferentially at guanines, adenines and guanines equally, at cytosines and thymines equally, and at cytosines alone. Resolving the product of these four reactions by electrophoresis on a polyacrylamide gel followed by autoradiography will detect the DNA sequence which was examined.

The Sanger method is an enzymatic method and involves the synthesis of DNA by a DNA polymerase using a single-stranded DNA template. This method is divided in three stages: first, template DNA is annealed to a oligonucleotide primer, secondly, primer/template duplex is used as a substrate for chain extension where dNTPs and radioactive labelled dATP are incorporated into the growing strand, finally, the reactions are terminated by addition of dideoxynucleotides into the growing strands in four separate reactions. This results in the production of a family of DNA fragments which can be visualised by autoradiography after separation by electrophoresis on denaturing polyacrylamide gels. DNA sequencing defines the location and exact nature of the change and therefore is the necessary final step of any detection method.

Cycle sequencing is a newly developed sequencing technique where the DNA template is simultaneously amplified by the addition of dideoxy terminators to PCR reaction. Recently, a new protocol based on the cycle

sequencing and fluorescence detection technology has been developed (Rosenthal and Charnock, 1992). Fluorescently labelled dideoxy terminators are set with different fluorescent dye coupled to each of the four dideoxy nucleotide triphosphate (ddNTPs). This method appears especially useful when high throughput automated sequencing is available.

1.9 Aims of the study

1- To amplify the *BRCA1* and *BRCA2* coding sequences by using either DNA or cDNA as the template.

2- To develop an efficient screening strategy for the detection of mutations, utilising techniques such as single strand conformational polymorphism (SSCP) analysis, Chemical cleavage of mismatches and the protein truncation test (PTT).

3- To completely characterise the mutations identified by the above techniques by direct DNA sequencing.

4- To use the information obtained from this study for the genetic counselling of families with a heritable mutation

CHAPTER TWO

MATERIALS AND METHODS

2.1 Patients

Forty breast and ovarian cancer patients with between 1 and 6 affected first or second degree relatives and with onset of between 23 and 60 years of age, were selected in the cancer clinic of this department (table 2.1a). All those forty patients were screened for mutations in the *BRCA1* gene. More than 95% of the coding sequence was screened, of which 40% was screened using RNA as a template and the remainder was screened using DNA as a template. Forty patients were screened for mutations in *BRCA2* gene. More than 90% of the coding sequence was screened using DNA as a template.

2.2 Extraction of DNA from peripheral lymphocytes

DNA from peripheral blood lymphocytes was isolated according to the method described by Kunkel *et al.* (1977). 10 ml of whole blood was put in a 50ml falcon centrifuge tube, then topped with ice cold lysis buffer (0.32M Sucrose, 10 mM Tris-HCL, pH 7.5, 5 mM $MgCl_2$, 1% Triton X-100). This was incubated on ice for 10 minutes then centrifuged at 2500 r.p.m. for 10 minutes at 4°C in an ICE DRP-6000 centrifuge. The supernatant was discarded and the pellet was resuspended in 3ml of nucleic lysis mix (10mM Tris-HCl, pH 8, 0.4M NaCl, 2 mM EDTA), 200 μl 10% SDS, followed by the addition of 100 μl of proteinase K (Boehringer Mannheim; 10mg/ml) to catalyse the digestion of histones and other proteins, and was incubated overnight at 37°C. 1ml of 6M NaCl was then added to overnight incubation and the sample was vigorously mixed and centrifuged at 2,500 r.p.m. for 10 minutes. The top layer was transferred into a 10ml tube. The samples were extracted twice with phenol/chloroform, followed by a chloroform/isoamyl alcohol extraction to remove trace of phenol. DNA was precipitated from the aqueous phase with a two volume of 100% ethanol. The DNA was spooled out using a sealed pasteur pipette, washed in 75% ethanol, air dried and

dissolved in a suitable volume of T.E. buffer (10mM Tris-HCl pH 8.0, 1mM EDTA). The DNA was stored at 4°C until required.

	No. of cases (mean age at diagnosis)		
Family	breast cancer	breast/ovarian or ovarian cancer	Other cancers
1	2 (48)	-	-
3	4(48)	---	2 (46)
4	4(44)	-	-
5	3 (48)	-	2(56)
6	2 (52)	-	1 (34)
7	3 (34)	-	--
8	3 (47)	2 (47)	--
9	3 (49)	-	-
10	4 (43)	3 (44)	2 (56)
11	5 (55)	-	3 (72)
12	2 (38)	-	-
13	8 (50)	1 (47)	8 (65)
14	6 (41)	1(64)	6 (65)
15	3 (38)	-	-
16	2 (44)	-	2
17	3 (42)	-	-
18	4 (42)	1 (40)	-
19	3 (45)	-	1 (45)
20	3 (36)	-	2 (59)
21	4 (39)	-	2 (52)
22	2 (46)	-	-
23	3 (36)	-	-
24	4 (58)	-	-
25	2 (49)	-	-
26	3 (41)	-	-
27	3 (55)	-	-
28	3 (39)	-	2
29	5 (51	-	2 (60)
30	3 (47)	-	-
31	2 (45)	-	-
32	2 (51)	-	1
33	3 (56)	-	-
34	3 (42)	3 (43)	2 (60)
35	7 (35)	-	-
36	3 (56)	-	-
37	6 (33)	-	3 (55)
38	2 (39)	1 (44)	-
39	1 (52)	2	-
40	3 (37)	2 (56)	-

Table 2.1a. Characteristic of cancer families which have been tested for *BRCA1* and *BRCA2* mutations.

2.3 Extraction of RNA from peripheral lymphocytes

The RNA extraction from peripheral lymphocytes involved the initial separation of lymphocytes from whole blood, followed by using either the acid-guanidinium thiocyanate- phenol/chloroform method or TRIzol™ (Total RNA Isolation Reagent) techniques.

To prevent RNA degradation by ribonuclease, all tubes, tips and solution (except organic solution) were treated with a solution of 0.1% DEPC (diethyl pyrocarbonate). Gloves were used all the time and were frequently changed.

Separation of lymphocytes from whole blood

Five ml of Histopaque-1077 (SIGMA) was poured in a universal tube, then 5 ml whole blood was added. The tube was centrifuged at 1500 r.p.m. for 30 minutes at room temperature. The interphase layer, consisting of lymphocytes, was transferred into a fresh tube. The cells were washed with 10ml of cold phosphate buffer saline (PBS) and pelleted by centrifugation at 1400 r.p.m for 10 minutes. The supernatant was discarded and this process was repeated once more. The pellet was subjected to total RNA extracting using one of the following methods:

2.3.1 Extraction of RNA using the acid guanidinium thiocyanate method

500µl of solution D (4M guanidinium thiocyanate, 25mM sodium citrate, 0.5% sarcosyl and freshly added 0.1mM β-mercaptoethanol) was added to the cells and thoroughly mixed until viscous, the mix was then transferred to a 1.5ml microfuge tube and extraction was carried out by adding 50µl of 2M sodium acetate (pH 4.0), 100µl chloroform-isoamyl alcohol (49:1), and 500µl phenol; the final volume was mixed by vortexing followed by incubation on ice for 15 minutes. The tube was then centrifuged on a bench top centrifuge for 20 minutes. The upper aqueous phase which contained RNA was transferred to a fresh microfuge tube and equal volume of isopropanol was added, the contents were mixed well and kept at -20 for

at least one hour to let the RNA to be precipitate out. The tube was then centrifuged at full speed in a bench top centrifuge for 20 minutes and the supernatant was discarded. The RNA pellet was dissolved in 400µl of solution D and an equal volume of isopropanol was added. The mixture was kept at -20 for one hour, centrifuged at full speed for 15 minutes and the supernatant discarded. The pellet was washed in 75% ethanol and air dried and dissolved in 55 µl of DEPC water and stored at -20.

2.3.2 RNA extraction from whole blood using TRIzol Reagent (Total RNA Isolation Reagent)

TRIzol Reagent is a ready-to-use reagent for the isolation of total RNA from cells and tissues supplied by Gibco BRL (cat. No. 15596-026). The advantages of this method are; saving time, less contact to hazardous materials and are carrying out all the steps at room temperature . For RNA extraction from lymphocytes, as acid guanidium thiocyanate method, first we separated the lymphocytes from whole blood then continued the extraction by TRIzol reagent. After isolation of the lymphocytes from whole blood, using the Histopaque as described above, 1 ml of TRIzol was added to the pellet (obtained from 5ml blood), mixed gently by pipetting and then transferred to a DEPC treated 1.5 ml microfuge tube and left for 5 minutes at room temperature to let the complete dissociation of nucleoprotein complexes. 200 µl chloroform per 1 ml was added to the reagent; shaken vigorously by hand for 15 seconds and incubated at room temperature for about 2 to 5 minutes. The sample was centrifuged at 12000 r.p.m at the bench top centrifuge at 4^0C for 15 minutes. After centrifugation, the mixture was separated into three different phases: the upper colourless aqueous phase, the red lower phase and an interphase. The upper colourless aqueous phase was transferred to a new 1.5ml microfuge tube. To avoid DNA contamination, we tried not to take the interphase which contained DNA. In order to precipitate the RNA, 500 µl isopropanol alcohol was added to the aqueous phase and mixed gently by inversion. After 10 minutes incubation at room temperature the

tube was centrifuged at 12000 r.p.m for 15 minutes at 4^0C and supernatant was discarded. The RNA was precipitated at the bottom of the tube as a gel like pellet. The pellet was washed with 75% ethanol (prepared with DEPC treated water), then the pellet was air dried, dissolved in 70µl DEPC treated water and kept at -40^0C for further analysis. Alternatively we can keep the RNA pellet in 75% ethanol and keep in -70 until use.

2.4 Qualitative assessment of RNA

The quality of extracted RNA was determined by running the RNA on agarose gel. To prepare a 1.5% agarose gel, 0.45g agarose was added to 22ml water in a flask and then boiled until the agarose dissolved. The gel was then allowed to cool to 55°C, onto which 3ml of 10x MOPS buffer [3-(N-morpholino) ethanesulphonic acid] was poured containing (200mM MOPS, 50mM sodium acetate pH 7, 10mM EDTA) and 5ml formaldehyde to make up a total volume of 30ml. To prepare the RNA sample, 1µl RNA solution was mixed to 5µl of formamide, 1.6µl (37%) formaldehyde, 1.3µl of H_2O and 1µl of 10x MOPS buffer. The mixture was heated to 55^0C for 10 minutes then quenched on ice and 2µl of gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 40% sucrose in water) was added. The sample was then immediately loaded. 1µg of *Escherichia coli* ribosomal RNA (Boehringer) was also loaded as markers and electrophoresed at 75V until the bromophenol blue dye reached the bottom of the gel. 1x MOPS was used as a running buffer. After electrophoresis was complete, the formaldehyde was washed out by soaking the gel for one hour in water; then stained the gel for 5 minutes in 5µg/ml ethidium bromide. The gel was destained in water overnight and viewed under U.V. light to check the integrity of the ribosomal RNA bands and hence the quality of the RNA preparation.

2.5 Design of oligonucleotide primers

Some of the oligonucleotides used in this study have been published and the rest have been designed for this study. Oligonucleotide primers were generally synthesised in the range of 18-24 bases except for those that were used for PTT analysis which were 65-70 base pairs. Two oligonucleotide primers were designed for each PCR reaction. Each of them was complementary to one strand of DNA template. The forward primer (F), was complementary to sense strand and the reverse primer (R) was complementary to the antisense strand .In this project oligonucleotides were designed using the computer programme, OLIGO version 3.4 (© Wojciech Rychlik; Medprobe).

The programme allowed the design of PCR primers that: **(i)** were highly specific for the intended target sequence and so as not to base-pair to other regions within the template, **(ii)** were not self-complementary and did not form dimers, **(iii)** as a pair, were compatible in PCR i.e., they were not complementary at their 3′ ends, to avoid primer dimer formation and had similar melting temperatures and duplex stabilities.

All the oligonucleotides were synthesised on APPLIED BIOSYSTEM (ABI) 391 DNA SYNTHESISER. Ammonium hydroxide was used to elute the primers from the solid support and deprotection was carried out at 55^{0}C for 12-16 hours. The tube was left for 15 minutes on ice. To purify deprotected oligonucleotides, 150 μl of aliquot of primer was taken and left in the fume hood for rapid evaporation of ammonia then left in -20 until further use .The rest was stored in their ammonia eluant at -20°C as stock.

2.6 The polymerase chain reaction (PCR) using genomic DNA as a template.

Depending on the purpose of study, the PCRs were performed in a total volume of 100, 50 or 25μl PCR reaction mix using 1.5-3 units AmpliTaq^R DNA polymerase (Perkin Elmer cat No: N801-0060) in 1x PCR

buffer (Perkin Elmer cat No: N808-0006), 200 μM of each dNTP, 10-40 pmol of each primer, 1.5mM $MgCl_2$ and 0.1 μg genomic DNA. Where necessary the concentration of each of the materials was optimised to get the best PCR result. To minimise contamination, buffer, primers and dNTPS were added into pre-labelled 0.5 ml microfuge tube on ice then the volume was made up to the desired volume with distilled water, the mixture was subjected to 10 minutes of UV radiation (GRI-Amplirad) then DNA and enzyme were added, the mixture was vortexed, centrifuged, overlaid with two drops of mineral oil and subjected to PCR. All the above stages were carried out on a bucket of wet ice. In general, the profile for the PCRs were; two minutes at 94^{0}C for one cycle (initial denaturing) followed by 30-40 cycles at 94^{0}C for 30-60 seconds (denaturation), 53 -60^{0}C (depending on the primers) for 30-60 seconds (primer annealing), and 72^{0}C for 1-3 minutes (primer extenuation). The final extension was 10 minutes at 72^{0}C, for one cycle. PCR products were visualised by agarose gel electrophoresis (in 1x TBE), ethidium bromide staining and transillumination on a UV-lamp (Fotodyne,UV-transilluminator). Permanent records of these gel were made using a gel documentation system (UVP Imagestor 5000) linked to a thermal printer. The primers used for amplification of exon 11 of the *BRCA1* gene in order to do either chemical cleavage mismatch analysis or protein truncation test and the condition for amplification were as described in Tables 2.1 and 2.4. Primers used for amplification of exon 11 of the *BRCA1* gene in order to do single strand conformational analysis were, as described in table 2.2 (Friedman *et al.* 1994). Primers for amplification of exon 1-10 and 12-24 of the *BRCA1* gene in order to do single strand conformation analysis and the conditions for amplification were as described in Table 2.3 (Friedman *et al.* 1994). The condition for amplification of exons were set according to Friedman *et al.* (1994). Primers used for amplification of *BRCA2* gene in order to do protein truncation test and the condition for amplification were as described in Tables 2.5 and 2.6. Primers used for amplification of exons

1-10 and 12-27 of the *BRCA2* gene were, as described by Tavtigian *et al.* (1996).

Segment	primer name	position	primer sequence
A(F)	ALG 10F	INTRON	5′ GGAATTAAATGAAAGAGTATGAGC 3′
A(R)	ALG10R	1656	5′ TGTGAGGGGACGCTCTTG 3′
B(F)	ALG15F	1501	5′ TTGGGAAAACCTATCGGAA 3′
B(R)	ALG15R	2366	5′ ATCTTTGGGGTCTTCAGCA 3′
C(F)	ALG16F	2252	5′ GTGTTCAAATACCAGTGAACTTA 3′
C(R)	ALG16R	3287	5′ GGAGCCCACTTCATTAGTAC 3′
D(F)	ALG17F	3178	5′ CCAAGTACAGTGAGCACAATTA 3′
D(R)	ALG17R	INTRON	5′ GTGCTCCCAAAAAGCATAAA 3′
F1(F)	BR11F1	793	5′ *CTTGTGAATTTTCTGAGACGG 3′
F1(R)	BR11R1	2125	5′ ATCAGTTGTAGGTTTCTGCTGCTGTG 3′
F2(F)	BR11F2	1921	5′ *ACAATTCAAAAGCACCTAAAAAG 3′
F2(R)	BR11R2	3383	5′ AACCCCTAATCTAAGCATAGCATTC 3′
F3(F)	BR11F3	3061	5′ *CACCACTTTTTCCCATCAAGTC 3′
F3(R)	BR11R3	4183	5′ TTATTTTCTTCCAAGCCCGTTC 3′

* means that: The T7 primer with promoter and sequence for initiation of translation (5′ GCTAATACGACTCACTATAGGAACAGACCAACCATGG) was added to the 5′ site of the primer for the protein truncation test.

(F): Forward primer. (R): Reverse primer.

Table 2.1b: Sequence of primers used in this study for amplification of exon 11 of the *BRCA1* gene in order to do CCM and PTT analysis.

EXON 11	FORWARD PRIMER	REVERSE PRIMER
Ai	5′GGAATTAAATGAAAGAGTATGAGC	5′CTTCCAGCCCATCTGTTATGTTG
A	5′AACACCACTGAGAAGCGTGCAG	5′CTCACACAGGGGATCAGCATTC
B	5′CAACATAACAGATGGGCTGGAAG	5′ACGTCCAATACATCAGCTACTTTGG
B2	5′CCTAGAGATACTGAAGATGTTCCTTGG	5′GCCAGTAAGTCTATTTTCTCTGAAGA
C	5′GGTTCTGATGACTCACATGATGG	5′TCTGTGGCTCAGTAACAAATGCTC
D	5′GAAAACCTATCGGAAGAAGGCAAG	5′TCATCACTTGACCATTCTGCTCC
D2	5′GAGCCACAGATAATACAAGAGCGT	5′GCAGATTCTTTTTCGAGTGATTCTAT
E	5′ATCAGGGAACTAACCAAACGGAG	5′CGCATGAATATGCCTGGTAGAAG
E2	5′TCAGGGAACTAACCAAACGGAG	5′CCATGAGTTGTAGGTTTCTGCTG
F	5′AGGCTGAGGAGGAGGTCTTCTACC	5′CAGCTCTGGGAAAGTATCGCTG
G	5′GCAACTGGAGCCAAGAAGAGTAAC	5′CCTGAGTGCCATAATCAGTACCAGG
H	5′ CAGCGATACTTTCCCAGAGCTG	5′TCTGTTTTTGCCTTCCCTAGAGTG
H2	5′AAGTGTCTAATAATGCTGAAGACCCC	5′CCCAATGGATACTTAAAGCCTTCT
I	5′GCACTCTAGGGAAGGCAAAAACAG	5′CATTCCTCTTCTGCATTTCCTGG
I2	5′GAAGGCTTTAAGTATCCATTGGG	5′CTTATCTTTCTGACCAACCACAGG
J	5′ GCCAGTCATTTGCTCCGTTTTC	5′CGTTGCCTCTGAACTGAGATGATAG
K	5′ TGCAGGCTTTCCTGTGGTTG	5′GGCTAATTGTGCTCACTGTACTTGG
K2	5′GCAACGAAACTGGACTCATTACTC	5′AATACTGGAGCCCACTTCATTAGTA
L	5′TCAATGTCACCTGAAAGAGAAATGG	5′CAGGATGCTTACAATTACTTCCAGG
M	5′TTGAATGCTATGCTTAGATTAGGGG	5′GACGCTTTTGCTAAAAACAGCAG
N	5′GTTTGTTCTGAGACACCTGATGACC	5′AGTGTTGGAAGCAGGGAAGCTC
O	5′GAGTCCTAGCCCTTTCACCCATAC	5′GTGATGTTCCTGAGATGCCTTTG
P	5′CGTTGCTACCGAGTGTCTGTCTAAG	5′ AGCCCGTTCCTCTTTCTTCATC
S	5′ TTCAAGGTTTCAAAGCGCC	5′ AATGAGTCCAGTTTCGTTGC

Table 2.2: Sequence of primers used in this study for amplification of exon 11 of the *BRCA1* gene in order to do SSCP analysis (Friedman *et al.* 1994).

EXON	FORWARD PRIMER	REVERSE PRIMER
1	5′ TAGCCCTTGGTTTCCGTG	5′ TCACAACGCCTTACGCCTC
2	5′GAAGTTGTCATTTTATAAACCTTT	5′ TGTCTTTTCTCCCTAGTATGT
3	5′TCCTTGACACACCAGACATTTA	5′ TTGGATTTTCGTTCTCACTTA
5	5′ CTCTTAAGGGCAGTTGTGAG	5′ TTCCTACTGTGGTTGCTTCC
6	5′CTTATTTTAGTGTCCTTAAAAGG	5′TTTCATGGACAGCACTTGAGTG
7	5′CACAACAAAGAGCATACATAGGG	5′ TCGGGTTCACTCTGTAGAAG
8	5′TGTTAGGTGACTGATGATGGT	5′ATCCAGCAATTATTATTAAATAC
9	5CCACAGTAGATGCTCAGTAAATA	5′TAGGAAAATACCAGCTTCATAGA
10	5′ TGGTCAGCTTTCTGTAATCG	5′GTATCTACCCACTCTCTTCTTCAG
12	5′AATGGAAAGCTTCTCAAAGTA	5′ATGTTGGAGCTAGGTCCTTAC
13	5′CATAACCTGATAAAGCTCCAGCAGG	5′GATGACCTTTCCACTCCTGGTTC
14	CTAACCTGAATTATCACTATCA	5′GTGTATAAATGCCTGTATGCA
15	5′ TGGCTGCCCAGGAAGTATG	5′AACCAGAATATCTTTATGTAGGA
16	5′AATTCTTAACAGAGACCAGAAC	5′AAAACTCTTTCCAGAATGTTGT
17	5′ GTGTAGAACGTGCAGGATTG	5′ TCGCCTCATGTGGTTTTA
18	5′ GGCTCTTTAGCTTCTTAGGAC	5′ GAGACCATTTTCCCAGCATC
19	5′ CTGTCATTCTTCCTGTGCTC	5′CATTGTTAAGGAAAGTGGAGC
20	5′ ATAGACGTGTCTGCTCCAC	5′ GGGAATCCAAATTACACAGC
21	5′AAGCTCTTCCTTTTTGAAAGTC	5′GTAGAGAAATAGAATAGCCTCT
22	5′ TCCCATTGAGAGGTCTTGCT	5′ GAGAAGACTTCTGAGGCTAC
23	5′ CAGAGCAAGACCCTGTCTC	5′ ACTGTGCATCTCAAGCACCA
24	5′ATGAATTGACACTAATCTCTGC	5GTAGCCAGGACAGTAGAAGGA

Table 2.3: Sequence of primers used in this study for amplification of exons 1-10 and 12-24 of the *BRCA1* gene (Friedman *et al.* 1994).

Table 2.4: PCR conditions for amplification of exon 11 of the *BRCA1* gene.

SEGMENT	DENATURARION	ANNEALING	EXTENTION	CYCLES
A	94 - 1 min	58- 1 min.	72- 2 min.	35
B	94 - 1min	58 - 1 min.	72- 2 min.	36
C	94 -1 min	54 - 1 min.	72 - 2 min.	36
D	94 - 1 min	55 -1 min.	72 -2 min.	36
F1	94 - 1 min	56 -1 min.	72 -2.5 min	36
F2	94 - min	55 -1 min.	72 - 2.5 min.	36
F3	94 - min	55 -1 min.	72 -2.5 min	36

Table 2.5: Sequence of primers used in this study for amplification of exon 11 of the *BRCA2* gene.

SEGMENT	PRIMER NAME	POSITION	PRIMER SEQUENCE
A	BR2.AF	2162-2182	*5 GAAGCTGTTCACAGAATGATT 3
A	BR2.AR	3651-3639	5 TGTACTCTTCTGCAATATGTAGC 3
B	BR2.BF	34228-3450	*5 CTGTATCTGCACATTTACAGAGT 3
B	BR2.BR	4737-4715	5 TAGTTGATTTCCAGTACCAACTG 3
C	BR2.CF	4610-4634	*5 CCTTAAATTCTGAATTACATTCTG 3
C	BR2.CR	5913-5891	5 CTCGTAACAACCTGCCATAATTT 3
D	BR2.DF	5825-5848	*5 TTACAGACAGTTTCAGCAAAGTAA 3
D	BR2.DR	5987-5966	5 AAGAGAATGTGTGGCATGACTT 3

*The T7 primer with promoter and sequence for initiation of translation (GCTAATACGACTCACTATAGGAACAGACCACCATGG) was added to the 5′ site of primer when protein truncation test was carried out.

Table 2.6: PCR condition for amplification of the *BRCA2* gene.

SEGMENT	DENATURARION	ANEALING	EXTENTION	CYCLES
A and D	95- 1MIN	55 - 1 MIN	72 - 2.5 MIN	36
B and C	95 -1 MIN	558 -1 MIN	72 - 2.5 MIN	36

2.7 Reverse transcriptase PCR (RT-PCR)

Reverse transcriptase PCR (RT-PCR) was carried out to obtain part of the cDNA of *BRCA1* gene.

To synthesise the first strand of cDNA, reverse transcription was carried out using one of these three primers: **(i)** specific oligonucleotides to prime the mRNA of interest, **(ii)** oligo (dT_{12-18}) (Pharmacia) to get random cDNA from any RNA, **(iii)** random hexamers.

Total RNA isolated from lymphocytes was used as a template for reverse transcription. The cDNA, in turn, was used as a template for RT-PCR using primers to amplify the selected cDNA region. The random primer was the best for synthesising the first strand cDNA. The disadvantage of oligonucleotide was its inability to amplify further than 1.2 Kb from the 3′ end of the RNA. The 5′ end of *BRCA1* encompassing exon 1 to 10 and 3′ end encompassing exons 12-24 amplified by nine sets of primers six of them were used as a nested primers.

Three different ways were used to prime the RNA for cDNA synthesis. In the first, a 3′ (antisense) gene specific antisense primer was annealed to the RNA and extended with reverse transcriptase. This generated a cDNA template for the 5′ primer (sense) allowing PCR amplification to occur. When using a gene specific primer, a number of exprimental parameters needed to be optimised, including primer concentration and annealing temperature.

In the second and third methods, the entire population of mRNA molecules was first converted into cDNA by priming with either oligo (dT_{12-18}), or random hexamers. Two gene specific PCR primers were then added for amplification.

Reverse transcription was carried out as follows: 6µl (0.5-1µg) of total RNA was mixed with 4µl of reverse primer (random hexamer at 50ng/µl) in a DEPC treated microfuge tube, this was heated to 95°C for 5 minutes

followed by 10 minutes incubation at 65°C. The tube was then quenched on ice and then the following was added: 2μl of 0.1 M DTT, 1μl of 10mM dNTPs solution, 4μl of 5x transcriptase buffer (Gibco BRL 250mM Tris-Hcl, pH 8, 375mM KCl, 15mM Mg Cl_2) and 200U of M-MLV transcriptase (Gibco, BRL cat No: 28025-013), then the volume was made up to 20μl using DEPC treated water, the tube was left in a 42°C water bath for 60 minutes. Reverse transcriptase was inactivated by heating at 95°C for 5 minutes half of this transcribed was used as a template for PCR analysis.

To improve the sensitivity and specificity of RT-PCR, nested RT-PCR was used. For amplifying each segment of the cDNA, two sets of primers were used. The reverse transcriped mixture obtained from reverse transcription was subjected to two consecutive PCRs. The first PCR contained an external pair of primers, while the second contained two nested primers which were internal to the first primer pair. The larger fragment produced by first reaction was used as template for the second PCR. In this project half of the transcript obtained from reverse transcription was used for a 100μl reaction of first round PCR then 10μl of PCR from the first round was used for the second round of PCR. Table 2.7 lists the primers used for amplification of the *BRCA1* gene using RNA as a template.

SEGMENT	NAME	POSITION	SEQUENCE
I	BR1.1F*	30-51	5′ CAGGCTGTGGGGTTTCTCAGA 3′
I	BR1.1 R*	1650-1631	5′ CTGATGTAGGTCTCCTTTTA 3′
II	BR1.3F	83-103	5′ CCTCTGCTCTGGGTAAAGTT 3′
II	BR1.3R	1057-1037	5′ GTTATGTTGGCTCCTTGCTA 3′
III	2F15	187-207	5′ GTGTCCCATCTGTCTGGAGT 3′
III	2R10	876-857	5′ CACGCTTCTCAGTGGTGTTC 3′
IV	C2F	212-235	5′ CAAGGAACCTGTCTCCACAAAGTG 3′
IV	C2R	530- 511	5′ AAGTCTTTTGGCACGGTTTCTG 3′
V	C4F	581- 605	5′CCAACTCTCTAACCTTGGAACTGTG 3′
V	C4R	878-857	5′ TGCACGCTTCTCAGTGGTGTTC 3′
VI	BR11F4*	4011-4032	5′ TCACAGTGCAGTGAATTGGAAG 3′
VI	BR24R1*		5′ GTAGCCAGGACAGTAGAAGGA 3′
VII	BR11F5*	4153-4173	#5′ AAGAAAGAGGAACGGGCTTGG 3′
VII	BR24R2*	5693-5672	5′ GATCTGGGGTATCAGGTAGGTG 3′
VIII	BR1.4F	4240-4257	5′ GTGAAACAAGCGTCTCTG 3′
VIII	BR1.4R	5039-5022	5′ TGTCAATTCTGGCTTCTC 3′
IX	BR1.5F	4852-5651	5′ ATCCTTCTGAAGACAGAGCCC 3′
IX	BR1.5R	5651-5630	5′ GAGTGCCTACACTGTCCAACACC 3′

* Primers were described by Frans B.L. Hogervorst *et al.* (1995).

 # The T7 primer with promoter and sequence for initiation of translation (GCTAATACGACTCACTATAGGAACAGACCACCATGG) was added to the 5′ site of primer when protein truncation test was carried out.

Table 2.7: Primers used for amplification of the *BRCA1* mRNA.

2.8 Identification of PCR products

PCR product was visualised by mixing 6µl of PCR product with 1µl of loading mix (0.25% bromophenol blue, 0.25% xylene cyanol, and 40% sucrose) and loading on 1% agarose gel containing ethidium bromide. A 1% agarose gel was prepared by adding 0.5g agarose powder (SEA KEM GTG, FMC Bioproduct) in 50 ml 1x TAE buffer. The mixture was then boiled and let to be cool down to 55^{0}C and then ethidium bromide (5µg/ml) was added and

the gel was poured the gel on casting tray. The sample was then loaded on gel and subjected to electrophoresis at constant 100 volts for 30 minutes. The DNA was then visualised using a standard UV transilluminator and photographed using a gel documentation system (UVP Imagestor 5000) linked to a thermal printer.

2.9 Purification of PCR products

At the end of PCR, the amplified DNA was purified by removing the excess primers, dNTPs, and salts using different techniques. These included the **Geneclean^R II KIT** (BIO 101) and isopropanol precipitation.

2.9.1 Geneclean^R II KIT purification

Purification of PCR product was carried out using **Geneclean^R II KIT** (BIO 101). This kit was used to clean PCR product of over 500 bp in size. After removal of the mineral oil, the total PCR product was transferred to a 1.5 ml eppendorf tube and 3 volume of the supplied stock solution of NaI were added. 15µl of the provided glassmilk suspension was then added, mixed and kept on ice for 15 minutes with occasional mixing to let the DNA bind the molecule. The glassmilk-DNA complex was pelleted by centrifuging for 5 second and the supernatant was removed. The pellet was then washed three times in 300µl NEW wash provided in the kit. The DNA was then eluted into 15µl T.E. buffer by incubation at 65°C for 5 minutes. The mixture was then centrifuged for 5 minutes and the supernatant, which contained the DNA, was transferred to a fresh microfuge tube.

2.9.2 Purification of PCR products using an agarose gel and a Geneclean^R II KIT (BIO 101)

At the end of each PCR the product was checked on 1% agarose gel. If non-specific products of amplification were seen the entire PCR product with 1/6 volume of loading buffer was loaded on a low melting agarose gel (Nusive, Flowgen) and electrophoresed at 100 volts till the bromophenol blue dye came to the end of the gel. After visualisation on a UV

transilluminator the desired band was cut out from the gel and placed into 1.5 ml autoclaved eppendorf tube, then 3 volume of supplied NaI stock solution was added and incubated at 55°C in a water bath for 10 minutes to dissolve the agarose. To this, 15µl of glassmilk was added, vortexed and placed on ice for 10 minutes with occasional mixing. The procedure was then continued as described in section 2.9.1.

2.9.3 Isopropanol precipitation of PCR products

This method was used to remove excess dNTPs, salts and primers from PCR products. Mineral oil was removed from the top of the PCR product. Then one volume of 4M ammonium acetate and two volumes of isopropanol was added, the mixture was vortexed and left on ice for 10 minutes, followed by 10 minutes centrifugation at high speed. The supernatant was discarded and the DNA pellet was washed in 70% ethanol, air dried and dissolved in appropriate amount of T.E. buffer pH 7.0.

2.10 Methods for mutation detection(screening)

2.10.1 Detection of mutations by single strand conformational polymorphism analysis (SSCP)

This technique was used to screen part of the *BRCA1* gene and exons 1-10 and 12-27 of *BRCA2.*

This technique allows the detection of single base change in short DNA fragments, based on mobility differences of single stranded molecules. The primary sequence difference alter the intra-molecular intractions that generate a three- dimentional folded structure. The molecule may thus move at different rates through a non-denaturing polyacrylamide gel. In PCR-SSCP analysis, the region of DNA thought to contain mutations were amplified using polymerase chain reaction. For detecting the product, either isotope was incorporated into PCR product itself or silver stain method was used. The PCR was then heated to generate single-strand and then fractionated on MDE™ gel solution (AT BIOCHEM cat No. 1-500-00) to

detect any conformational change due to sequence difference. The technique involves different stages as follows:

(A) Radiolabelled PCR

A 15μL radiolabelled PCR was carried out by mixing; 7.8μl water, 1.5μl of each primers (10 pmol/μl), 1μl dNTPs mix (2.5mM/μL each). This mixture was then subjected to 10 minutes UV irradiation to minimise contamination. To this mixture, 1.5μl 10x PCR buffer (Perkin Elmer) containing 15mM $MgCl_2$,0.5μl [α-^{32}PdCTPs) and 0.3μl Taq polymerase (Perkin-Elmer) was added, overlaid with one drop mineral oil and subjected to 30 cycles of PCR. The PCR condition depended on the primers used. Tables 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, and 2.7 list primers and PCR conditions used to amplify *BRCA1* and *BRCA2*.

(B) Gel preparation and pouring

The sequigene sequencing gel apparatus (Bio Rad) with 0.4mm spacers and comb was used for preparation and detection of radiolaballed PCR product.

The glass plates were cleaned by soapy water, then wiped and dried with ethanol. To ensure that the gel will not stick to the glass plate, one of the plates (usually the back plate) was treated with anti stick product such as dimethyldichlorosilane. The apparatus was assembeled according to manufacturer's instructions. The bottom of the gel was sealed using the sealing apparatus a wick of 3mm Whatman paper and 30ml of a 10% acrylamide solution. After addition of 40μL TEMED and 150μl of 20% ammonium per sulfate (APS) which allowed polymerisation, the gel was poured at the bottom of the Gel apparatus to seal the bottom.

The gel was prepared using MDE™ gel solution (AT BIOCHEM cat No: 1-500-00) as follows: 12.5 ml MDE gel solution, 6ml 10x TBE buffer, 2.5 ml glycerol, and 32 ml water was mixed. Then 40μl TEMED and 200μl 10% fresh APS was added to the mixture, filled up to 50 ml with water, mixed well and poured between two glass plates, the 20 well comb was inserted and the gel was allowed to polymerise for 1 hour. After the gel had set, the sealing

apparatus and the well forming comb were removed, the buffer chambers were filled with 0.6x TBE and the wells were washed with buffer.The samlpes were then prepared and loaded onto the gel and electrophoresis was carried out at room temperature for 18-20 hours at constant 5-6 Watts.

(C) Preparation of the samples

1μl of the radiolabelled PCR product was diluted to 10μl of stop solution (95% Formamide, 10mM NaOH, 0,25% Bromophenol blue, 0.25% Xylene Cyanol) and heated for 4 minutes at 95°C then cooled on ice for 2 minutes and 5μl of each sample was loaded on to MDE gel.

(D) Autoradiography

When the run was completed the plates were removed from the apparatus allowed to cool briefly before separation. The gel was transferred on to Whatman 3mm filter paper, covered with saran wrap, dried under a vacuum for 30 minutes at 80°C on a gel dryer (model 583 Bio Rad) and subjected to autoradiography using KODAK X-Omat diagnostic AR imaging film.

Alternatively the silver stain method was used to detect the PCR-SSCP. The procedure was as follows:

15μl unlabelled PCR product was carried out as described earlier, then the MDE gel was prepared using standard sequencing apparatus with 1mm spacer and comb. The sample was prepared and electrophoresis was carried out under the same condition as above. When the run was completed the gel was removed to a nonmetallic container and the gel was then silver stained to visualise the bands.

Silver stain method

Following electrophoresis the gel was fixed in a solution of 10% ethanol /0.5% acetic acid for 10 minutes at room temperature, then the fix was poured out and the gel was stained with 0.1% silver nitrate (made from 1% stock) (Sigma) for 10 minutes followed by twice rinsing with distilled water. The gel was developed with a solution of 1.5% NaOH (made from 15% stock) 0.048% formaldehyde for 20 minutes at room temperature. The

stain was fixed with solution of 0.75% $NaCO_3$ (made from 7.5% stock) for 10 minutes. The gel was then photographed and covered by a sheet of plastic paper.

2.10.2 Chemical cleavage of mismatches (CCM)

Mutation screening by chemical cleavage method is based on the fact that mismatches cytosine(C) and thymine (T) are much more reactive with compounds hydroxylamine and osmium tetroxide, respectively, than are Watson and Crick-paired cytosine and thymine bases. In this protocol, an excess of unlabelled target DNA was hybridised with labelled wild type DNA probe and heteroduplexes were formed. The heteroduplex was then divided into two parts, one aliquot was treated with hydroxylamine, which reacts with mismatched C bases; another aliqout was treated with osmium tetroxide which reacts with mismatched T bases. The reactions were then mixed with piperidine to cleave the sites where have been reacted with hydroxylamine or osmium tetroxide. Cleaved fragments were then electrophoresed and sized on polyacrylamide gel, in order to identify the point of cleavage, and hence the position of the mutation. The technique involved five different stages: **(A)** preparation of target DNA and labelled probe **(B)** heteroduplex formation **(C)** chemical modification **(D)** chemical cleavage of mismatches and **(E)** electrophoresis and autoradiography.

(A) Preparation of the target DNA and radiolabelled probe

The DNA segment for CCM was amplified by PCR as described in section 2.6. Tables 2.1 and 2.4 list the primers and conditions of PCR used in this project. To prepare the probe the same fragment from a normal individual was amplified by PCR and [α-^{32}P]dCTP (Amersham) incorporation into the PCR as described for SSCP. The PCR products were checked on a 1% agarose gel, if nonspecific products of amplification were seen, the entire PCR product was electrophoresed in a low melting gel and the required band was cut out and subjected to Geneclean as described in section 2.9.2. If nonspecific products of amplification were not seen, the PCR was directly

genecleaned and dissolved in the appropriate amount of 1x T.E. buffer (10mM Tris HCl, 1mM EDTA, pH 7.0).

(B) Heteroduplex formation

200ng of tested DNA was mixed with 1/10 volume of labelled probe in a 20μl volume of 1x prehybridisation buffer (10x prehybridisation contain 3M NaCl and 1M Tris-HCl pH 8.0) topped with one drop mineral oil and then placed in boiling water bath for 5 minutes. The tubes were then immediately transferred to 65°C water bath and incubated 5-16 hours to allow hybrid formation to occur. The mix was then transferred to a siliconized 1.5ml eppendorf tube and 3μl of glycogen (20mg/ml) and 750μl of stop/precipitation mixture (63mM sodium acetate, 20 μM EDTA and 80% ethanol) was added then mixed well and chilled on dry-ice for 15 minutes; followed by centrifugation at high speed for 10 minutes, the supernatant was then discarded and the pellet was rinsed with 70% ethanol, air dried and resuspended in 14μl of $TE_{0.1}$ buffer (10mM Tris-HCl pH 7.4 0.1mM EDTA). This was used as a substrate for chemical modification by osmium tetroxide and hydroxylamine.

(C) Chemical modification using osmium tetroxide and hydroxylamine

Each 14μl of hybrid sample was split into 2 x 7 μl aliquots in 1.5ml siliconised eppendorfs tube for each chemical modification.

(i) Osmium tetroxide preparation:

1.44g of 4% w/v commercially available osmium tetroxide (from Aldrich cat No. 25175-5) was added to 6.7μl of pyridine and 15 μl of $T_{o.1}E$ buffer. 18 μl of this mix was added to the 7μl of hybrid from last stage, mixed well and incubated at 37°C for 2 hours.

(ii) Hydroxylamine preparation

A 4M solution of hydroxylamine hydrocholoride adjusted to pH 6.0 with diethylamine was prepared as follows:

1.3g of hydroxylamine hydrocholoride (BDH) was dissolved in 1.5ml of prewarmed (37°C) distilled water. The pH of the solution was adjusted to 6.0 by adding diethylamine (about 1.5ml) and the solution was stored at 4°C for

up to 6-10 days. 20 μl of this solution was added to the 7μl sample of hybrid from last stage and the mixture was incubated at 37°C for 2 hours.

The reactions were stopped by adding 750 μl of stop/precipitation mix, chilled on dry ice for 10 minutes then spun down at high speed for 10 minutes. The supernatant was then discarded and the pellet was rinsed with 70% ethanol and briefly air dried.

(D) Chemical cleavage of mismatches

A fresh 10% solution of piperidine was made by adding 90 μl of piperidine to 810 μl water. 50 μl of this solution was added to each pellet from last stage and vortexed to resuspend the pellet. They were then incubated at 90°C for 30 minutes, the tubes were then chilled on ice and 750 μl of stop/ precipitation mixture was added, chilled on ice for 10 minutes, spun down at high speed for 10 minutes; the supernatant was then discarded and the pellet was rinsed with 70% ethanol and briefly air dried. The pellet was resuspended in 7 μl of formamide loading buffer (95% formamide, 10mM EDTA,10mg/ml bromophenol blue, 10mg/ml xylene cyanol).

(F) Gel electrophoresis and autoradiography

Sequencing gel apparatus from BioRad was used to run the gel. The apparatus was set up as described for SSCP. Then 50 ml of 8% denaturing polyacrylamide gel (NBL Ltd.) was made as follows: 12ml 0f 40% polyacrylamide-bisacrylamide (19:1) was added to 25.3g urea and 5ml of 10x TBE filled up to 50ml with water. The urea was then dissolved by stirring and then 200 μl fresh APS and 40 μl TEMED was added. The gel was poured and let to be polymerized for 1 hour.

The gel was prewarmed to 50°C and 10 μl of resuspended sample which was heated for 5 minutes at 90°C and then chilled on ice for 2 minutes was loaded. A labelled 1Kb ladder (Gibco BRL) was also denatured and electrophoresed on the gel in order to estimate the size of the cleaved products. Electrophoresis was carried out at constant 50 Watts till the bromophenol blue dye reached the end of the gel, then the gel was

transferred to 3mm Whatman paper and autoradiographed using Kodak-X-Omat AR film overnight.

2.10.3 Protein truncation test (PTT)

The protein truncation test is an efficient method for detecting nonsense or frameshift mutations in *BRCA1* and *BRCA2* genes. When only genomic DNA is available, exon 11 of *BRCA1* and *BRCA2* can be conveniently screened by PTT in 3 and 4 overlapping segments respectively. In this protocol PCR is performed using forward primers containing the T7 promoter and a eukaryotic translation initiation sequence. Protein products are then synthesised in a couple in vitro transcription/translation reaction and analysed by gel electrophoresis. The technique involves four different stages: **(A)** preparation of the forward primer with T7 prompter, **(B)** preparation of target DNA, **(C)** linked transcription-translation, **(D)** SDS-PAGE.

(A) Synthesising the upstream primer with the T7 promoter

Modified forward primers, containing a T7 promoter and an eukaryotic translation initiation sequence was prepared to generate PCR products suitable for PTT analysis. The T7 promoter sequence that was added to the 5′ end of the forward primer is presented below:

5′ GCTAATACGACTCACTATAGGAACAGACCACCATGG

(B) Preparation of target DNA

50µl PCR reaction was performed as described earlier using forward primer containing the T7- promoter sequence and a eukaryotic translation initiation sequence at 5′ end. 5µl of this PCR was resolved on a 1.5% agarose minigel prepared in 1x TBE buffer to confirm the size and quality of PCR product. The PCR was then purified using isopropanol precipitation as described in section 2.9.3. The pellet was then dissolved in 5µl RNase -free water.

(C) Linked transcription- translation

Linked T7 transcription-translation system from Amersham (RPN 3152) was used in this experiment. All experiments were carried out in 1/4 scale of manufacturer's instructions to save the materials.

The procedure contained two stages:

1-transcription was carried out by mixing 2µl of T7 transcription mix and 0.5µl cleaned DNA from last step in an DEPC treated tube, gently mixed by pipeting up and down followed by incubation at 30°C in water bath for 15 minutes.

2-translation, the tube was then removed from water bath and 1.8µl nuclease-free water, 0.7µl ^{35}S-Methionin and 7.5µl translation mix were added, mixed gently and incubated at 30°C water bath for another 60 minutes. The tube was then placed on ice to terminate the reaction. The product was then analysed on a 12-15% SDS-PAGE.

(D) SDS polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using protean^{R}II xi cell kit (Bio Rad). The apparatus was assembled according to manufacturer's instruction. The SDS-PAGE composed of two different sections; a 16 cm seperating gel which was 8-15% depending on the size of translated product (the effective separation range of SDS-PAGE is shown in Table 2.8 and a 4cm of 5% stacking gel as shown in figure 2.1. Separating gel was made by mixing reagents shown in Table 2.9 the reagents were mixed gently ensuring no air bubbles were formed. The gel was then poured into the space between glass plates, left the 4 cm for stacking gel, overlaid the gel with 0.1% SDS to ensure a flat surface and to exclude air bubbels. After gel was set the SDS was washed with water and stacking gel was made as shown in Table 2.10, and poured at the top of the separating gel, the comb was then inserted and the gel allowed to set. The apparatus was then assembled and the tank filled with electrophoresis buffer (196mM glycine, 0.1% SDS, 50mM Tris-HCl pH 8.3) this buffer was used for both top and bottom tanks. The sample was then prepared and loaded onto the gel.

(E) Sample preparation

A 5μl aliquot from the translation reaction was diluted with 20μl of sample buffer (0.5ml of 1M Tris-HCl pH 6.8, 0.8μl glycerol, 0.4ml 2-mercaptoethanol and 4.6ml water, this buffer is stable for up to 4 weeks at room temperature), mixed well and boiled for 4 minutes to denature the protein (the remainder of the transcription- translation product was stored at -20 for future use). The sample was then loaded and run for 4 hours at constant 200 volts. To determine the size of the translated product 5μl of the standard protein size marker (rainbow, Amersham) was mixed to 15μl of sample buffer and was denatured at the same condition and loaded as marker. The electrophoresis was then stopped when the dye reached to the bottom of the gel. The gel was transferred to a plastic tray and fixed for 30 minutes (the fixative was isopropanol, water, acetic acid with the proportion of 26:65:10). The fixing solution was then poured out and gel was soaked in "amplify" (Amersham cat No. NAMP 100) for 30 minutes with agitation. This was removed from the solution transferred to a 3mm Whatman paper, covered with saran wrap and dried under vacuum at 60°C for 60 min. The gel was then exposed for 1-7 days and developed according to the manufacturer's instruction.

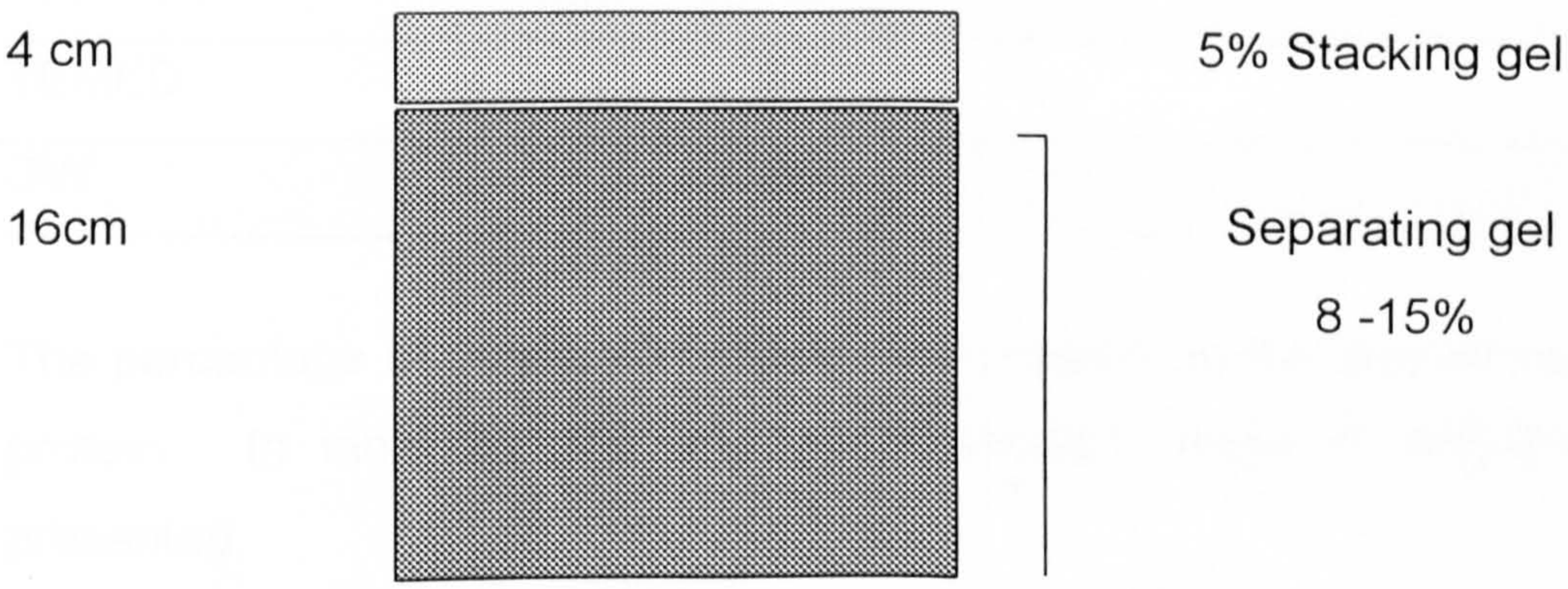


Figure 2.1 Schematic diagram of SDS-PAGE. The top part is always 5% stacking gel followed by different concentrations of separating gel.

Table 2.8 The effective separation range of SDS-PAGE.

% Acrylamide	linear range of separation (kd)
15	12-45
10	15-70
5	25-200

Table 2.9 The formulation of SDS-PAGE with different concentrations.

Component	8%	10%	12%	15%
30% acrylamide	5.3	6.7	8.0	10.0
1.5 M Tris pH8.8	5.0	5.0	5.0	5.0
10% SDS	0.2	0.2	0.2	0.2
10% APS	0.2	0.2	0.2	0.2
TEMED	0.012	0.008	0.008	0.008
DW	9.3	7.9	6.6	4.6

Table 2.10 The formulation of 5% stacking gel

component	Volume (ml)
30% acrylamide	1.7
1M Tris pH6.8	1.25
10%SDS	0.1
10% APS	0.1
TEMED	0.01
DW	6.8

The percentage of acrylamide chosen will depend on the size of translated protein. In table 2.8 the effective separation range of SDS-PAGE is presented.

2.11 Direct sequencing

Samples that gave positive screening results were subjected to sequencing of PCR products from the corresponding exon(s) or cDNA segments to detect any sequence alterations. Direct sequencing of PCR product was carried out using dideoxy chain termination method described by Sanger *et al.* (1977). Three different sequencing strategies were used in this study. (1)- Direct sequencing of the asymmetric PCR products using sequenase version 2.0 sequencing kit USB (Amersham). (2)- Direct sequencing of PCR product using Thermo sequenase radiolabaled terminator cycle sequencing (Amersham product No. 79750). (3)- PCR sequencing using **PRISM™** Ready Reaction DyeDeoxy Terminator cycle sequencing Kit (Perkin Elmer).

2.11.1 Direct sequencing of asymmetric PCR products

T7 DNA polymerase (sequenase version 2.0) was used to sequence asymmetric PCR products. This protocol involves different stages which are as follows:

(A) Asymmetric PCR to generates single stranded template

A well purified single stranded DNA was needed to perform a sequencing under optimal conditions. Asymmetric PCR was carried out. The PCR of corresponding region was done as described earlier. The PCR product was run on 1.5% low melting gel and appropriate band was cut out from the gel. This was then soaked in 50µl T.E. buffer for overnight to let the DNA be eluted from the gel. A 10µl of aliquoted DNA was used in 100µl PCR reaction containing 50 pmol of one primer and 1/75 dilution of the other primer (limiting primer), 6µl of dNTPs (2.5mM each), 10µl of 10x buffer (Perkin Elmer) 1.5 mM $MgCl_2$ and 2.5 unit Tag polymerase (Perkin Elmer), made up to 100µl with distilled water, overlaid with two drops of mineral oil and subjected to 40 cycles of PCR using appropriate annealing temperature. At the end of PCR, 5µl of PCR product was loaded on 1% agarose gel to check the quality and quantity of PCR. 5µl of 1kb ladder as well as double

stranded control was loaded to monitor the amount of single stranded PCR. The PCR product was purified by using isopropanol precipitation. The pellet was then dissolved on 7μl of distilled water and then used for sequencing reaction.

(B) Annealing template and primer

A 1μl of limiting primer (1pmol/μl) and 2μl of 5x sequencing buffer (supplied by kit) was added to 7μl of purified asymmetric PCR, mixed gently and annealed by heating the tube to 65^{0}C for 2 minutes then allowed the tube to cool slowly to <35°C over 30 minutes period. The tube was then centrifuged briefly and chilled on ice. While annealing, labelled and filled 4 tubes each with 2.5μl of each termination mixture (G,T,C and A), then prewarmed at 42°C for at least 3 minutes.

(C) labelling reaction

To ice cold annealed DNA mixture the following was added; 1μl DTT(0.1M), 2μl of 1x labelling mix (diluted from 5x stock), 0.5μl [α-^{35}S]dATP and 2μl diluted sequenase DNA polymerase (made by mixing 25μl sequenase DNA polymerase, 25μl inorganic pyrophosphatase, 150μl glycerol enzyme dilution buffer), then mixed thoroughly and incubated 5 minutes at room temperature.

(D) Termination reactions

While annealing was carried out, 4 tubes labelled G, A, T, and C was prepared, then 2.5 μl of the ddGTP Termination Mix was placed in the tube labelled G, similarly filled the A, T and C tubes with 2.5 μl of ddATP, ddTTP and ddCTP Termination Mixes, respectively. Capped the tubes to prevent evaporation, then let the tubes be prewarmed before the beginning of the termination reaction. After the labelling reaction was completed, 3.5μl of mixture was transferred to each prewarmed (A,T,C and G) tube and the incubation continued for a further 5 minutes at 42°C. The reaction was then stopped by addition of 4μl of stop solution (provided in the kit). The sample was stored at -20 until further use.

(E) Sequencing gel electrophoresis and autoradiography

The sequencing gel apparatus (Bio Rad) with 0.4mm spacers and comb was used. The kit was prepared and assembled as described for SSCP. 50ml of 8% denaturing polyacrylamide gel was prepared by mixing; 12ml of 40% acrylamide-bis-acrylamide (19:1), 25.5g urea, 3ml of 20x glycerol tolerant buffer (216g Tris-Base, 72g Taurin, 4g EDTA up to 1 litre with water) then water up to 50ml. The gel was then mixed and 200µl of 10% fresh APS and 40µl of TEMED was added. The gel was then poured and allowed to set for 1 hour. The sample was heated for 3 minutes then chilled on ice for 3 minutes and loaded on to the prewarmed gel, the gel was run at constant 52 Watts for 2-4 hours. At the end of electrophoresis the glass plates were gently pulled apart and the gel was transferred to a 3mm Whatman paper covered with saran wrap then dried, exposed and developed as described for CCM analysis.

(F) Modifications in sequencing protocol

Depending on the region of DNA to be sequenced some modifications were made in order to get the better result. These modifications include:

1) Reading sequences close to the primer: To provide maximum information of sequences close to the primer, two methods were used; one was to reduce the nucleotide concentration in the labelling step. This was possible by diluting the labelling mix to a 1:10 or 1:20 of the stock reagent instead of 1:4. When reading sequences within 20 nucleotides of the 3′ end of the primer it was essential that sufficient DNA and primer be present therefore the DNA and primer was also doubled. The other modification was to use Mn buffer which affects the reactions in the termination step. The addition of Mn^{+2} (1µl of supplied buffered $MnCl_2$ solution) to sequencing reaction reduces the average length of DNA synthesised in the termination step, intensifying bands less than 20 nucleotides from the primer.

2) Elimination of compressions: For running sequences where compressions are a problem, the dITP labelling-mix (diluted 1:5) was substituted for the dGTP labelling mix and the dITP and the dITP termination

mixture were used in the termination reactions. The reactions containing dITP were run alongside dGTP reactions, since dITP may potentiate pausing artefacts.

3) Extending sequences farther from the primer: Two different ways were used for extending sequences farther from the primers (more than 300 bp); the first one was the extending reaction in the labelling step by increasing the concentrations of the dNTPs in the labelling reactions 3-5 fold and extending the labelling reaction to 5 minutes and also the amount of {α-35S}dATP was increased (1-2μl instead of 0.5μl). The second involved the alteration of the nucleotide mixture used in the termination step. This was achieved by replacing the usual volume of termination mix (2.5μl) by 1.5μl of sequencing extending mix (supplied with the kit) and 1μl of termination mix. Then the gel was electrophoresed 4-10 times longer and was run at 40°C instead of 50°C for an improved result.

2.11.2 Sequencing using Thermostable sequenase and [α-^{33}P] dideoxynucleotides (ddNTPs)

In this protocol the label is incorporated into the DNA sequencing reaction product by the use of four [α -^{33}P] dideoxynucleotide (ddNTPs) terminators (G, A, T, and C). This technique involves different stages as follows:

(A) Preparation of target DNA

100μl of corresponding region of DNA was carried out as described in section 2.6. A 4μl of PCR product was resolved on the 1% agarose gel to check for quality and quantity of PCR which was then purified by isopropanol precipitation as described in section 2.9.3, the pellet was then dissolved in 7μl water. This was then used for cycle sequencing.

(B) Preparation of termination mix:

Four 0.5 ml eppendorf tubes were labelled as (G, T, A, and C) and 2μl of termination master mix was added to each of them. After that 0.5μl of

ddNTPs (ddGTP to G tube, ddATP to A tube, ddTTP to T tube and ddCTP to C tube) were added and mixed gently.

(C) Preparation of reaction mixture

A 20μl of reaction mixture was prepared by mixing 2μl of reaction buffer, 7μl of DNA (cleaned DNA), 2μL of primer (1pmol/μl) and 9μl water.

(D) Cycling termination reaction

4.5μl of reaction mixture was transferred to each termination tube (G, A, T and C), mixed well and overlaid with 20μl of mineral oil, then subjected to 40 cycles of PCR . The PCR condition was 95°C for 30 sec, 55°C for 30sec and 72°C for 2min. At the end of PCR, 4μl of stop solution (supplied by the kit) was added to each termination reaction.

(E) Gel electrophoresis and autoradiography

The sequencing gel and condition of the gel electrophoresis and autoradiography were the same as described in section 2.11.1.(E).

2.11.3 PCR sequencing using the PRISM™ Ready Reaction DyeDeoxy Terminator cycle sequencing Kit (Perkin Elmer).

(A) DNA preparation and cycle sequencing

A 100 μL PCR reaction of corresponding region of DNA was carried out as described in section 2.6. 4μl of PCR product was resolved on the 1% agarose gel to check for quality and quantity of PCR which was then purified by isopropanol precipitation as described in section 2.9.3, the pellet was then dissolved in 7μl water. This was then used for cycle sequencing which was carried out as follows:

A 10.5μl PCR mix was made containing 2μl of the purified PCR product, 3.2 pmol of one of the primers set for initial PCR (optionally, nested primer can be used as well) and ddH_2O and kept on ice. 9.5μl of the terminator premix (supplied by the kit) (Prism™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit, Perkin Elmer) was added to each 10.5 μl PCR mix on ice, mixed well, overlaid with one drop of mineral oil and placed in a 96°C preheated PCR block (Perkin Elmer/Cetus PCR model 480). 25 PCR cycles

of 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes was then carried out. The PCR cycles were followed by a rapid thermal ramp to 4°C and held.

(B) Purification of the product

At the end of thermal cycling, 80µl of ddH_2O was added to each 20µl reaction volume under the oil and mixed by pipeting. The whole 100µl volume was transferred under the oil to a fresh 0.5 ml eppendorf tube. The product was precipitated by adding 15µl of 2M Na acetate, pH 4.5, and 300µl of absolute ethanol followed by incubation at -70°C for 15 minutes and centrifugation in a microcentrifuge at full speed for another 15 minutes at room temperature. Pellet was washed in 70% ethanol and dried using a vacuum centrifugation.

(C)Running of the sequencing reaction on the automated sequencer (ABI Prism 373 automated sequencing apparatus)

A 6% denaturing polyacrylamide gel containing 8% urea was prepared and run in 1x TBE buffer according to the parameters suggested by the ABI. Gel plates were carefully cleaned to avoid smearing which could interfere with data analysis. The plates were scanned for any signal artefacts caused by dirt. If such artefacts were observed , the gel- scanning area was cleaned once with a damp tissue then re-scanned, The gel was pre-run before loading for 10 minutes and re-scanned for signal artefacts as before. A 4 µl of gel loading mix was added to each sample pellet. The sample was denatured at 92°C for 2 minutes, rapidly quenched on ice then loaded onto the gel.

(D) Analysis of Results

The result obtained from each gel was analysed by the Macintosh computer attached to the PRISM 373 DNA system using 373A software version 1.2.2 (ABI). The sequencer which has a fluorscence detection system sends the collected data to the computer. The computer processes the data and presents it as a chromatogram with four coloured peaks.

Heterozygosity is represented by two superimposed peaks. However, the computer does not “call” the bases , but gives the designation “N”.

CHAPTER THREE

RESULTS

3.1 Strategy for amplification of the *BRCA1* coding sequence

More than 95% of *BRCA1* coding sequence was amplified by either reverse transcription of mRNA or DNA-PCR. *BRCA1* cDNA obtained by reverse transcription of mRNA was amplified by RT-PCR to yield a set of nine fragments (RNA fragments I-IX) figure 3.1. Genomic DNA was used for amplification of exon 11 and the other 23 exons if required.

3.1.1 Amplification of the BRCA1 cDNA by RT-PCR

Due to the large size of the *BRCA1* genomic DNA spanning the *BRCA1* gene and the large number of exons the initial strategy for amplification of the *BRCA1* coding sequence was using total cellular RNA from lymphocytes cells of patients with breast cancer. For this reason part of the *BRCA1* cDNA was first amplified by using two sets of primers spanning nucleotide positions 30-1650 and 4011-5711 (RNA fragments I and VI). Each of the fragments were then reamplified by using nested primers (fragments II-V and VI-IX) as shown in figure 3.1.

Optimisation of the RT-PCR

RT-PCR was carried out as described in section 2.7 using either random or specific down-stream primers for synthesising the first strand of cDNA followed by the PCR. Fragment VIII was then amplified by using primers B4F and B4R (Table 2.7). A 5µl of the PCR product was electrophoresed on 1% agarose gel to check the integrity of PCR product (figure 3.2a). All the PCR products were then loaded in to a 2% low melting agarose gel (Flowgen) and the expected products were then excised and eluted by soaking in 50µl of T.E. buffer overnight. A 10µl of aliquot of eluted cDNA was used for reamplification of the cDNA fragment using the same primers. Interestingly, no distinct band was observed as shown in figure 3.2b. The result of the amplification of the other fragments was not successful either.

This problem was solved by first amplifying fragments I and VI (nucleotide position 30-1650 and 4011-5711 respectively, (figure 3.3a and figure 3.4a) and then using nested primers inside those two fragments to amplify fragments II-V and VII-VIIII.

Fragment I was used as a template to amplify fragment II by using primers B3F and B3R (Table 2.7) figure 3.3b. Fragments III, IV and V were also amplified by using fragment I as a template and primers shown in Table 2.7 as nested primers where required.

Fragment VI was used as a template to amplify fragment VII by using primers B11F5F and B24R2 (Table 2.7), figure 3.4b. Fragments VIII and VIIII were also amplified by using fragment VI as a template and primers shown in Table 2.7, as nested primers where required.

3.1.2 Amplification of the BRCA1 *gene using genomic DNA*

Exon 11 of the *BRCA1* gene was amplified from genomic DNA by either 4 overlapping fragments A, B, C, and D using primers listed in Table 2.1 for chemical cleavage analysis (figure 3.5a) or 3 overlapping fragments F1, F2, and F3 using primers listed in Table 2.1 for protein truncation test analysis (figure 3.5b). The exons 1-10 and 12-24 were also amplified using primers listed in Table 2.3 where required. The PCR conditions and the expected size of PCR product for each fragments are summarised in Tables 2.1-6.

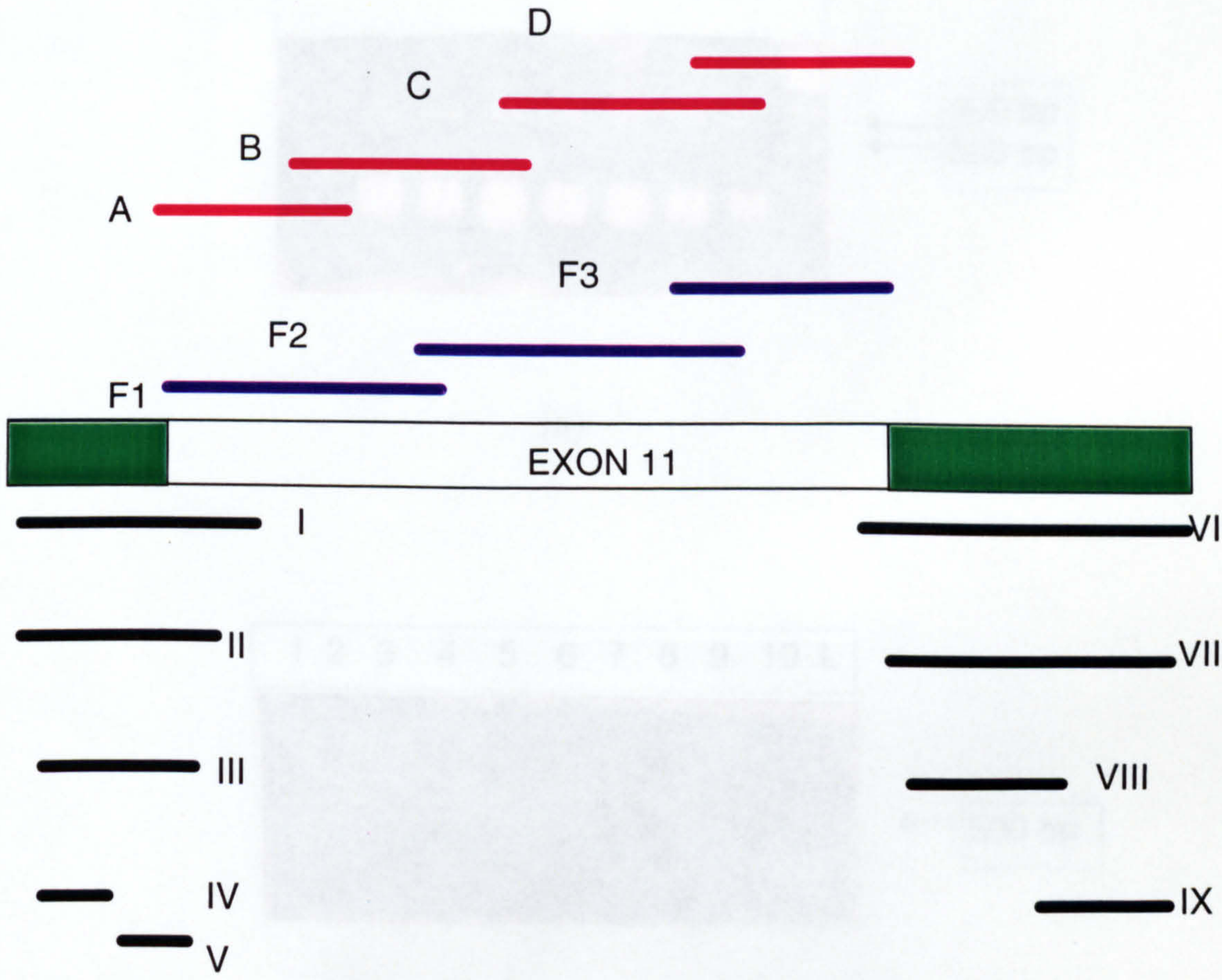
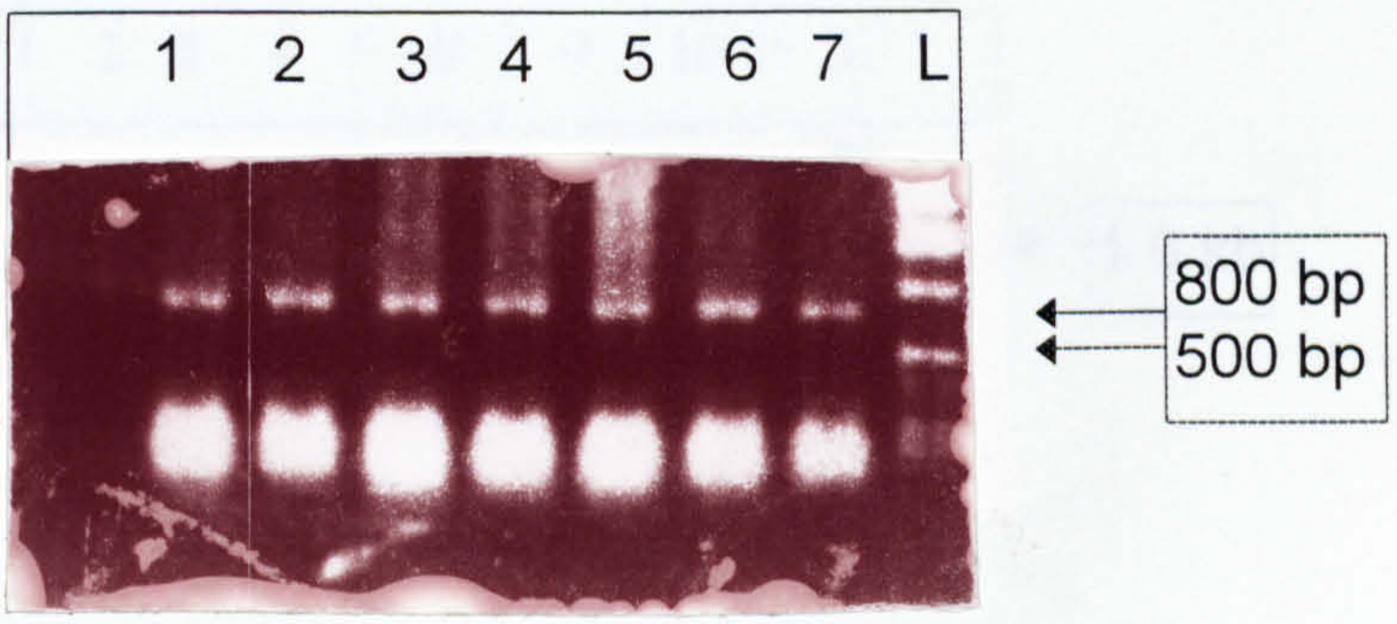
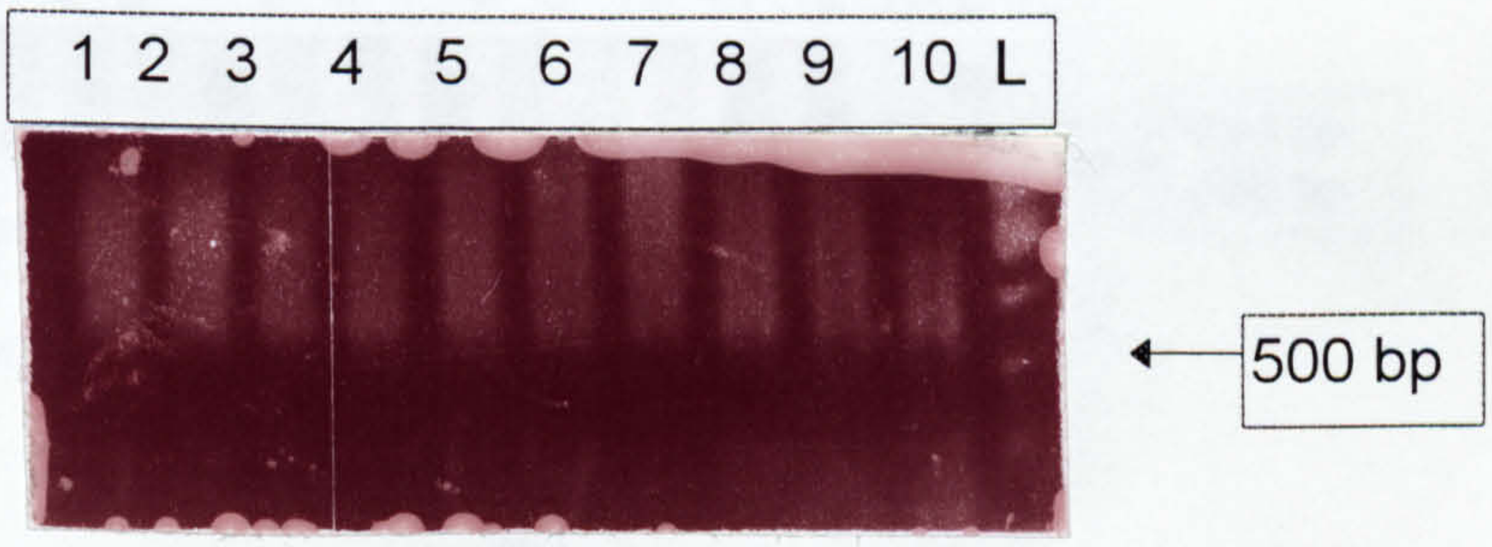


Figure 3.1

Diagrammatic representation of the *BRCA1* coding sequence. The filled boxes span cDNA nucleotides positions 1-788 (exons 1-10) and 4216-5711 (exons 13-24) which are amplified in nine fragments using the *BRCA1* mRNA as a template for RT-PCR and primers listed in Table 2.7. The unshaded box spans exon 11 (nucleotides positions 789-4215), amplified from genomic DNA by either using three fragments F1, F2 and F3 (in order to do PTT analysis) or four overlapping fragments A, B, C, and D (in order to do CCM). The sequences of primers are listed in Table 2.1b.



(a)



(b)

Figure 3.2 Agarose gel electrophoresis showing amplification products from *BRCA1* cDNA fragment VIII.

(a) Amplification of *BRCA1* cDNA fragment III showing 800 bp transcripts (marked by an arrow). L = 1kb ladder (Gibco BRL) with 500bp band indicated. Lane 1 = Normal control cDNA. Lanes 2-7 = Patients cDNA.

(b) Amplification of *BRCA1* cDNA fragment III using a 10µl of aliquot of the eluted cDNA from figure 3.2a, showing no clear band. L = 1kb ladder with 500bp band indicated. Lane 1 = normal control. Lanes 2-10 = patients cDNA

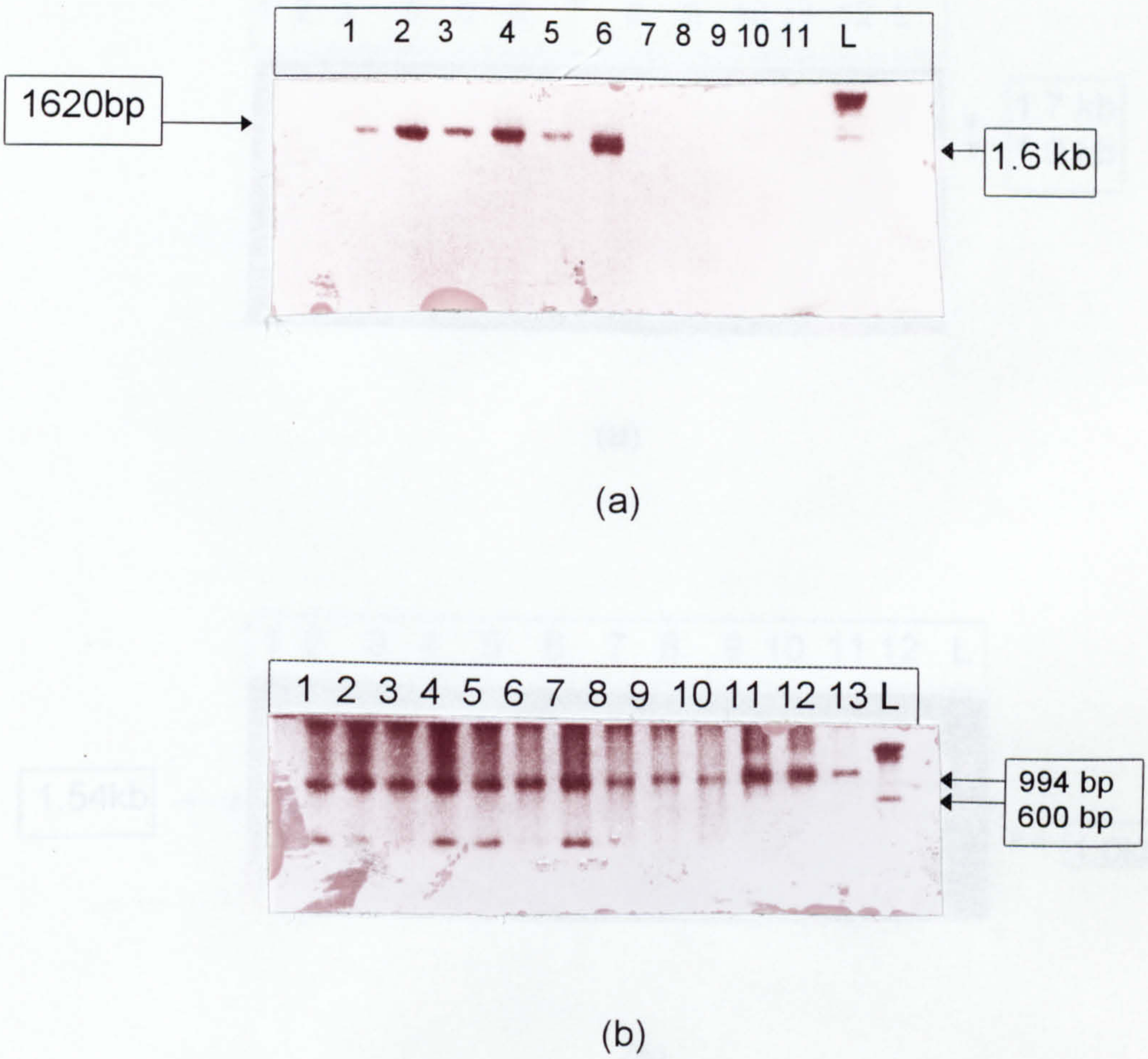
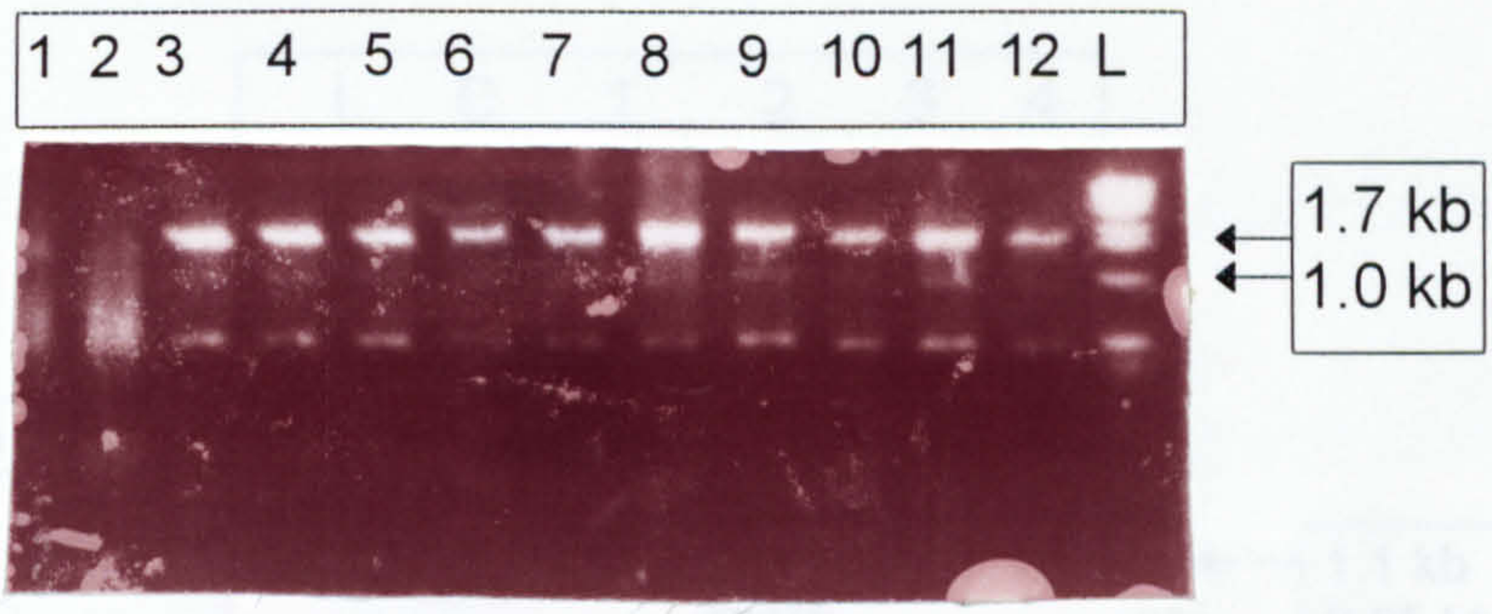


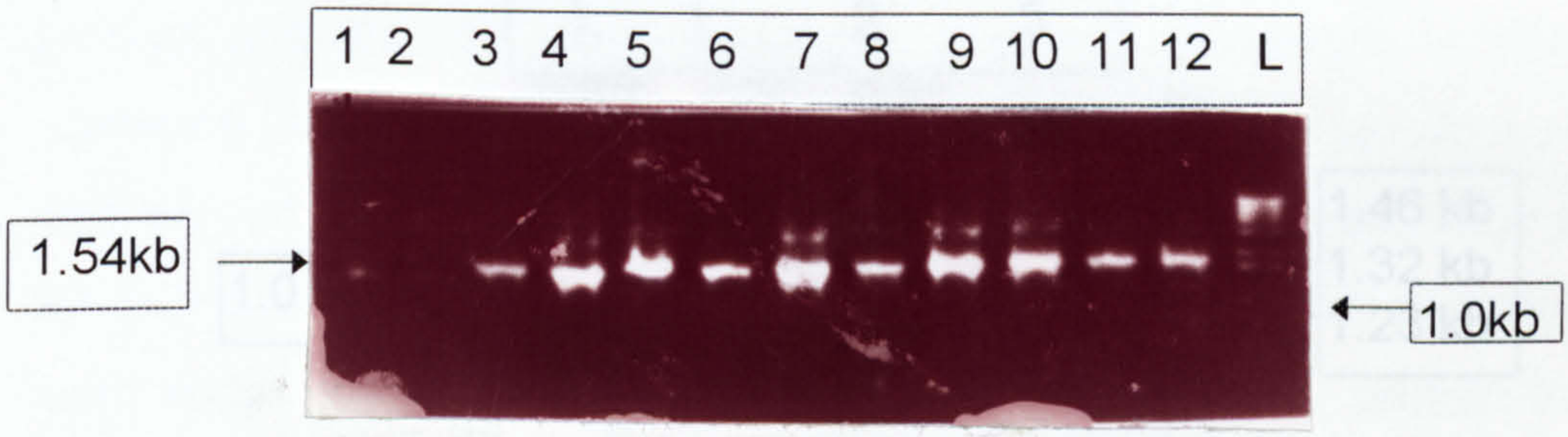
Figure 3.3 Agarose gel showing amplification products from *BRCA1* cDNA fragments I and II.

(a) Agarose gel showing amplification products (1620bp) of *BRCA1* cDNA fragment I (marked by an arrow). L = 1kb ladder with 1.6kb band indicated. Lane 1 = Normal cDNA. Lanes 2-11= Patient cDNA.

(b) Agarose gel showing amplification products of *BRCA1* cDNA fragment II (marked by an arrow) using a 5µl aliquot of the PCR product from fragment I as template and nested primer set B3F and B3R (Table 2.7). L= 100bp ladder with 600bp band indicated. Lane 1 = normal control. Lanes 2-13 = patients cDNA.



(a)

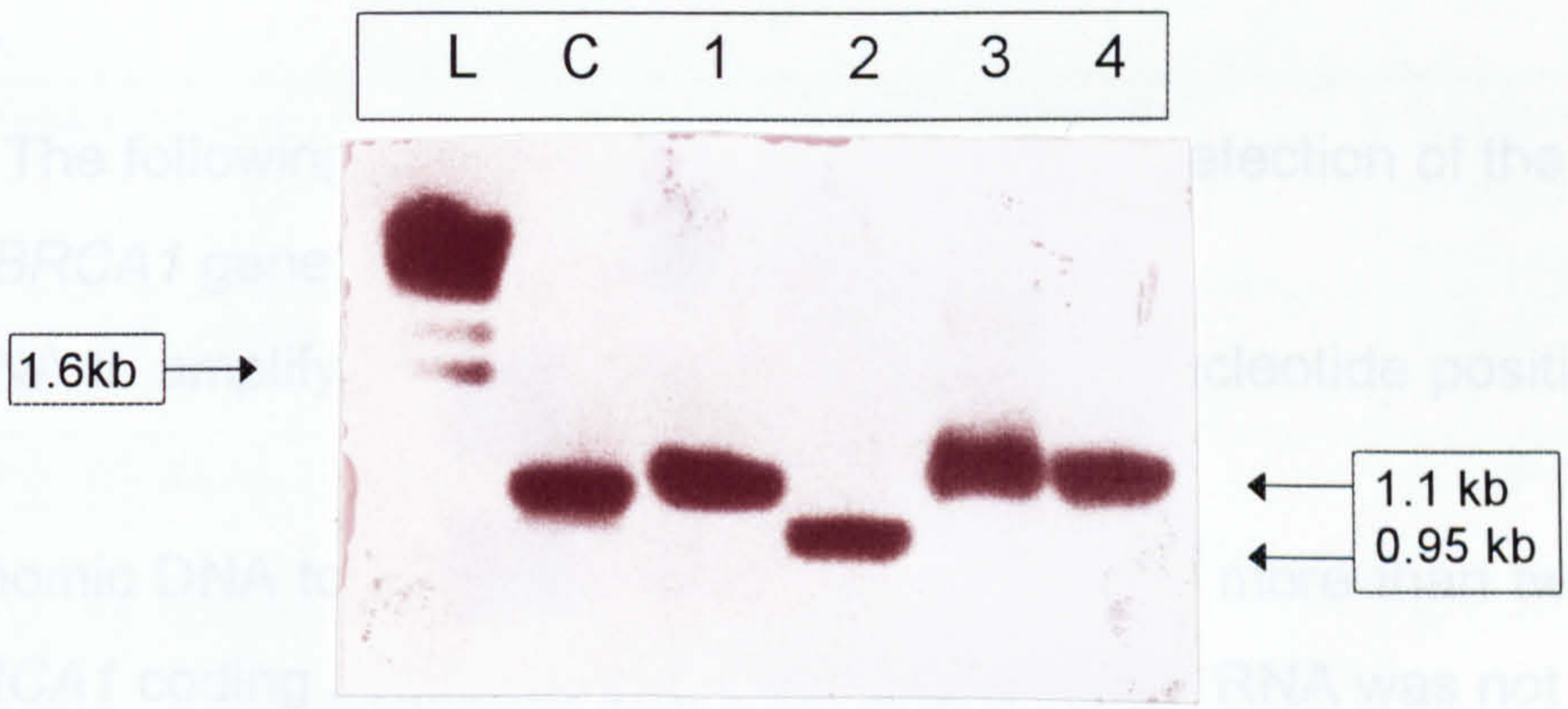


(b)

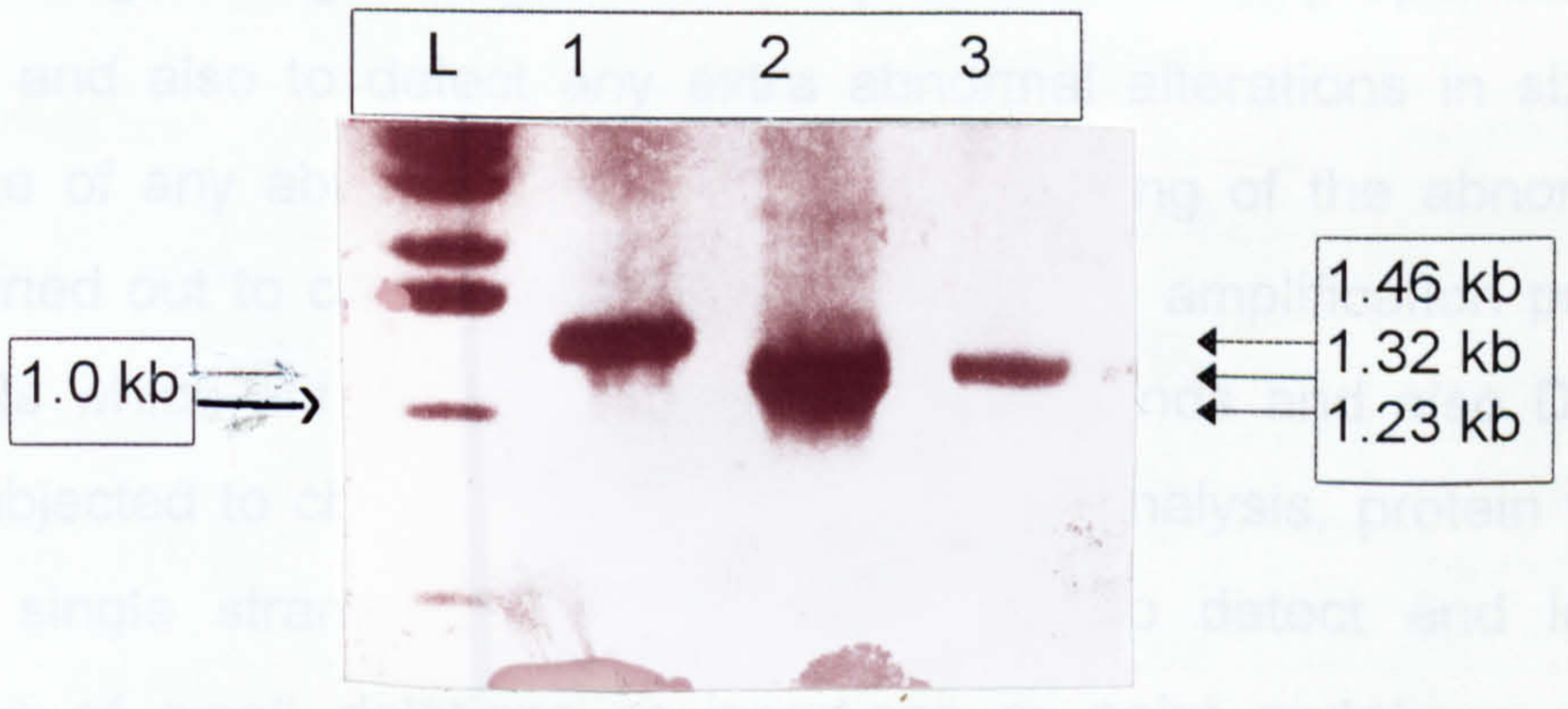
Figure 3.4 Agarose gel electrophoresis showing amplification products from *BRCA1* cDNA fragment VI and VII.

(a) Agarose gel showing amplification products of *BRCA1* fragment VI using primers BR11F4 and BR24R1(Table 2.7). The expected size of the PCR product is 1.7kb (marked by an arrow). L = 1kb ladder with 1.0 kb band indicated. Lane 1 = normal control. Lanes 2-12 = patients cDNA.

(b) Agarose gel showing amplification products of *BRCA1* cDNA fragment VII using a 5µl aliquot of the PCR from fragment VI as template and nested primer set BR11F5 and BR24R2 (Table 2.7). The expected size of the PCR product is 1.54 kb (marked by an arrow). L = 1kb ladder with 1.0 kb band indicated. Lane 1 = normal control. Lanes 2-12 = patients cDNA.



(a)



(b)

Figure 3.5 Agarose gel electrophoresis showing: Amplification products of four overlapping fragments A, B, C, and D of exon 11 from the *BRCA1* gene

(aThese were used for the chemical cleavage analysis using primers: AF and AR (fragment A), BF and BR (fragment B), CF and CR (fragment C) and DF and DR (fragment D). The sequences of the primers are shown in Table 2.1. L = 1kb ladder with 1.6kb band indicated. C = control DNA. Lane 1= fragment A (1.1kb). Lane 2= fragment B (0.95 kb). Lane 3= fragment C (1.1kb). Lane 4= fragment D (1.1kb).

(b) Amplification products of three overlapping fragments F1, F2 and F3 of exon 11 from the *BRCA1* gene. These were used for the protein truncation test analysis by using primers: BR11F1 and BR11R1 (fragment F1), BR11F2 and BR11R2 (fragment F2), and BR11F3 and BR11R3 (fragment F3). L= 1kb ladder with 1.0 kb band indicated. Lane 1= fragment F2 (1.463 kb). Lane 2= fragment F3 (1.23kb). Lane 3= fragment F1 (1.32 kb).

3.2 Strategy for detection of germline mutations in the *BRCA1* gene.

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

1- mRNA to amplify nucleotide positions 30-1650 nucleotide positions 4011-5711.

2- Genomic DNA to amplify exon 11 which contains more than two thirds of the *BRCA1* coding sequence and other exons where RNA was not available.

In the case of RNA the initial RT-PCR products were electrophoresed in a 1.5% agarose gel to check for the presence of the proper size of PCR product and also to detect any extra abnormal alterations in size. In the presence of any abnormal band, direct sequencing of the abnormal band was carried out to characterise the mutation. The amplification products of RT-PCRs which did not show any clear extra bands and also DNA-PCRs were subjected to chemical cleavage mismatch analysis, protein truncation test or single strand conformational analysis to detect and locate the presence of small deletions or insertions or point mutations within the *BRCA1* gene. Any truncated products or mismatches detected were fully characterised by asymmetric PCR and direct sequencing.

Every sequence change was checked by GCG package "MAP" enzyme to see if it created or abolished a restriction enzyme site. Digestion of the PCR products with appropriate restriction enzyme was then carried out to confirm the change in the sequence and also analyse other members of the family and other patients. Restriction digestion was used to screen normal controls for any changes which were found in patients to determine whether the changes are the cause of the disease or they are just a polymorphism. In the case of polymorphism the frequency of polymorphic allele was also determined in 58 patients and 50 normal controls using either restriction enzyme digestion or single strand conformational analysis. A

summary of sequence changes and the methods by which they were detected and characterised are tabulated below and each mutation is discussed in detail in the following pages.

sequence change	Location	Detection methods	Methods used for screening other individuals
Skipping of exon 5 (S)•	exon 5	RT-PCR, sequencing	RT-PCR
Skipping of exons 9-10 (S)•	exon 9-10	RT-PCR, sequencing	RT-PCR
Skipping of exons 5-7 (M)#	exons 5-7	RT-PCR, sequencing	RT-PCR
561 deletion CAG (S)•	exon 8	RT-PCR, CCM, sequencing	CCM
4477 deletion CAG (S)•	exon 14	RT-PCR, CCM, sequencing	CCM
2800 deletion AA (M)#	exon 11	PTT, sequencing	restriction enzyme analysis, SSCP
5382 ins C (M)#	exon 20	SSCP, sequencing	SSCP
3232 A to G (P)*	exon 11	CCM, sequencing	restriction enzyme analysis, SSCP
3667 A to G (P)*	exon11	CCM, sequencing	restriction analysis, SSCP
2201 C to T (P)*	exon 11	CCM, sequencing	restriction enzyme analysis, SSCP
2430 T to C (P)*	exon 11	CCM, sequencing	restriction, SSCP
4427 T to C (P)*	exon 13	SSCP, sequencing	SSCP
4956 A to G (P)*	exon 16	SSCP, sequencing	SSCP

#: Pathogenic mutations.
***:** Polymorphisms.
•: Splice site variants.
Table 3.1 Summary of the mutations and polymorphisms in the *BRCA1* gene and the methods by which they were detected in this study.

3.3. Optimisation of the protein truncation test

RT-PCR of fragment VII of the *BRCA1* gene was carried out using primers BR11F5 and BR24R2 (Table 2.7). Exon 11 was also amplified by three overlapping fragments F1, F2 and F3 using primers described in Table 2.1 and genomic DNA as template. All forward primers contained a T7 promoter and eukaryotic translation initiation sequence in order to generate PCR products suitable for PTT analysis. PTT analysis was carried out by adding 4μl of PCR product to the TnT7 coupled reticulocyte lysate system (Promega cat No: 14600) and following the manufacturer instructions. A positive control translation reaction using Luciferase DNA (supplied by the manufacturer) and negative control translation reaction by performing transcription/translation without adding DNA were also carried out to analyse the efficiency of the system. The synthesised protein products were separated on a 12% SDS-polyacrylamide minigel electrophoresis system for two hours then exposed for 16-48 hours for autoradiography. However, the efficiency of the PTT was not satisfactory. The efficiency of the PTT was improved as explained below:

1- PCR amplified products were purified using Gene clean kit (Biolab) and the concentration was estimated by spectrophotometry of 0.5μl of purified PCR product and comparing the intensity of the band with that achieved by a known concentration of 0.5μg/μl of control DNA supplied by the kit and using 1μl (0.5μg) of purified DNA for a total volume of 12.5μl of transcription/ translation reaction (figure 3.6).

2- Linked T7 transcription- translation system cat No: RPN 3152 (Amersham) was used (as described in section 2.10.3).

3- The translation reaction time was increased to 75 minutes.

4- The synthesised PCR products were separated on a 12-15% SDS polyacrylamide for 6-7 hours using Protean^{R} II cell kit from Bio Rad.

5- In order to increase the sensitivity of detection of ^{35}S, the gel was immersed in "Amplify" TM (Amersham) for 30 minutes. The gel was then dried and exposed to X-ray film for 1-7 days. Figures 3.7-9 show the PCR and optimised PTT results from fragments F1, F2 and F3. Therefore all further experiments were performed according to these and are described in section 2.10.3

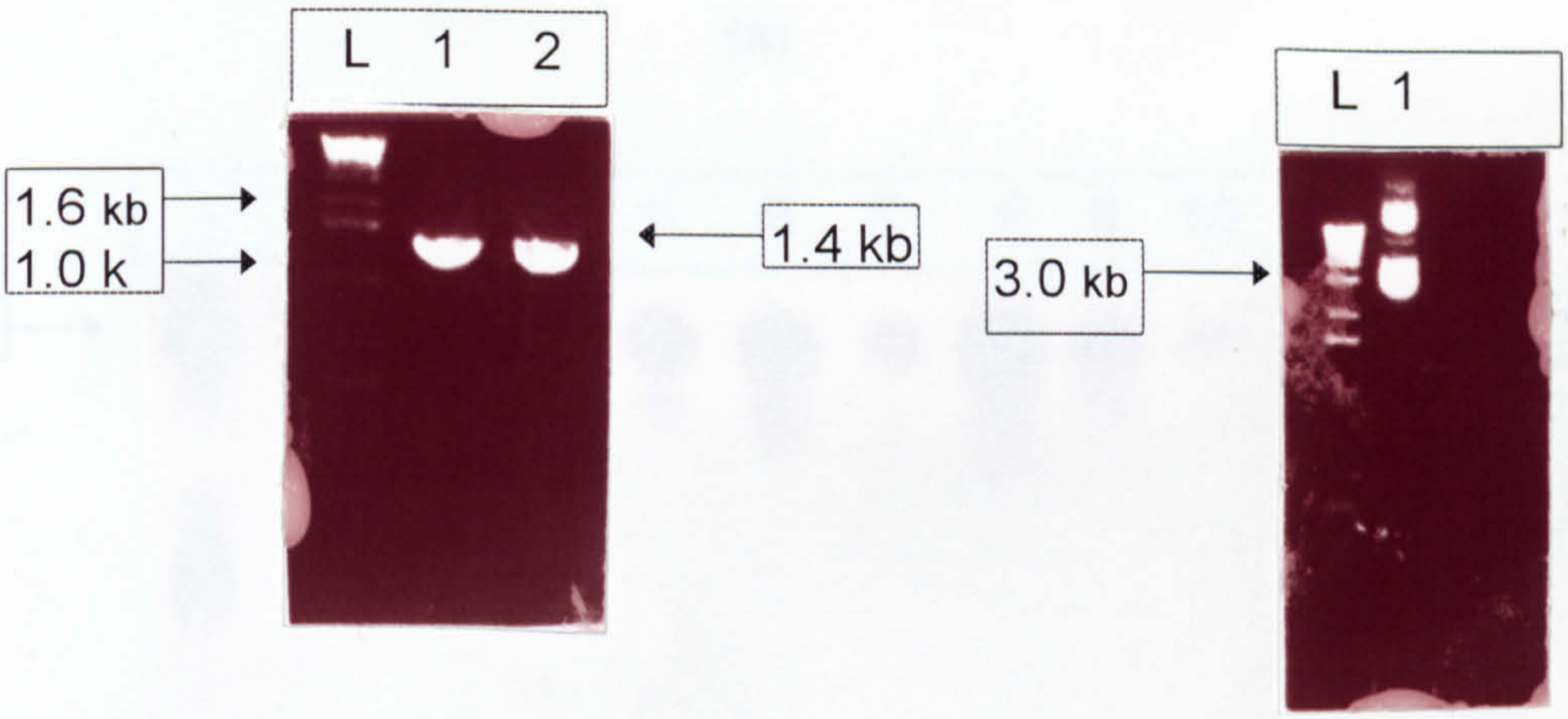
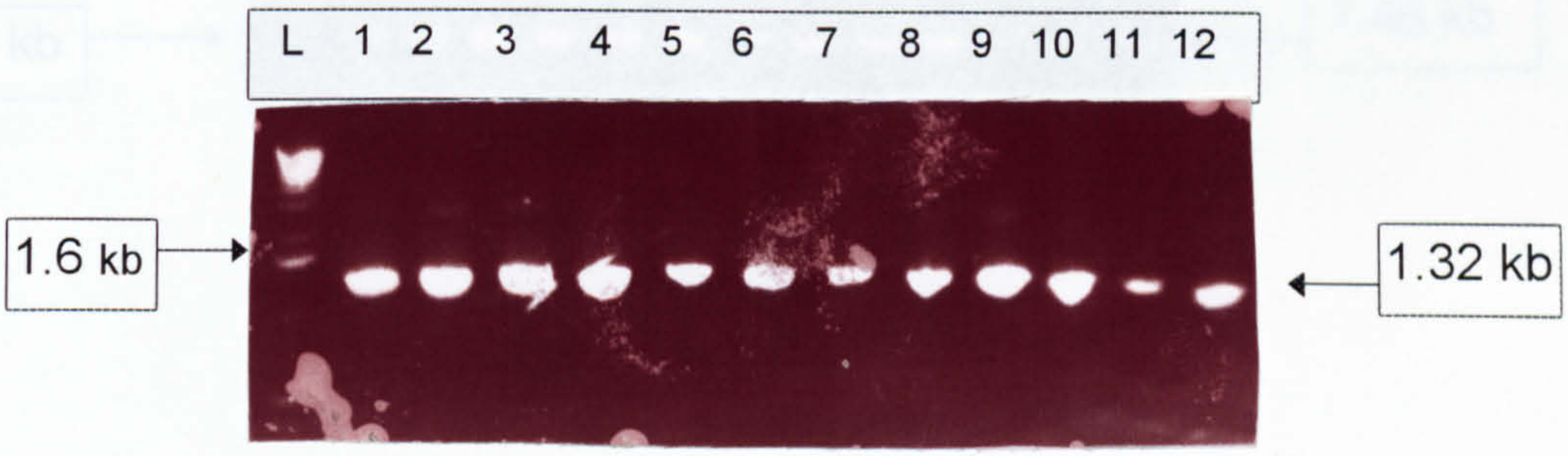


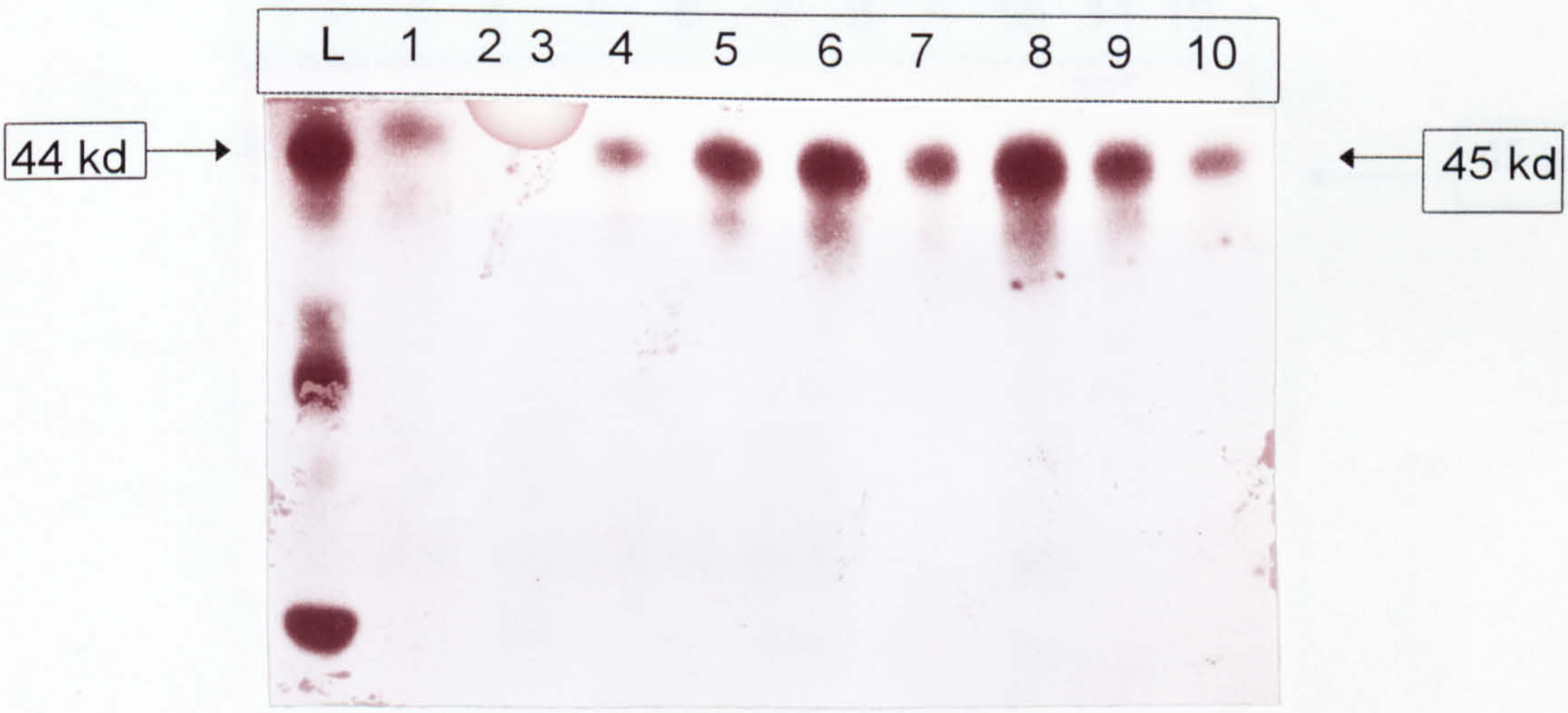
Figure 3.6 Agarose gel showing amplification products from fragment F1 of the *BRCA1* gene :

(a) 0.5µl (0.25µg) of purified PCR product (1.4kb) of the *BRCA1* gene (lanes 1-2). L= 1kb ladder with 1.6 and 1.0 kb band indicated.

(b) 1µl (0.5µg) of control DNA (lane 1). L=1kb ladder with 3.0 kb band indicated.



(a)

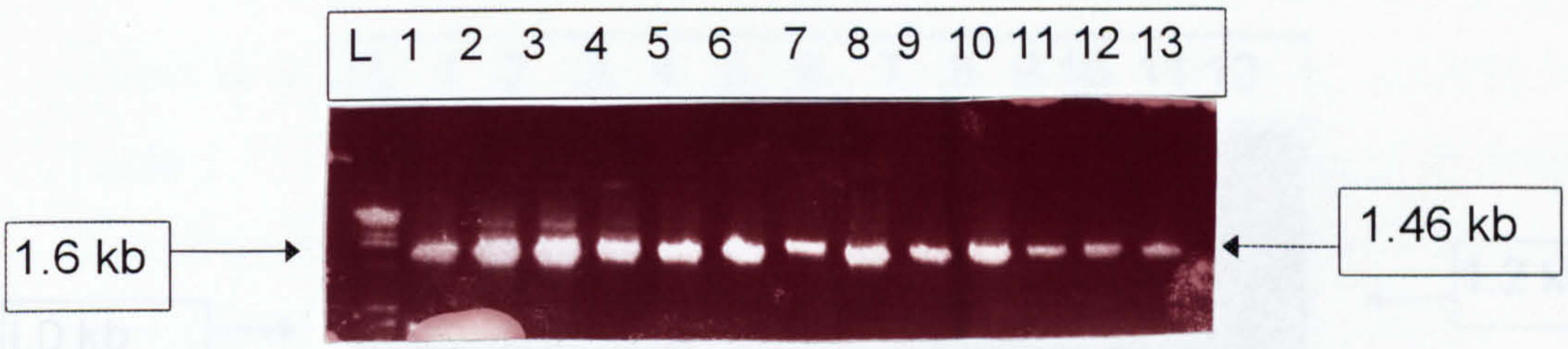


(b)

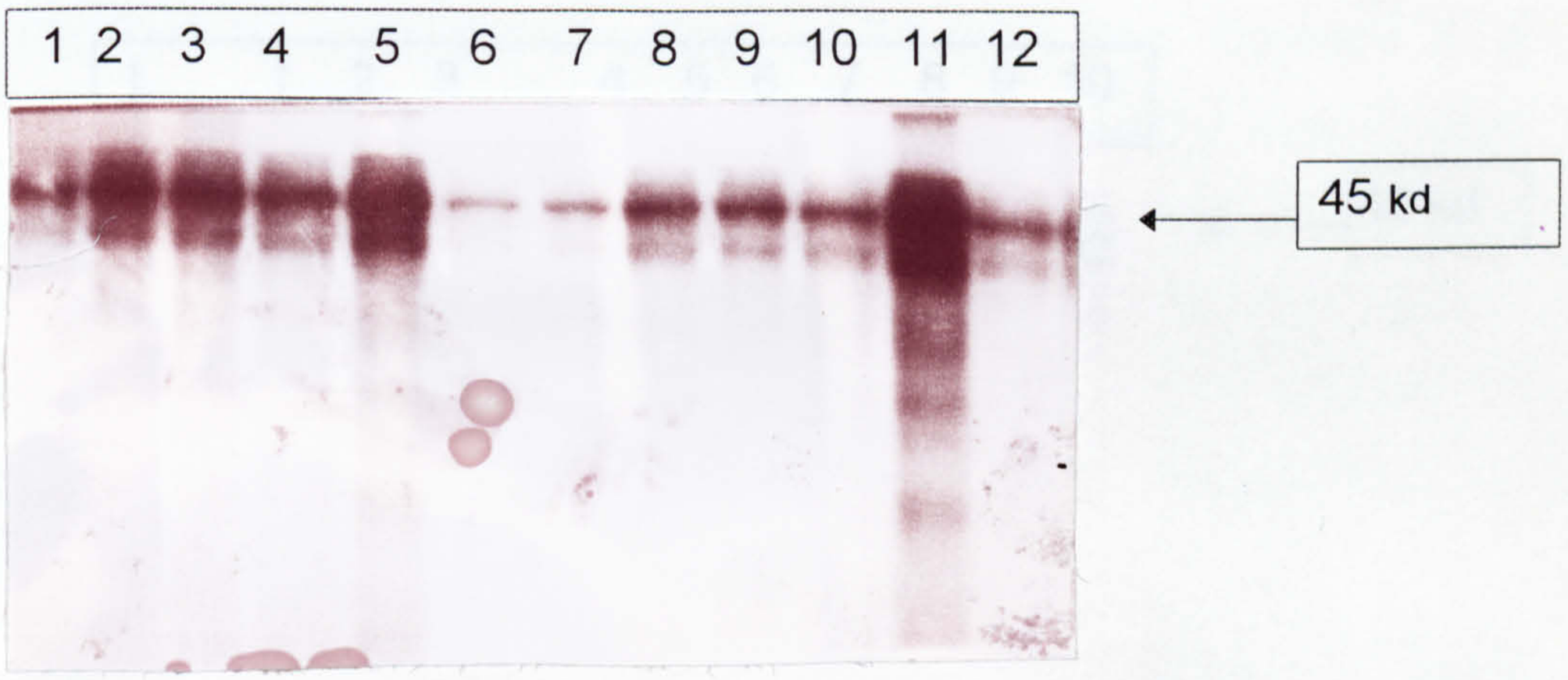
Figure 3.7 PCR amplifications and PTT analysis from fragment F1 of the *BRCA1* gene from several patients.

(a) Agarose gel showing 0.25μg of purified PCR products from fragment F1 (lanes 1-12) using primer set BR11F1 (Table 2.1). The expected size is 1.32kb (indicated by an arrow). L= 1kb ladder with 1.6kb band indicated.

(b) 15% SDS polyacrylamide gel showing optimised PTT from fragment F1 using 0.5μg of purified PCR product. The 45 kd protein is indicated by an arrow. L= Rain-bow ladder with 44kd band indicated.



(a)

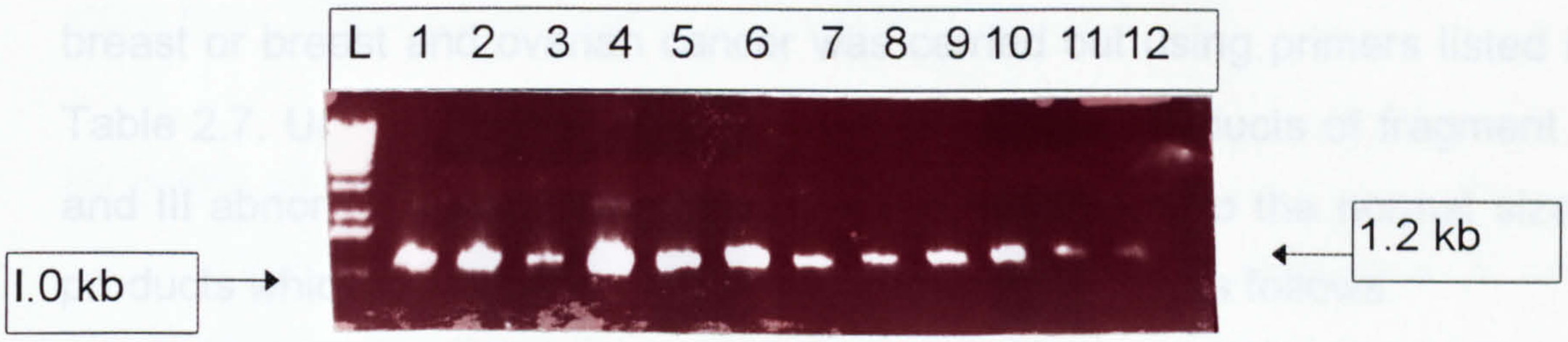


(b)

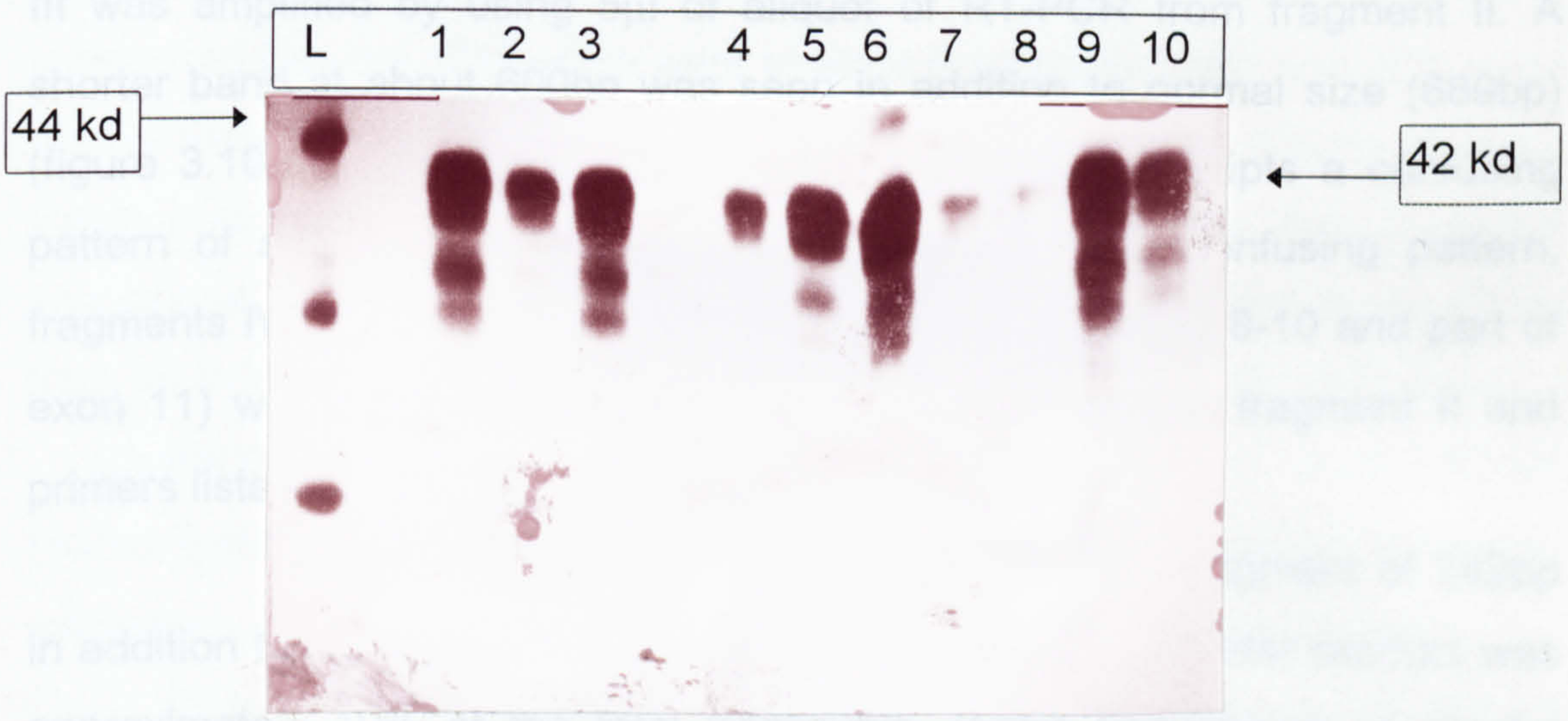
Figure 3.8 PCR amplifications and PTT analysis of fragment F2 of the *BRCA1* gene from several patients.

(a) Agarose gel showing 0.25μg of purified PCR product from fragment F2 (lanes 1-13) using primer set BR11F2 (Table 2.1b). The expected size is 1.46 kb (indicated by an arrow). L= 1kb ladder with 1.6kb band indicated.

(b) 15% SDS polyacrylamide gel showing optimised PTT from fragment F2 using 0.5μg of purified PCR product. The 45kd proteins is indicated by an arrow.



(a)



(b)

Figure 3.9 PCR amplifications and PTT analysis of fragment F3 of the *BRCA1* gene from several patients.

(a) Agarose gel showing 0.25μg of purified PCR product from fragment F3 (lanes 1-12) using primer set BR11F3 (Table 2.1). The expected size is 1.22kb (indicated by an arrow). L=1kb ladder with 1.0kb band indicated .

(b) 15% SDS polyacrylamide gel showing optimised PTT of fragment F3 using 0.5μg of purified PCR product. The 42kd protein is indicated by an arrow. L= Rain-bow ladder with 44kd protein indicated.

3.4 RT-PCR analysis of the *BRCA1 gene.*

RT-PCR of fragments II, III and VII from patients with family history of breast or breast and ovarian cancer was carried out using primers listed in Table 2.7. Upon electrophoresis of the amplification products of fragment II and III abnormal sized products were seen in addition to the normal sized products which were characterised and the results were as follows:

Skipping of exons 5 and 9-10 as a normal variant

RT-PCR of fragment II (figure 3.1) showed a prominent expected band of 994bp and a shadow like band close to the 994bp in almost all patients and normal control. To characterise the shadow like band, fragment III was amplified by using 5μl of aliquot of RT-PCR from fragment II. A shorter band at about 600bp was seen in addition to normal size (689bp) (figure 3.10). Upon direct sequencing of the short transcripts a confusing pattern of sequencing was seen. To characterise the confusing pattern, fragments IV (spanning exons 3-7) and V (spanning exons 8-10 and part of exon 11) were amplified by using 5μl of PCR product of fragment II and primers listed in Table 2.7.

RT-PCR of fragment IV revealed a smaller fragment of 242bp in addition to the normal sized fragment of 319bp. This shorter product was approximately 10% of the total transcripts. Direct sequencing of shorter transcripts revealed the absence of exon 5 (figure 3.11b). However, sequencing of the DNA in the vicinity of the exon-intron boundaries of exon 5 did not revealed any abnormal sequences.

RT-PCR of fragment V revealed a smaller sized fragment of 176bp in addition to the normal sized fragment of 298bp in most of the patients and control RNA (figure 3.12a). This shorter transcript was approximately 10% of the total transcript. Direct sequencing of shorter transcripts revealed the absence of exons 9-10 (figure 3.12b). SSCP/HA analysis of exon 9 using primers flanking exon 9 (Table 2.7) revealed a heteroduplex in addition to

single stranded DNA (figure 3.13a). Direct sequencing of DNA across intron exon boundary of exon 9 showed a one base pair deletion at position -54 in more than 50% of patients and control DNAs (figure 3.13b).

Skipping of exons 5-7

RT-PCR analysis of fragment III identified the presence of two abnormal sized products (612 and 483) in addition to a normal sized product of 689bp (figure 3.14) in individuals III.6 and III.7 from families 7 and 8 respectively (Appendixes 1 and 2). Direct sequencing of the 483bp product revealed the absence of exons 5-7 (figure 3.15), the 612 bp product was correspondent to the skipping of exon 5. Interestingly, 50% of the transcripts belonged to the skippings of exons 5-7, 25% were exon 5 skipping (compared with the 10% which were present in all transcripts) and 25% were normal transcripts. Direct sequencing of genomic DNA in the vicinity of exon-intron boundaries of exons 5, 6, 7, and 8 did not reveal any abnormal sequences. The absence of these three exons was not present in the cDNA from 38 index cases from the other families or in twenty healthy individuals.

Characterisation of single base substitutions and small rearrangements in products of RT-PCR, using either chemical cleavage mismatch or single strand conformational polymorphism analysis.

Chemical cleavage analysis of RNA-fragment VIII was carried out on the rest of the patients who showed a normal pattern of RT-PCR.

The presence of CAG at the beginning of exon 14

Chemical cleavage analysis of RNA-fragment VIII revealed the presence of cleavage band in almost 50% 0f RT-PCRs. Direct sequencing of cDNA revealed the absence of CAG at the beginning of exon 14 (figure 3.16). This change was also found in some of the normal control RNA hence proving that this is just a normal variation.

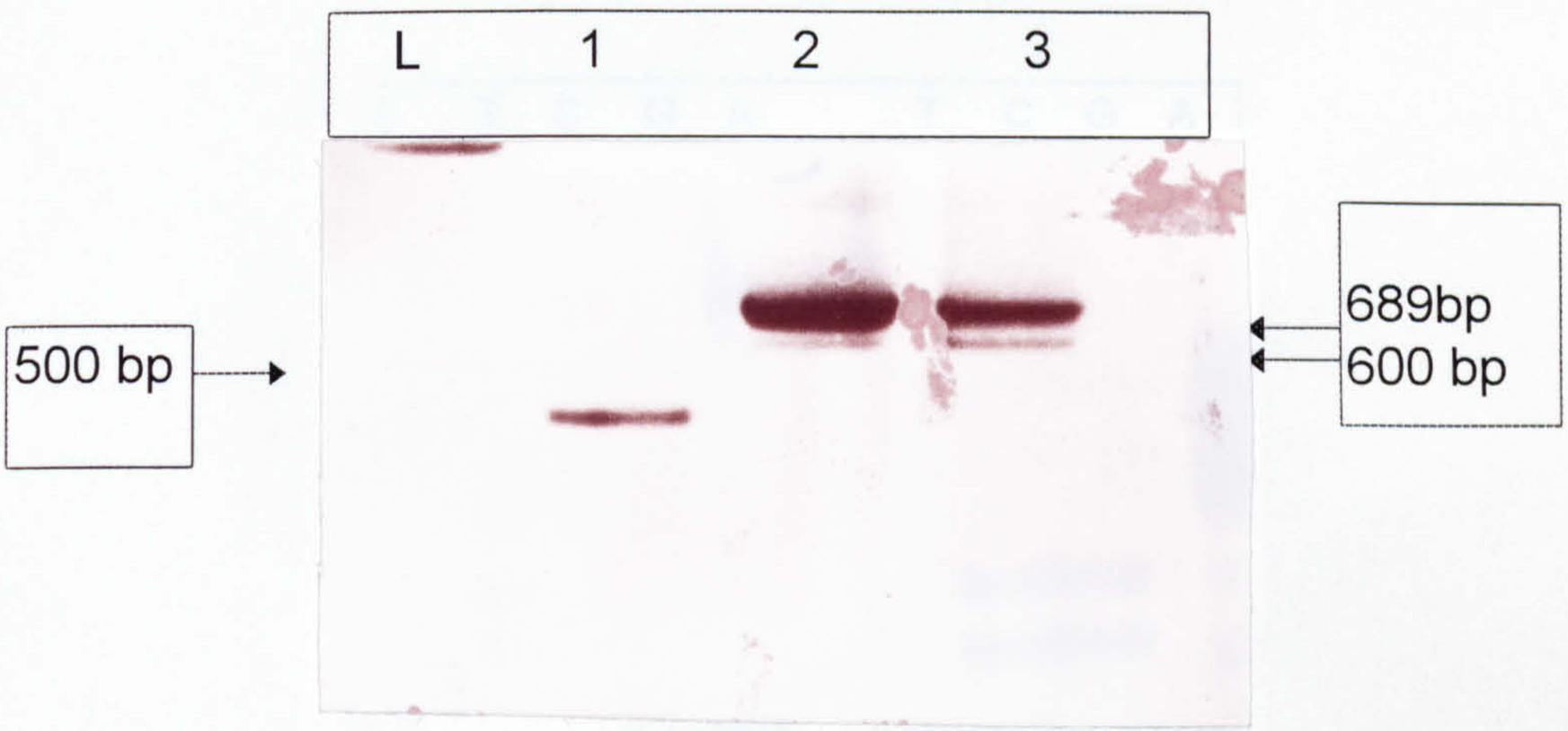


Figure 3.10 Agarose gel electrophoresis of RT-PCR products of fragment III of the *BRCA1* gene.

A smaller transcript (~600bp) is seen in addition to the normal sized transcript (689bp). L= 1kb ladder with 500bp band indicated. Lane 1= control PCR product. Lanes 2-3 = patients.

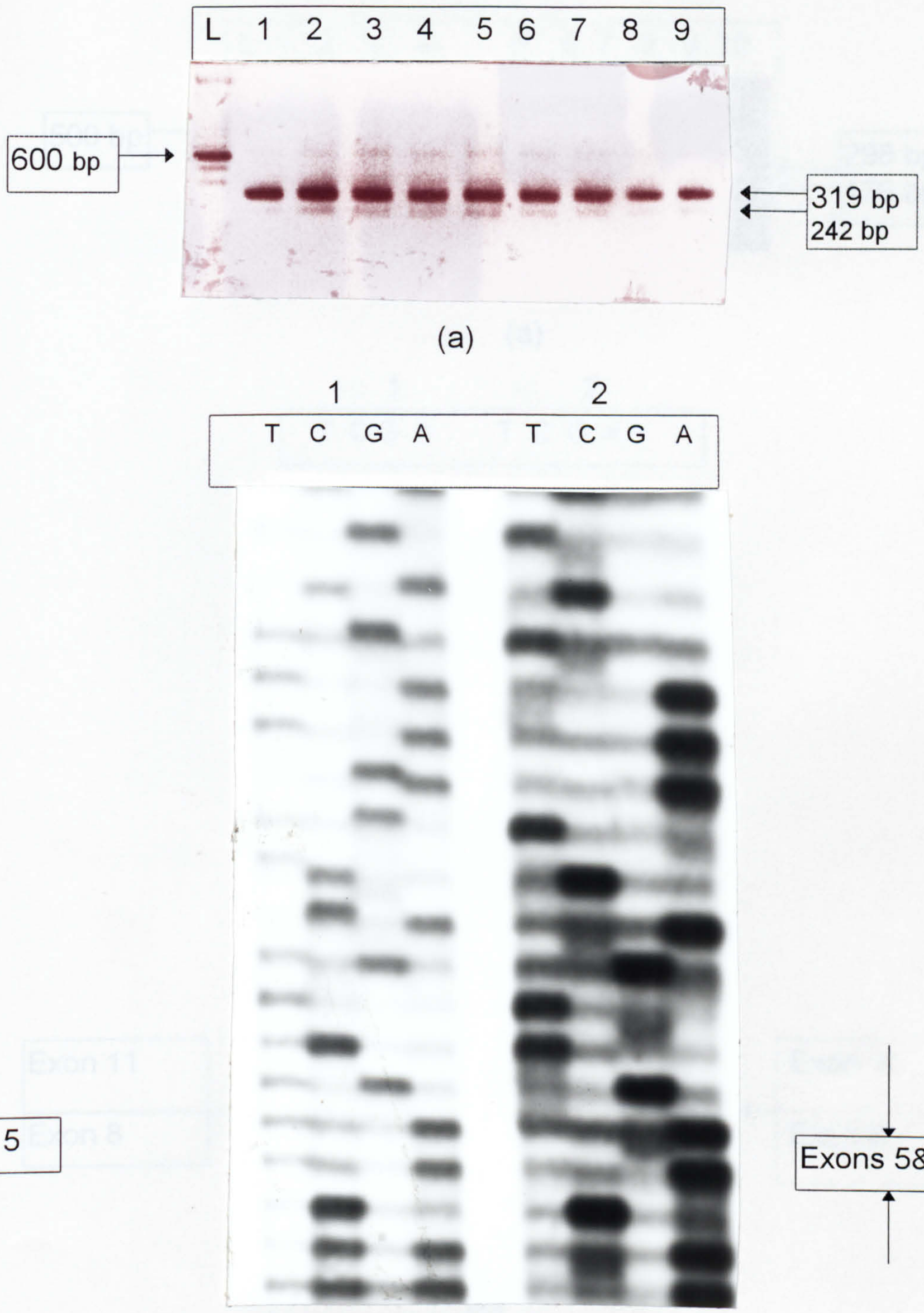
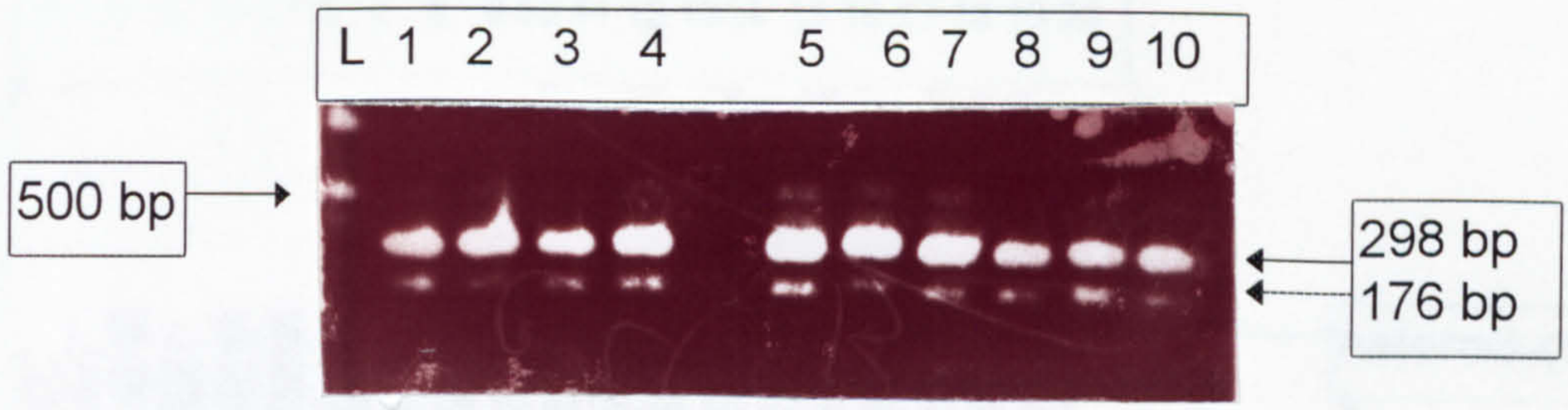


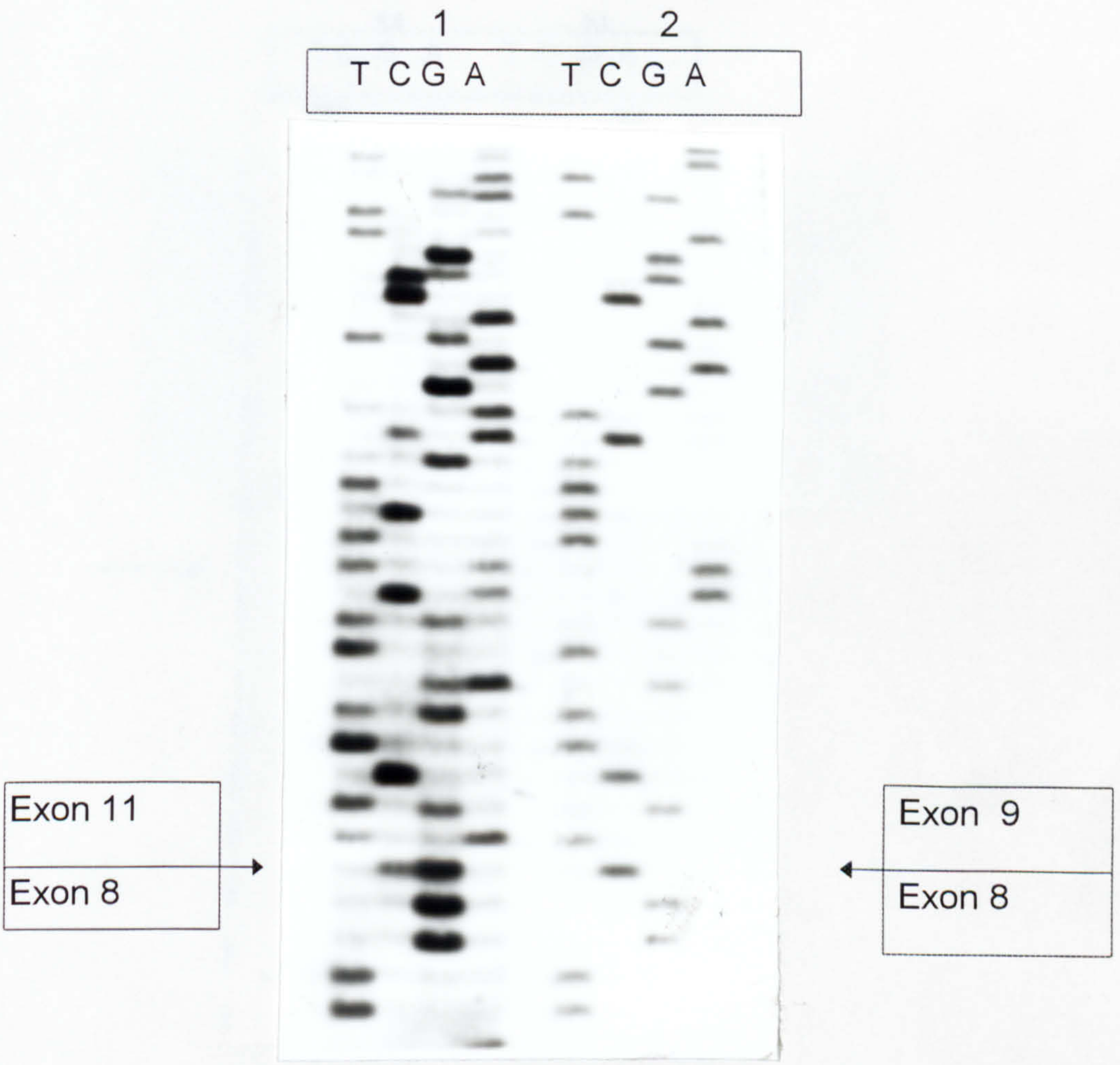
Figure 3.11 RT-PCR products and sequence analysis of the region showing exon 5 skipping.

(a) Amplification of fragment IV encompassing exons 3-7 using primers C_2F and C_2R showing a small transcript of 242bp in addition to the normal one of 319bp (marked by arrows). L=100kb ladder with 600bp band indicated. Lanes 1-9 = patients.

(b) Direct sequencing of the smaller transcripts reveals exon 5 skipping. 1=Upper band 2= Upper and lower band.



(a)



(b)

Figure 3.12 RT-PCR products and sequence analysis of the region showing exons 9-10 skipping in the *BRCA1* gene.

(a) Amplification of fragment V encompassing exons 8-10 and part of exon 11 using primers C_4F and C_4R showing a small transcript of 176bp in addition to the normal one of 298bp (marked by arrows). L= 1kb ladder with 500bp band indicated. Lanes 1-10 patients.

(b) Direct sequencing of smaller transcripts revealed the skipping of exons 9 and 10. 1 = Lower band. 2 = Upper band.

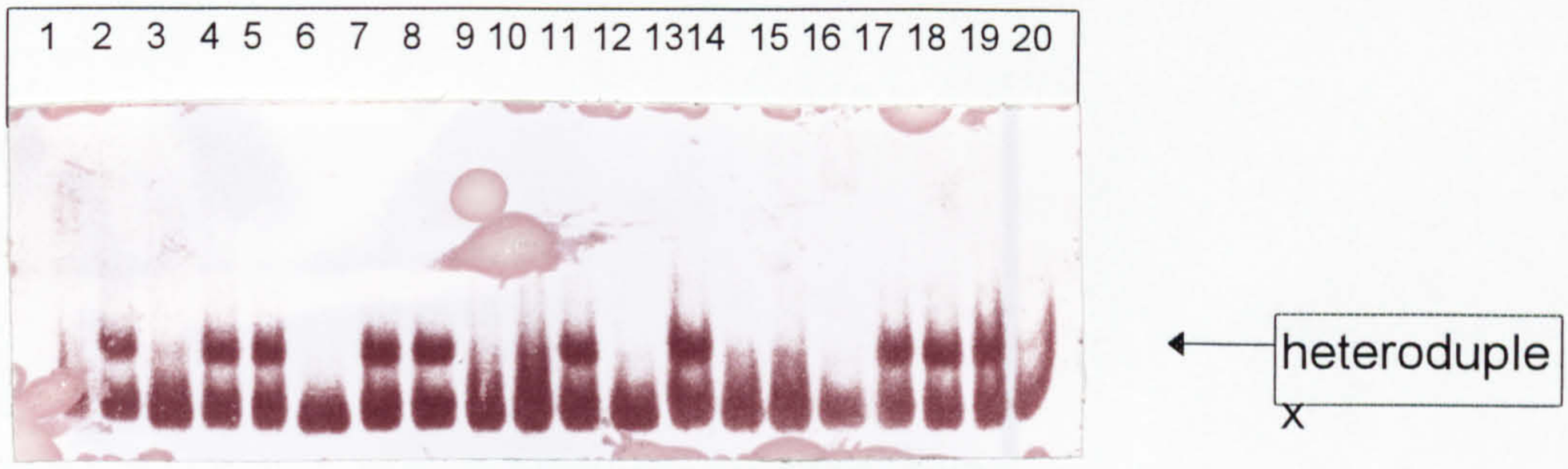


Figure 3.13 HA and direct sequence analysis of exon 9 of the *BRCA1* gene.

(a) Negative image of a HA analysis of exon 9 using flanking primers (Table 2.3) showing a heteroduplex band in some of the patients (indicated by an arrow).

(b) Direct sequencing of exon 9 and flanking DNA showing a single base deletion (A) at position -54 (marked by an arrow). N= normal. M= mutant.

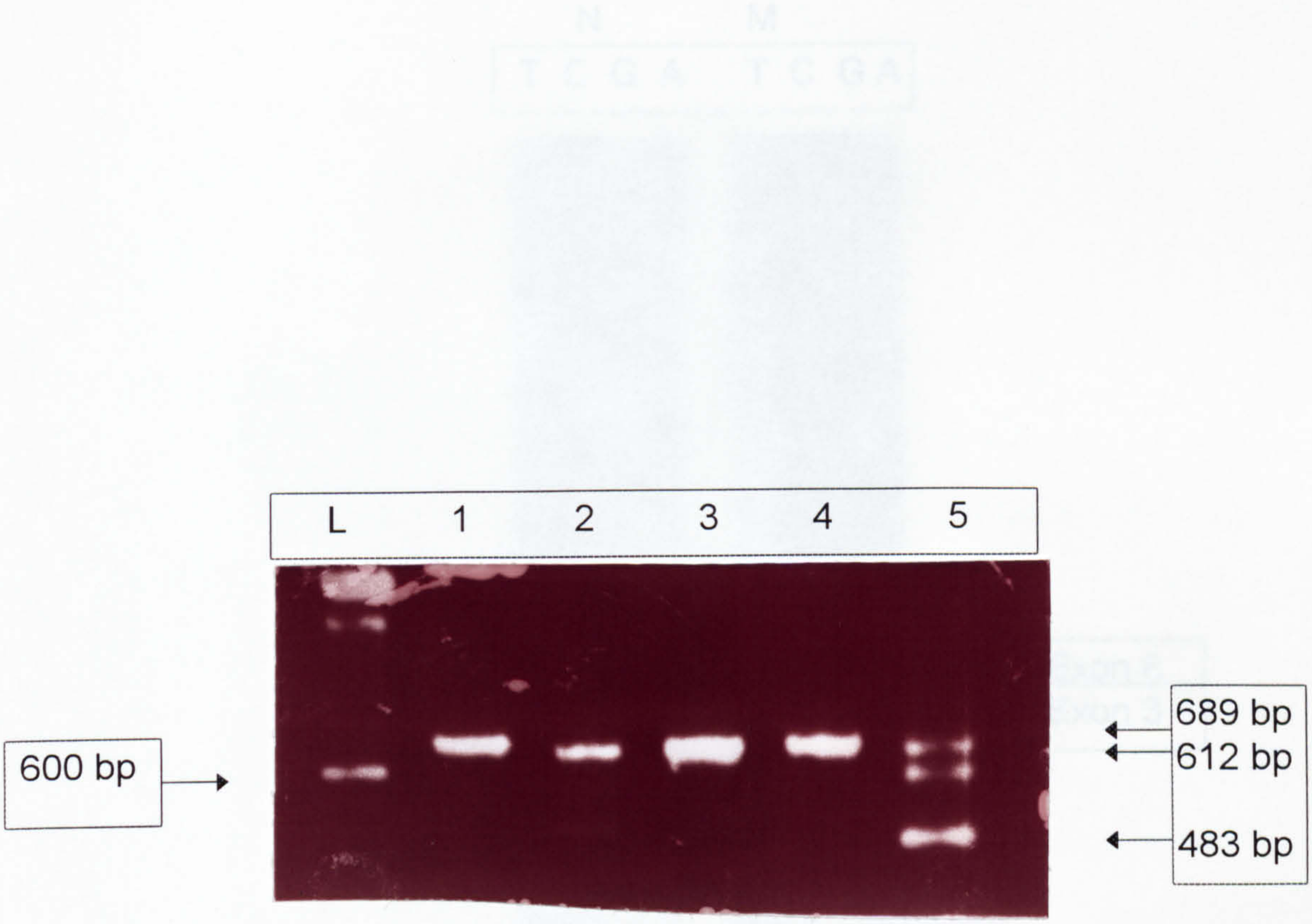


Figure 3.14 RT-PCR analysis of the region showing exons 5-7 skipping of the *BRCA1* gene.

Agarose gel showing RT-PCR products of fragment III with two shorter bands of 612 and 483bp in addition to the normal one of 689bp (lane 5). L = 100bp ladder with 600bp indicated. Lanes 1-4 = normal controls. Lane 5= Patient.

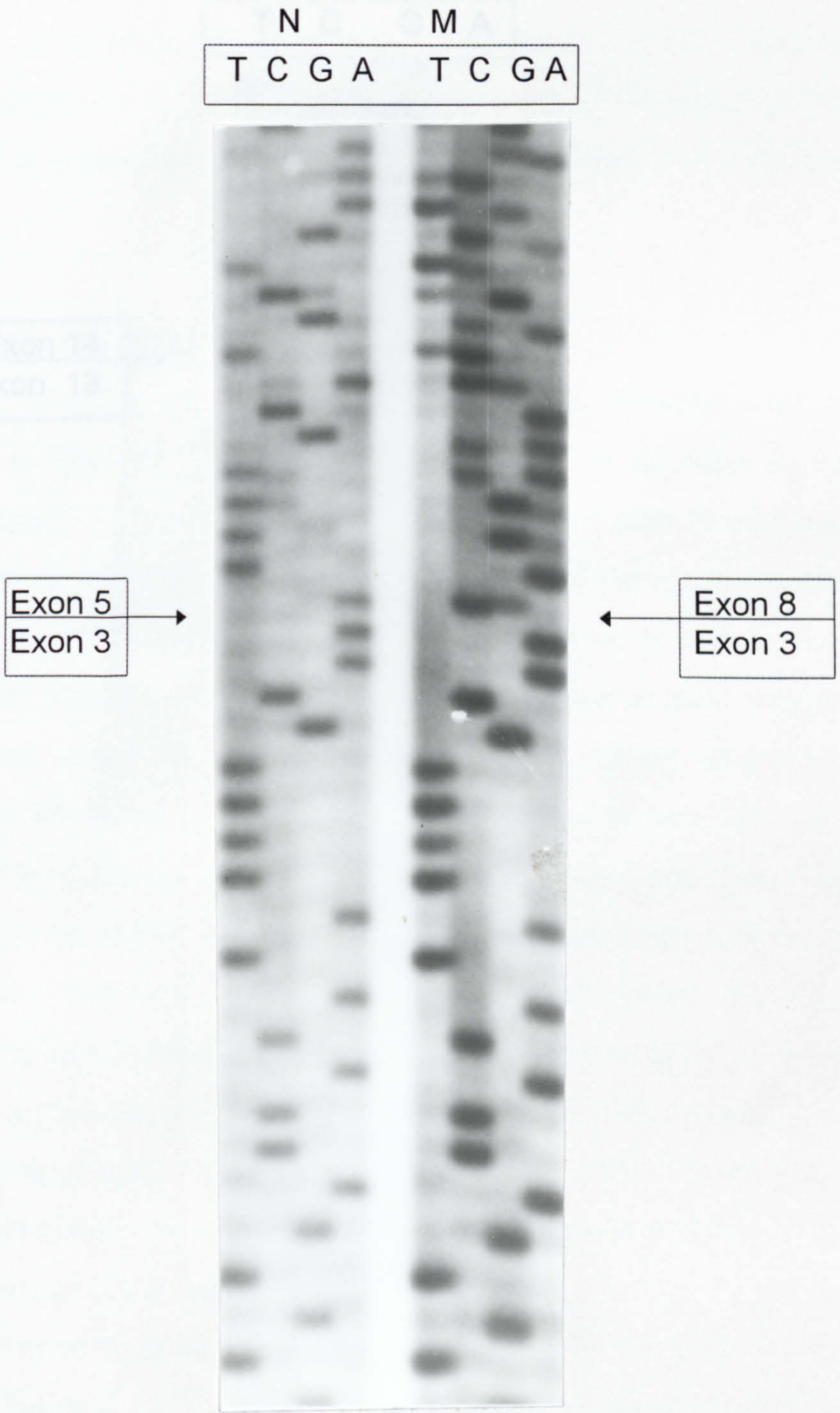


Figure 3.15 Sequence analysis of the region showing exons 5-7 skipping in the *BRCA1* gene.

Direct sequencing of the shorter 483bp product from figure 3.14 showing exon 3 spliced to exon 8 (indicated by an arrow). N= sequence of normal product. M= sequence of shorter product of 483bp.

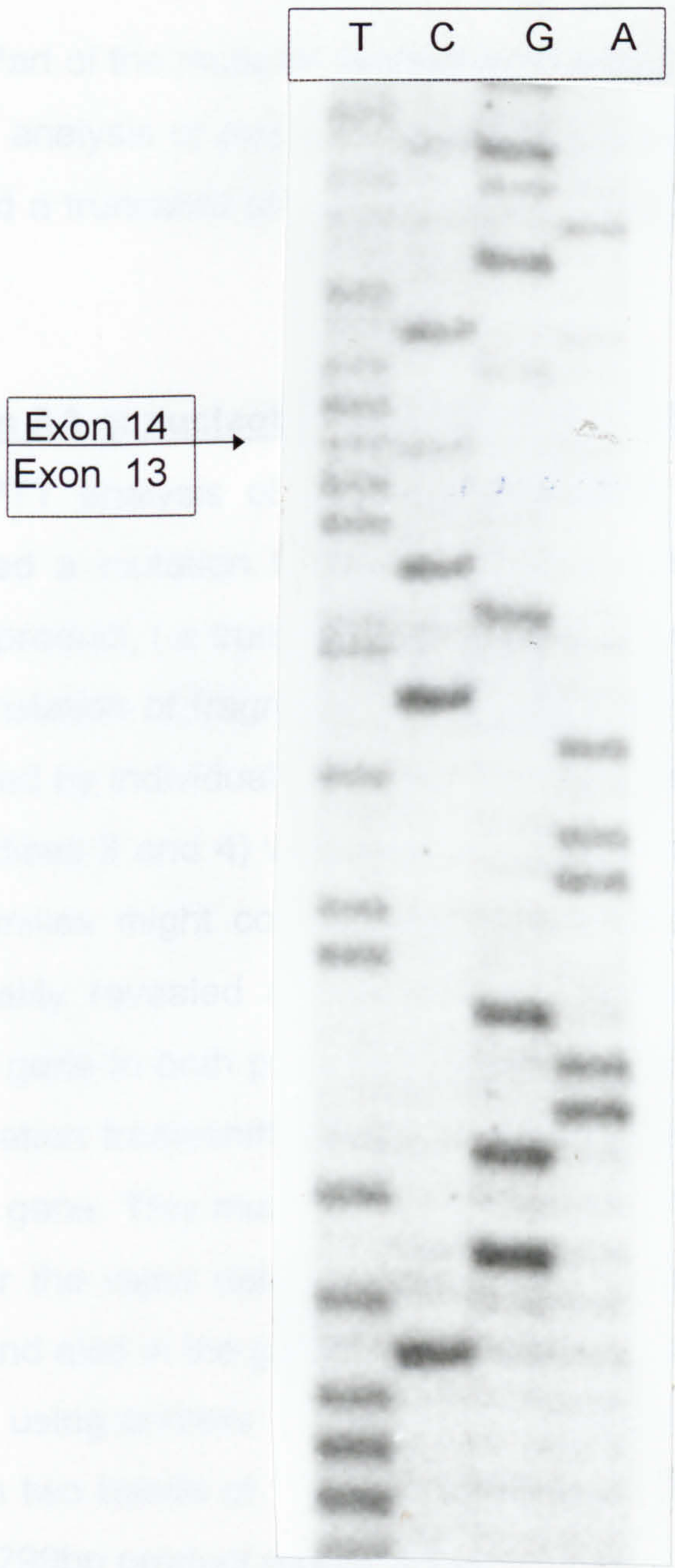


Figure 3.16 Partial sequence analysis of *BRCA1* cDNA around the junction of exons 13 and 14 (fragment VII).
The region of the gel containing exon 14 shows a complex sequence produced by a 3bp deletion (C A G) at the beginning of the exon 14.

3.5 Protein truncation test analysis

Part of the mutation screening strategy of the *BRCA1* gene was based on PTT analysis of exon 11 and RNA fragment VII. However, PTT analysis revealed a truncated product in fragment F2 from exon 11 and is presented below:

Deletion AA at nucleotide positions 2800-2801

PTT analysis of fragment F2 (figure 3.1) showed that two cases contained a mutation in one of *BRCA1* alleles in addition to the normal protein product, (a truncated protein was observed after *in vitro* transcription and translation of fragment (F2) (figure 3.17). The truncated products were generated by individuals III.1 and III.9 from families 10 and 35 respectively, (Appendices 3 and 4) which appeared to be similar in size, suggesting that both families might contain identical mutations. Direct sequencing of the abnormality revealed an AA deletion at nucleotide position 2800 of the *BRCA1* gene in both probands (figure 3.18). These base deletions produce a translation frameshift leading to a predicted termination at codon 901 of *BRCA1* gene. This mutation creates a *Tth*111I restriction site which can be used for the rapid detection of this mutation in the other members of the family and also in the general population (figure 3.19a). Digestion of a 299bp product using primers SF and SR (Table2.2) by *Tth*111I restriction enzyme result in two bands of 100 and 199bp from the mutant allele, in addition to normal 299bp product (figure 3.19).

Subsequent analysis of individual III.7 from family 35 by *Tth*111I digestion revealed no digested products even though she was an affected member of the family.

Restriction digest analysis using *Tth*III1 and DNA from 4 affected members from family 10 (individuals II.2, II.3, II.4, III,3) revealed that the above mutation is present in individuals II.2, 11.3, and II.4 but is not present in individual III.3. This means that this affected member may either have another mutation or that she suffers from sporadic breast cancer.

SSCP analysis could also be used to screen for the presence of this deletion if the restriction enzyme was not available (figure 3.20). Subsequent analysis of 68 patients with a family history of breast and breast/ovarian cancer and 50 control DNAs showed no alteration at this position.

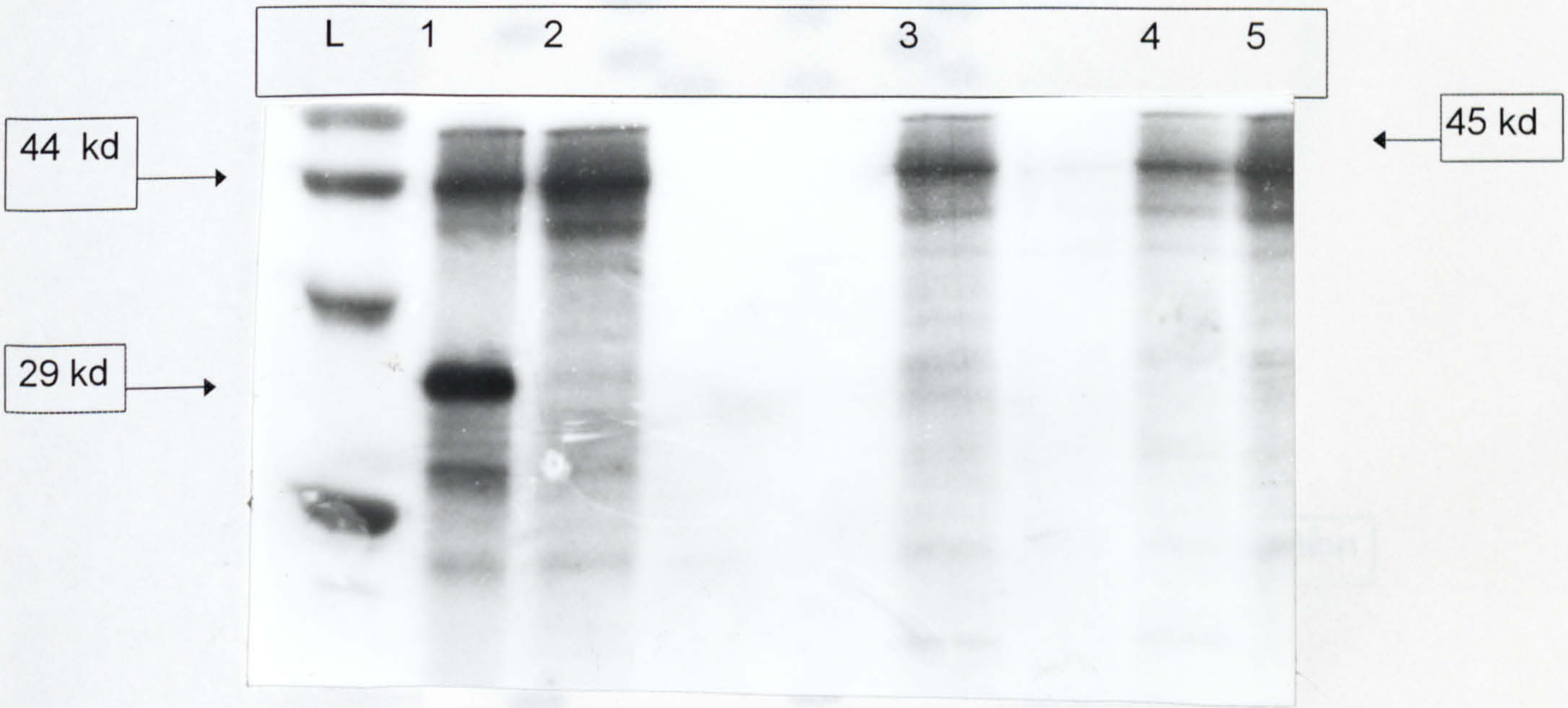


Figure 3.17 PTT analysis of fragment F2 of the *BRCA1* gene.
PTT analysis of fragment F2 showing a truncated protein of 29 kd in addition to the normal 45 kd protein (lane 1). Lanes 2-5 show the normal 45kd products. L = ladder with 44kd band indicated.

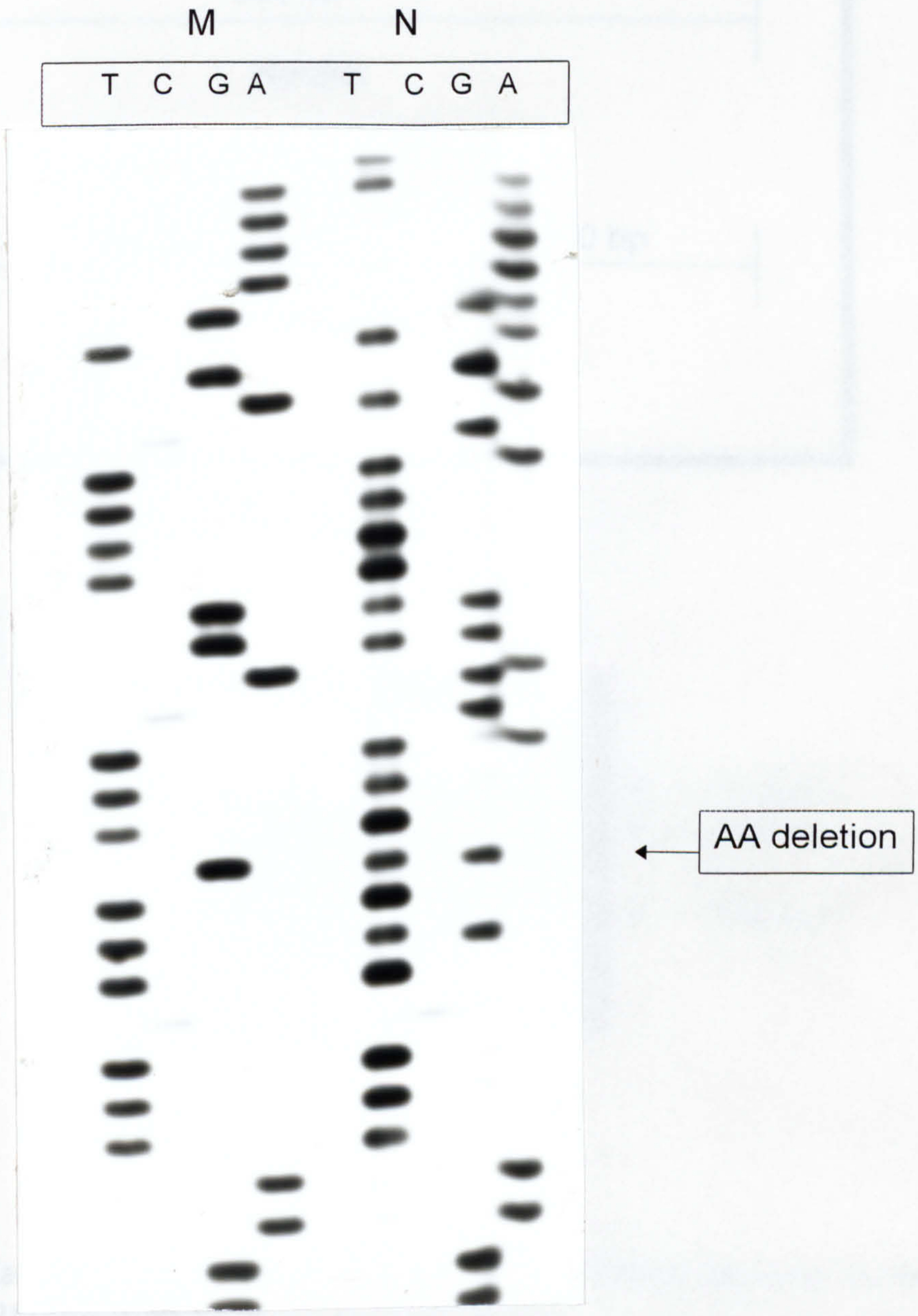
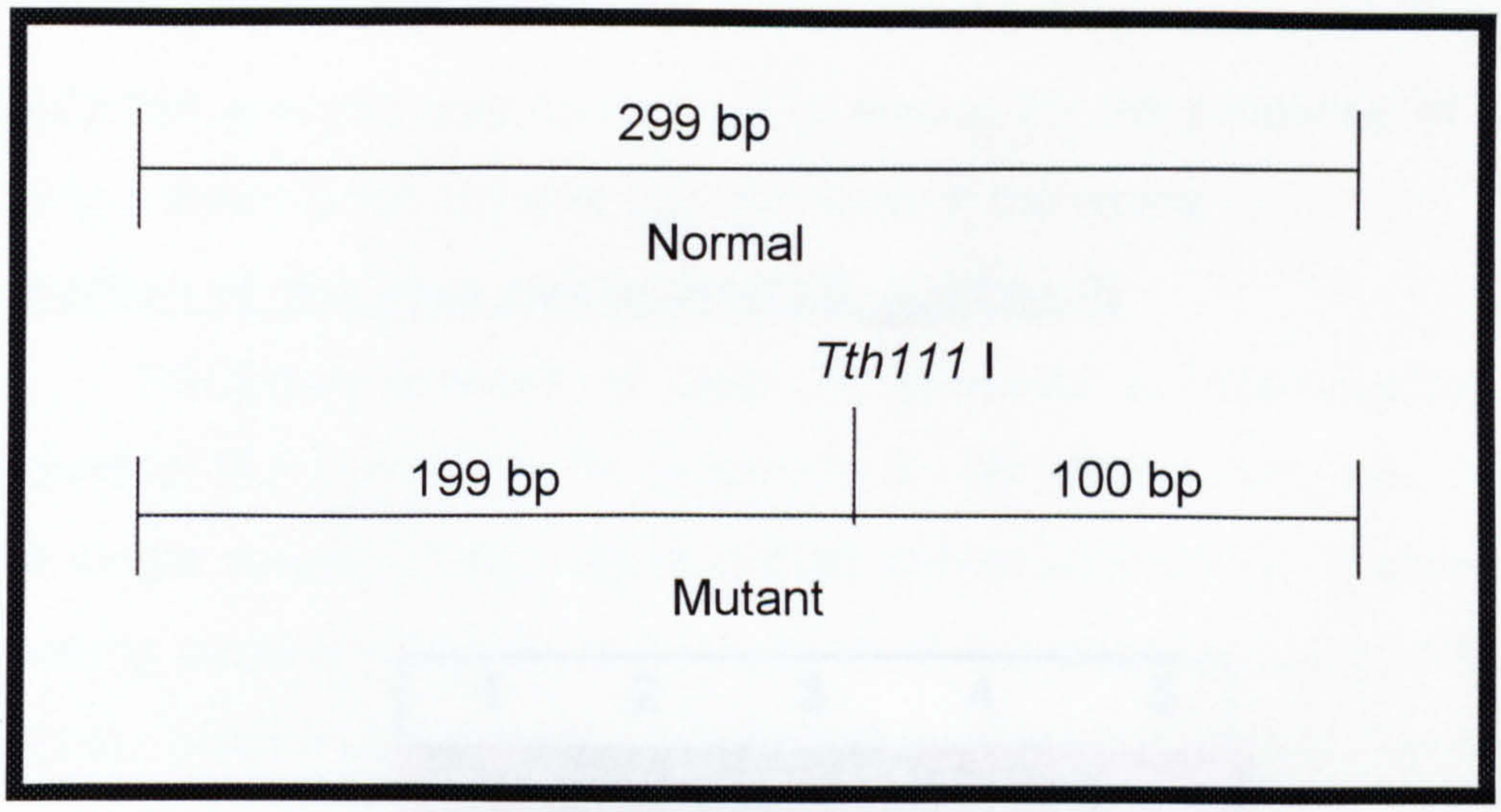
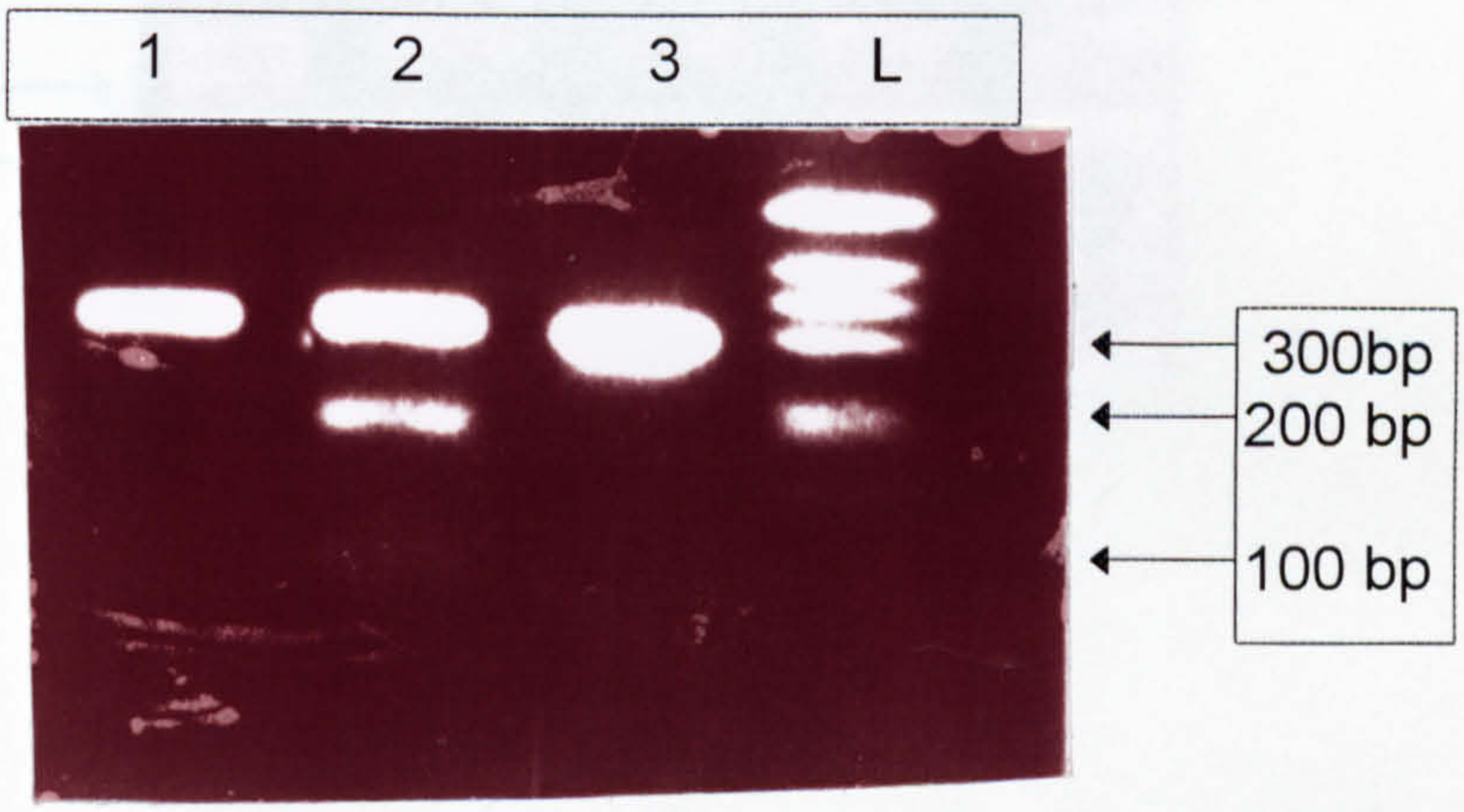


Figure 3.18 Partial sequence analysis of fragment B showing 2800delAA in the BRCA1 gene.

Beyond the point marked by the arrow a parallel sequence is seen along with the normal sequence. This is due to the presence of both the normal and mutant allele sequence which lacks two As at positions 2800-2801. N= normal sequence. M= mutant.



(a)



(b)

Figure 3.19 (a) Restriction map of *Tth*111I restriction enzyme to detect the *BRCA1* 2800delAA.

Restriction map of amplified DNA fragment S (Table 2.2) showing the restriction fragment size expected for the normal (299bp) and mutant (199 and 100bp) alleles upon digestion with *Tth*111I I restriction enzyme.

(b) Restriction analysis of the *BRCA1* 2800delAA with *Tth*111I.

Restriction enzyme digestion with *Tth*111I in a 2% SeaKem (GTG) agarose gel (Flowgen) indicate the creation of a *Tth*111I restriction site due to the mutation in patient No.10. L= 100bp ladder (Gibco BRL). Lane 1= patient's undigested PCR product. Lane 2= patient's digested product. Lane 3= Normal control PCR product.

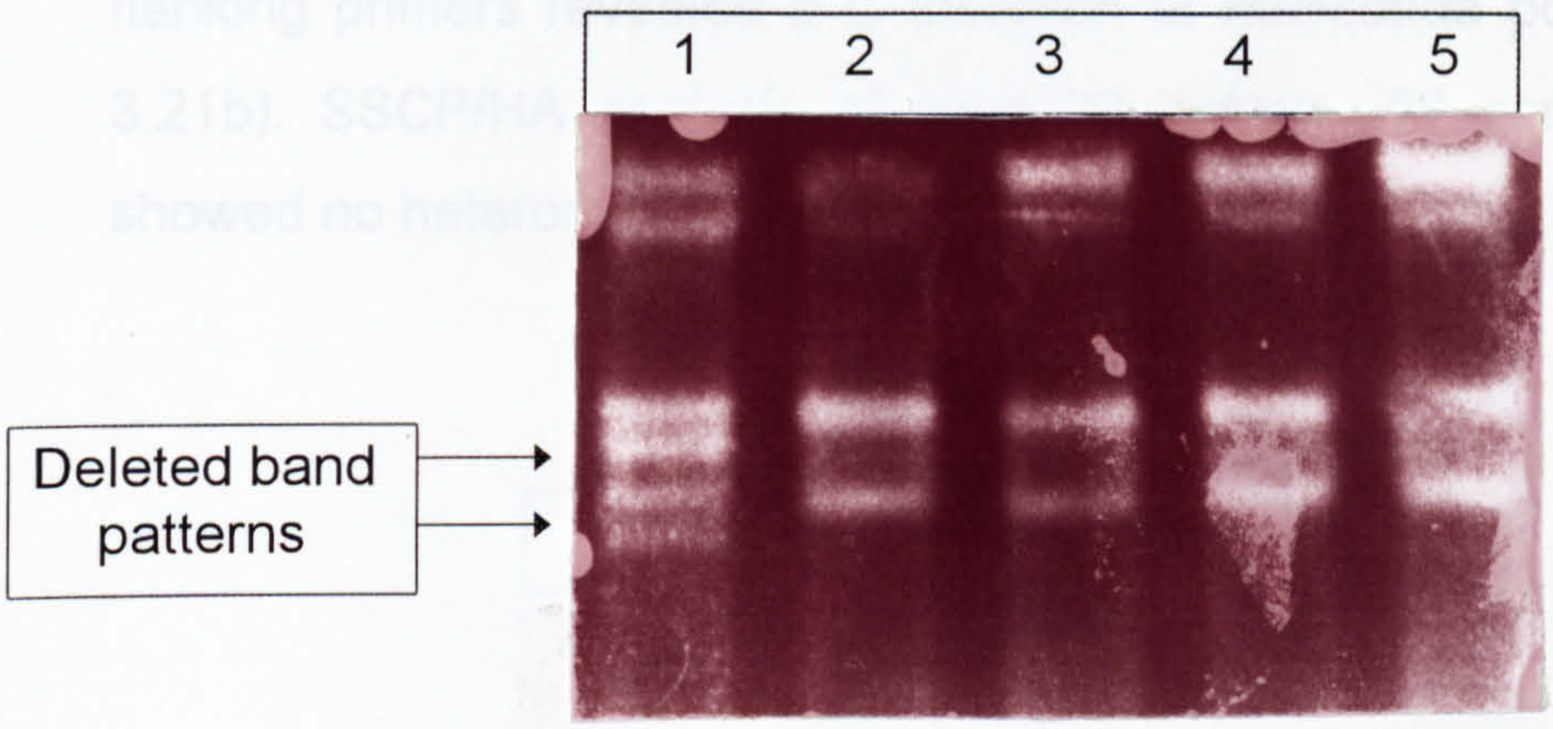


Figure 3.20 SSCP analysis of the *BRCA1* DNA fragment S.
Negative image of a silver stained MDE gel showing a shifted pattern at the level of the single strands in lane 1 compared with the level of single strands in the control lanes 2-5.

3.6 Mutation detection using SSCP/HA analysis

As RNA samples were not available from some of the families, SSCP/HA analysis was carried out to screen for the presence of mutations using primers listed in Table 2.2. for each of the exons.

Insertion of one base pair in exon 20 (5382insC)

SSCP/HA analysis of exon 20 revealed a heteroduplex band in individual III.1 from family 34 (Appendix 5). No shifted band was detected in the single stranded DNA (figure 3.21a). Direct sequencing of exon 20 using flanking primers revealed a C insertion at nucleotide position 5382 (figure 3.21b). SSCP/HA analysis of exon 20 using 50 control DNA samples showed no heteroduplex corresponding to this insertion.

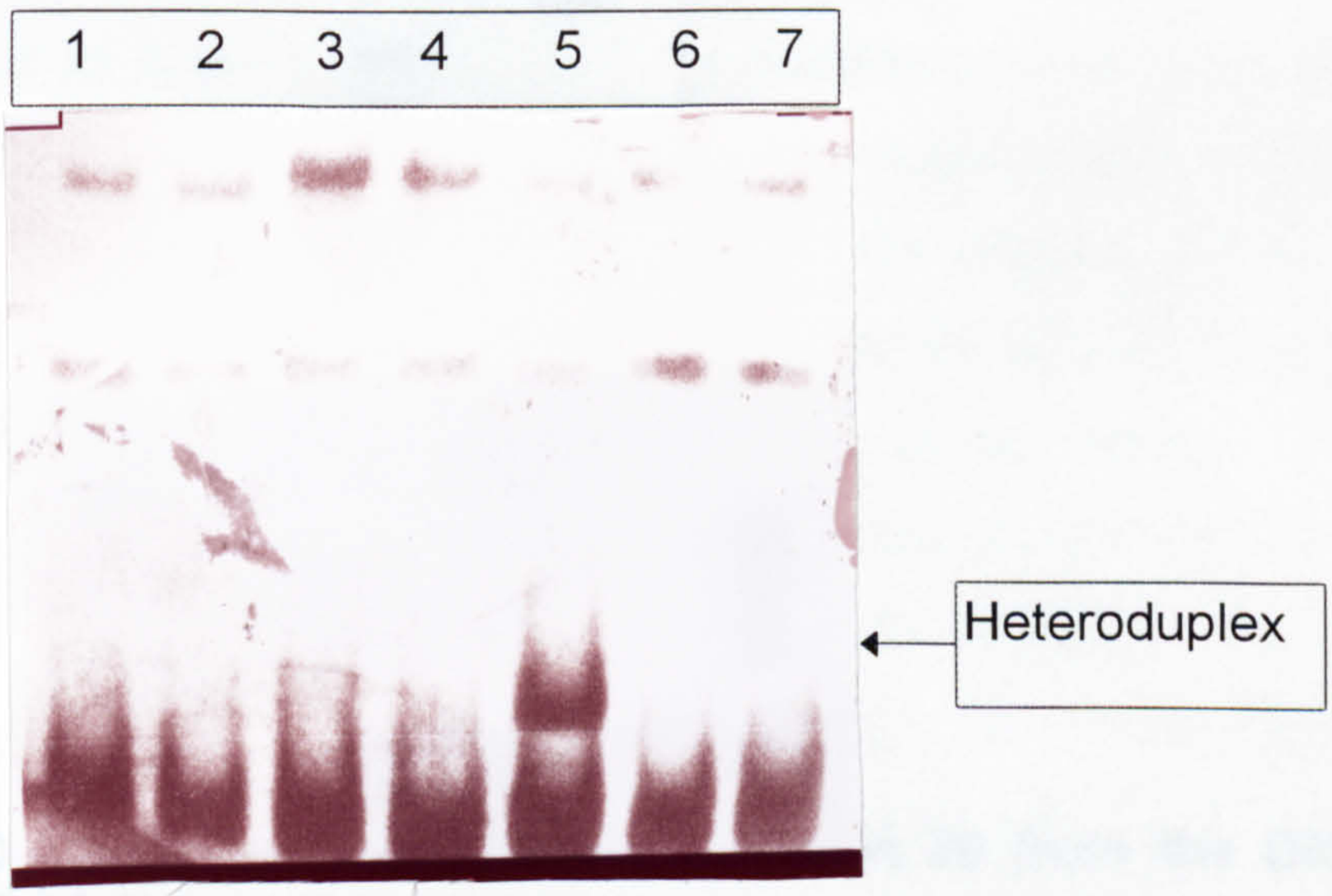


Figure 3.21a SSCP/HA analysis of exon 20 in *BRCA1*.
(a) Silver stained MDE gel of SSCP analysis of *BRCA1* exon 20, showing a heteroduplex band in lane 5 (marked by an arrow). There is no clear shifted band at the level of single strands in lane 5 compared with the level of single strands in the other lanes.

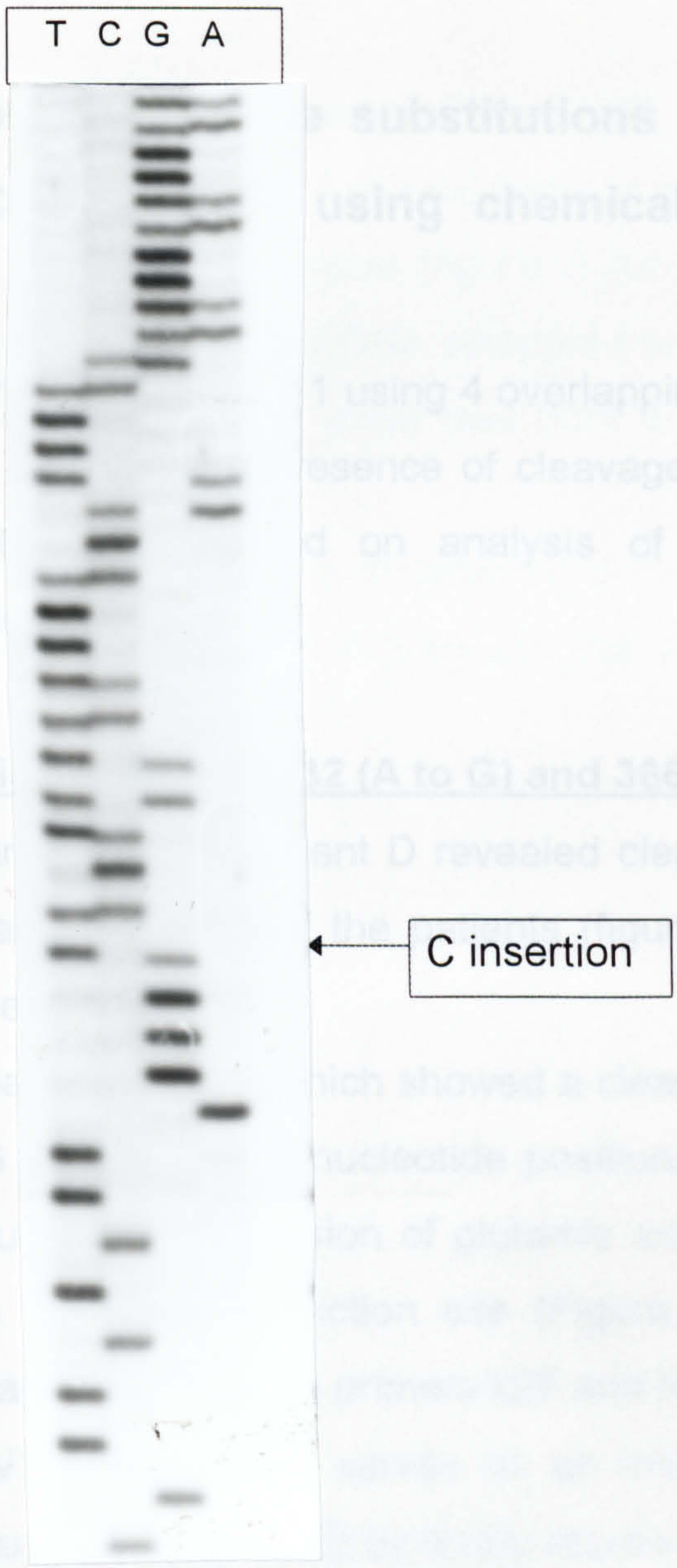


Figure 3.21b Direct sequencing analysis of exon 20 from the DNA in lane 5 (Figure 3.21a).
The sequence surrounding position 5382 and the point of insertion of cytosine (indicated by an arrow) is shown. As the patient is heterozygous for the mutation, both the normal and the mutated sequences run together beyond the point of insertion.

3.7 Characterisation of single base substitutions and small rearrangements in PCR products using chemical cleavage analysis.

Chemical cleavage analysis of exon 11 using 4 overlapping fragments A, B, C, and D (figure 3.1) revealed the presence of cleavage products in these fragments. The mutations identified on analysis of these DNA fragments are presented below:

Polymorphisms at nucleotide position 3232 (A to G) and 3667 (A to G)

Chemical cleavage analysis of fragment D revealed cleavage bands on modification of hydroxylation in some of the patients (figure 3.22). The patterns of cleaved bands were different.

Direct sequencing analysis of DNA which showed a cleavage product of 54bp revealed an A to G substitution at nucleotide position 3232 (figure 3.22b). This substitution causes the conversion of glutamic acid to glycine. This mutation also creates an *N1a*Iv restriction site (Figure 3.23a). The product of amplification of fragment k2 (using primers k2F and K2R) contains another normally present *N1a*Iv site which serves as an internal control. Digestion of the 296bp product of fragment k2 by N*1a*Iv results in two bands of 234bp and(34 and 30bp), from the mutant allele, in addition to the 266bp and 30bp bands from normal allele (figure 3.23b). Restriction enzyme digestion by *N1a*Iv enzyme of fragment k2 from 58 patients and 50 control DNAs revealed that the frequency of this substitution is 29% and 32% in patients and normal individuals, respectively.

SSCP analysis of fragment k2 showed additional bands (figure 3.24) and can therefore be used to detect this polymorphism.

Direct sequencing analysis of DNA which showed a cleavage band at position 489 revealed an A to G substitution at nucleotide position 3667 (figure 3.25a). This causes the conversion of the amino acid Lysine to Arginine. This mutation creates an *BseR1* restriction site. Digestion of the

253bp product of fragment N using primers NF and NR (Table 2.2), produces bands of 124bp and 129bp from the mutant allele in addition to the undigested 253bp band from the normal allele (figure 3.26b). Restriction digest analysis of 58 patients, and 50 normal DNA samples from fragment N revealed that the frequency of the mutant allele was 30% in the patients and 31% in the controls.

However SSCP analysis of fragment N showed no distinct additional band from those samples which are heterozygous for this polymorphism (figure 3.25b).

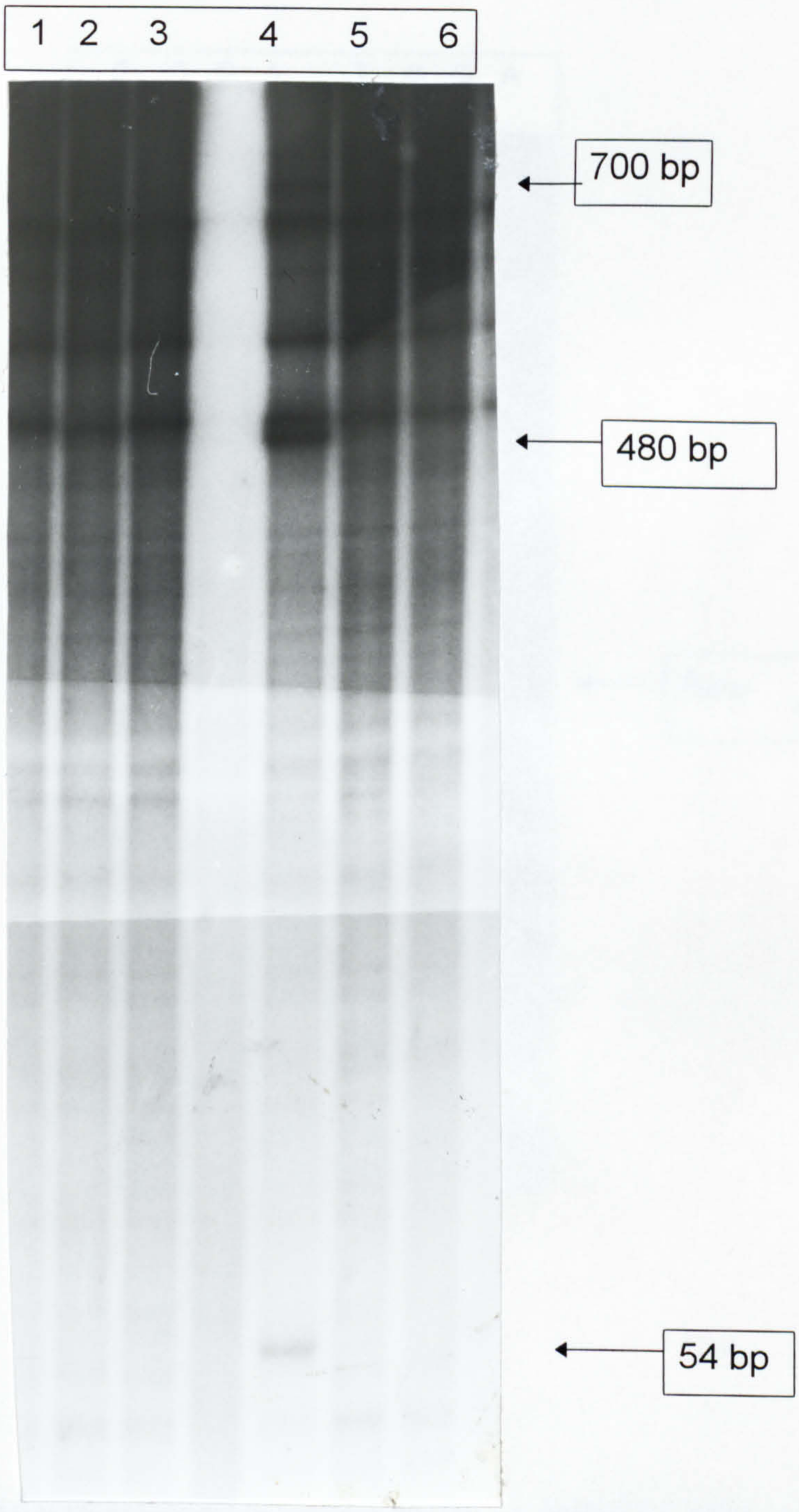


Figure 3.22a Autoradiograph of the chemical cleavage analysis of the *BRCA1* DNA fragment D after hydroxylamine modification.
Three cleavage products of 54, 480 and 700bp are seen in lane 4 (indicated by arrows).

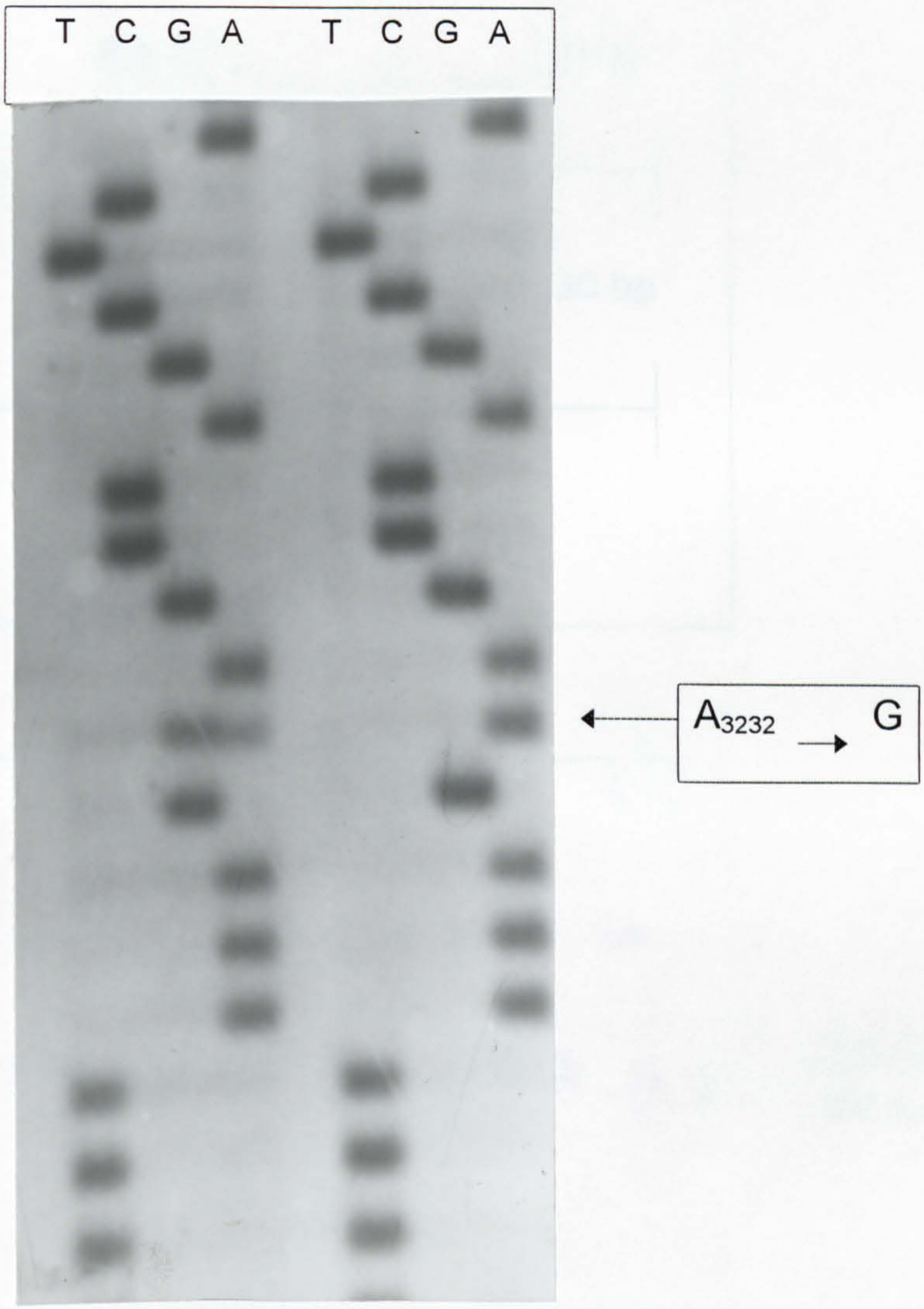
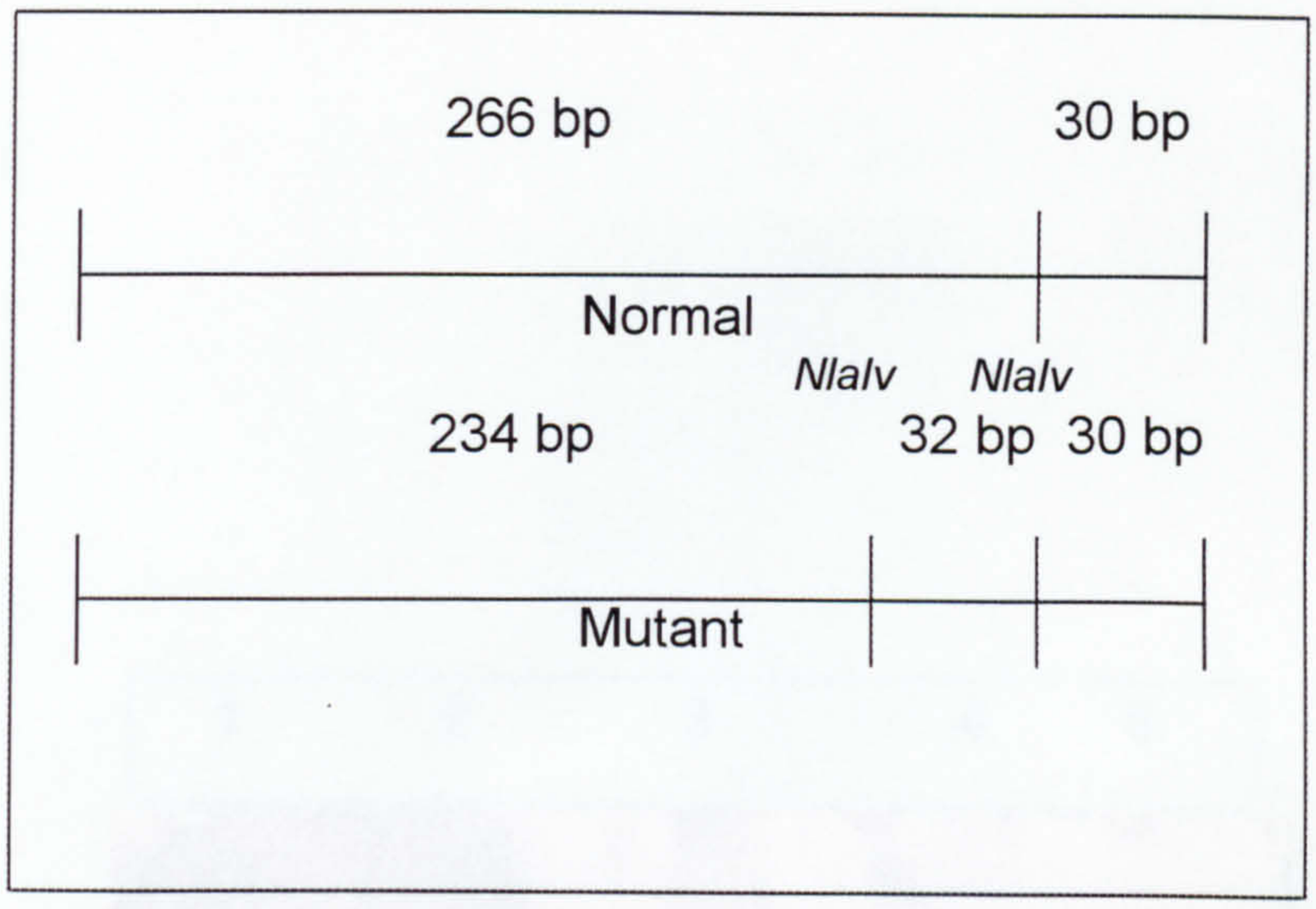
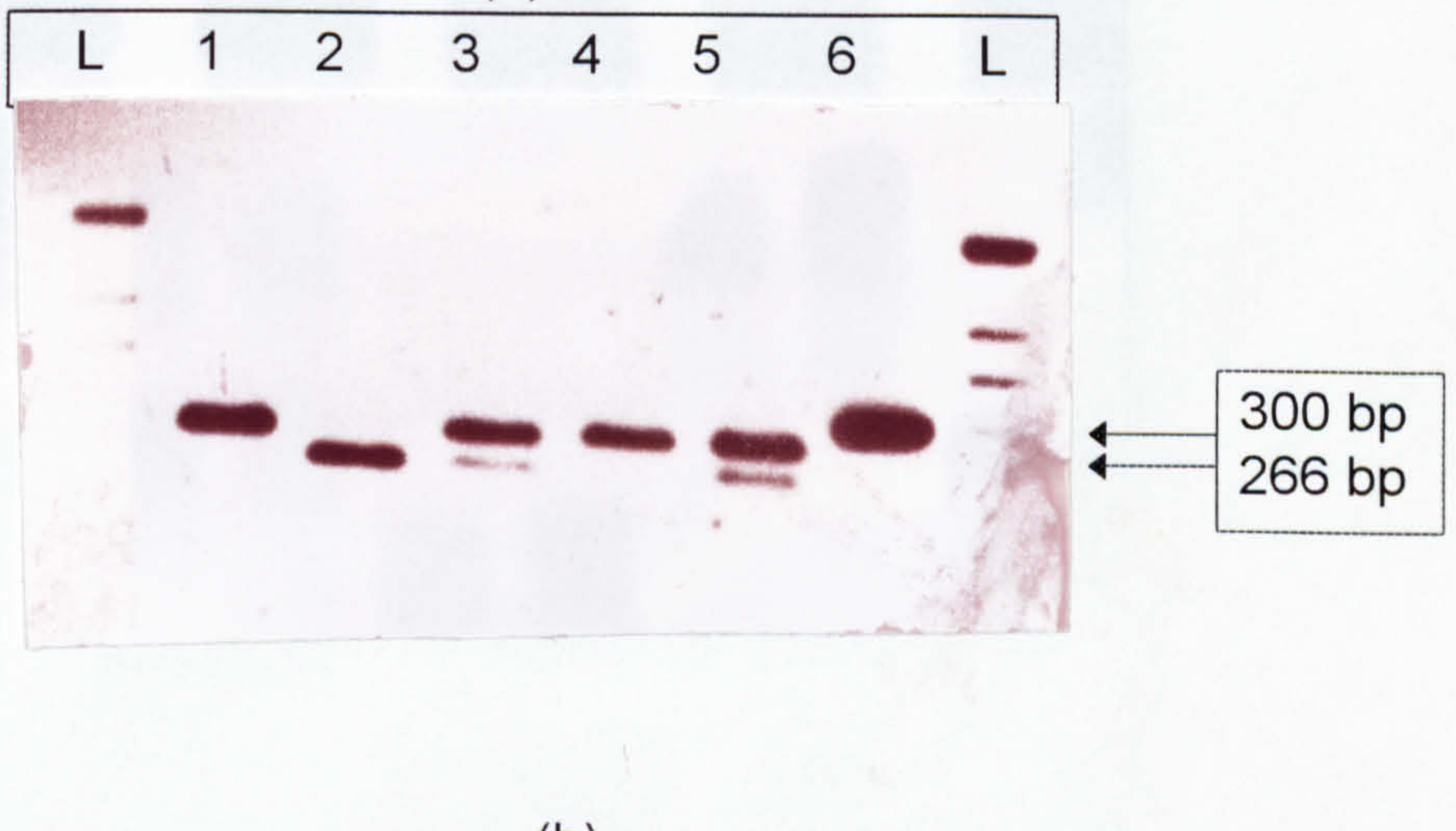


Figure 3.22b Partial sequence analysis of the *BRCA1* DNA fragment K2 to detect the 3232 A to G mutation.

DNA fragment K2 in the region of the 54bp (figure 22a) cleavage product revealed an A to G substitution at nucleotide position 3232 (indicated by an arrow). N= normal. M= mutant.



(a)



(b)

Figure 3.23 (a) Restriction map of N*la*IV to detect the *BRCA1* 3232 A to G mutation.

Restriction map of amplified DNA fragment k2 showing the restriction fragment sizes expected for the normal (266 and 30bp) and mutant (234, 30 and 34bp) DNA upon digestion with *NlaIV* restriction enzyme.

(b) Restriction analysis of the *BRCA1* 3232 A to G substitution with *Nla*IV.

Restriction enzyme digestion products in a 2% Sea Kem (GTG) agarose gel indicate the creation of a N*la*Iv restriction site due to the mutation (3232 A to G). L= 100 bp ladder. Lanes 1 and 6 = undigested PCR product. Lane 2 = homozygous for polymorphic allele G. 3 and 5 = heterozygous for polymorphic allele G. Lane 4 = normal.

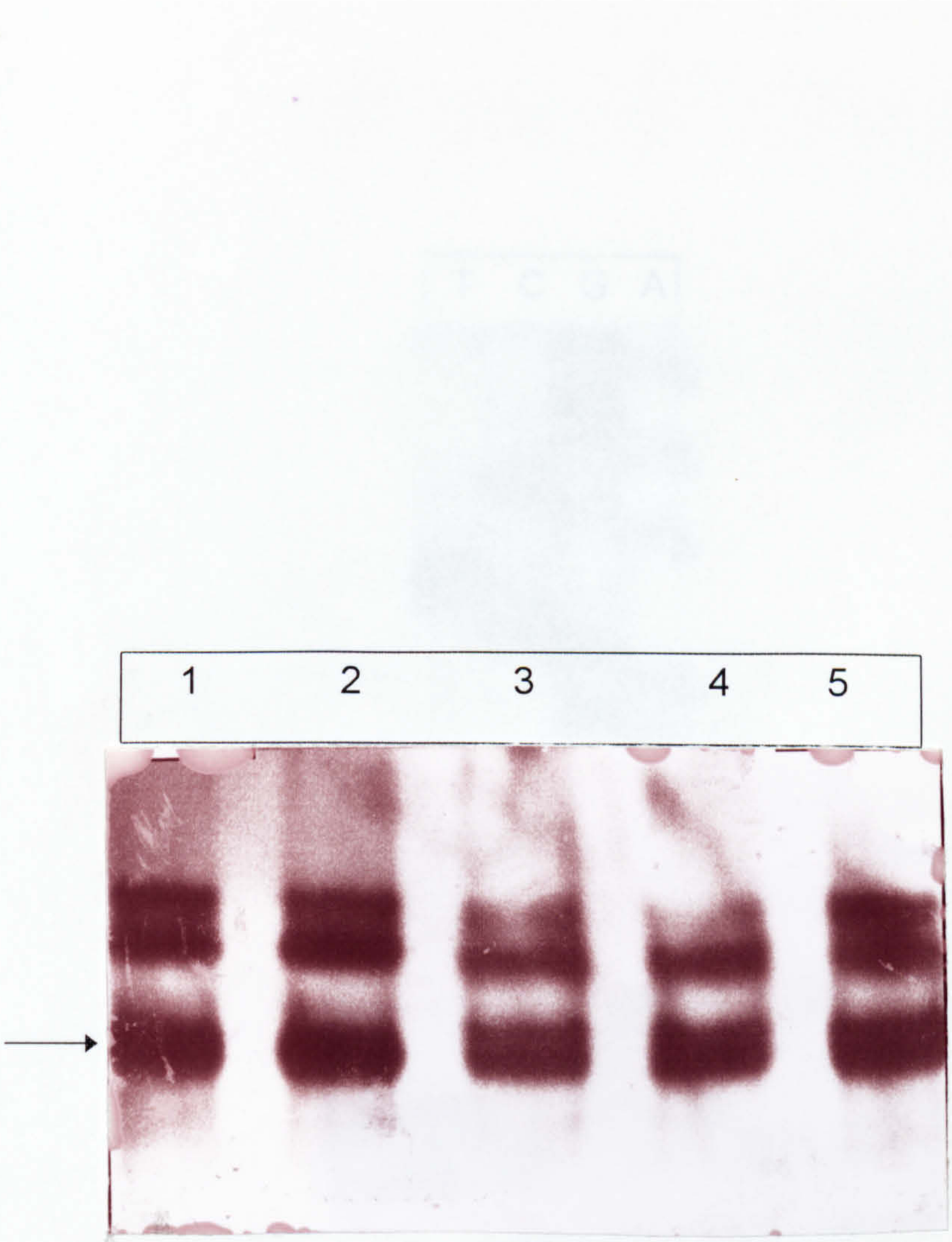


Figure 3.24 SSCP analysis of the *BRCA1* DNA fragment k2.
Silver stained MDE gel of DNA fragment k2 (Table 2.2), showing a shift at the level of the single strands in lanes 1 and 2, due to the mutation, compared with the pattern of the single strands in the normal controls in lanes 3-5.

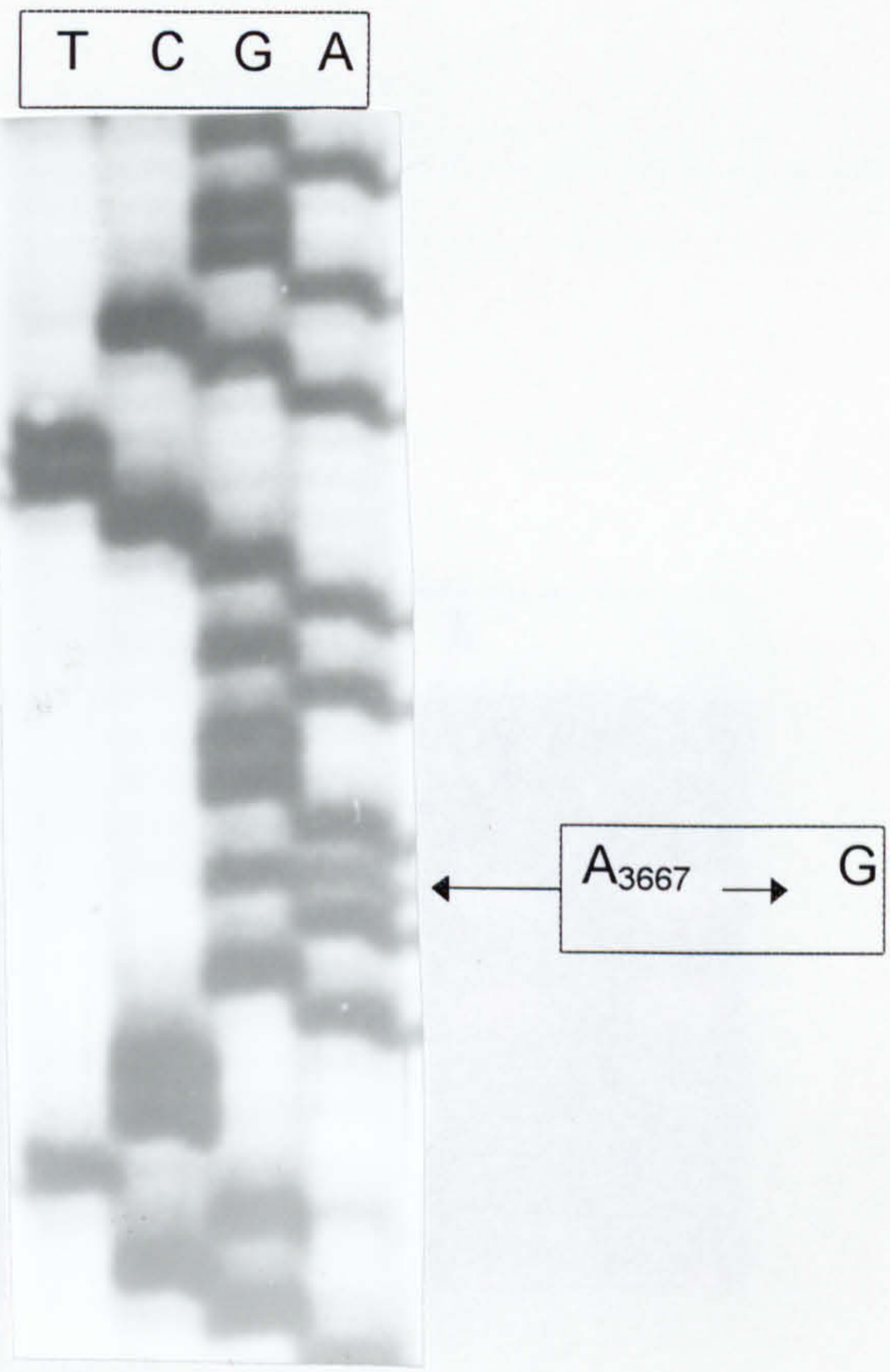


Figure 3.25a Partial sequence analysis of the *BRCA1* DNA fragment N showing the 3667 A to G mutation.

Fragment N in the region of the 489bp chemical cleavage product, revealed an A to G substitution at nucleotide position 3667 (indicated by an arrow).

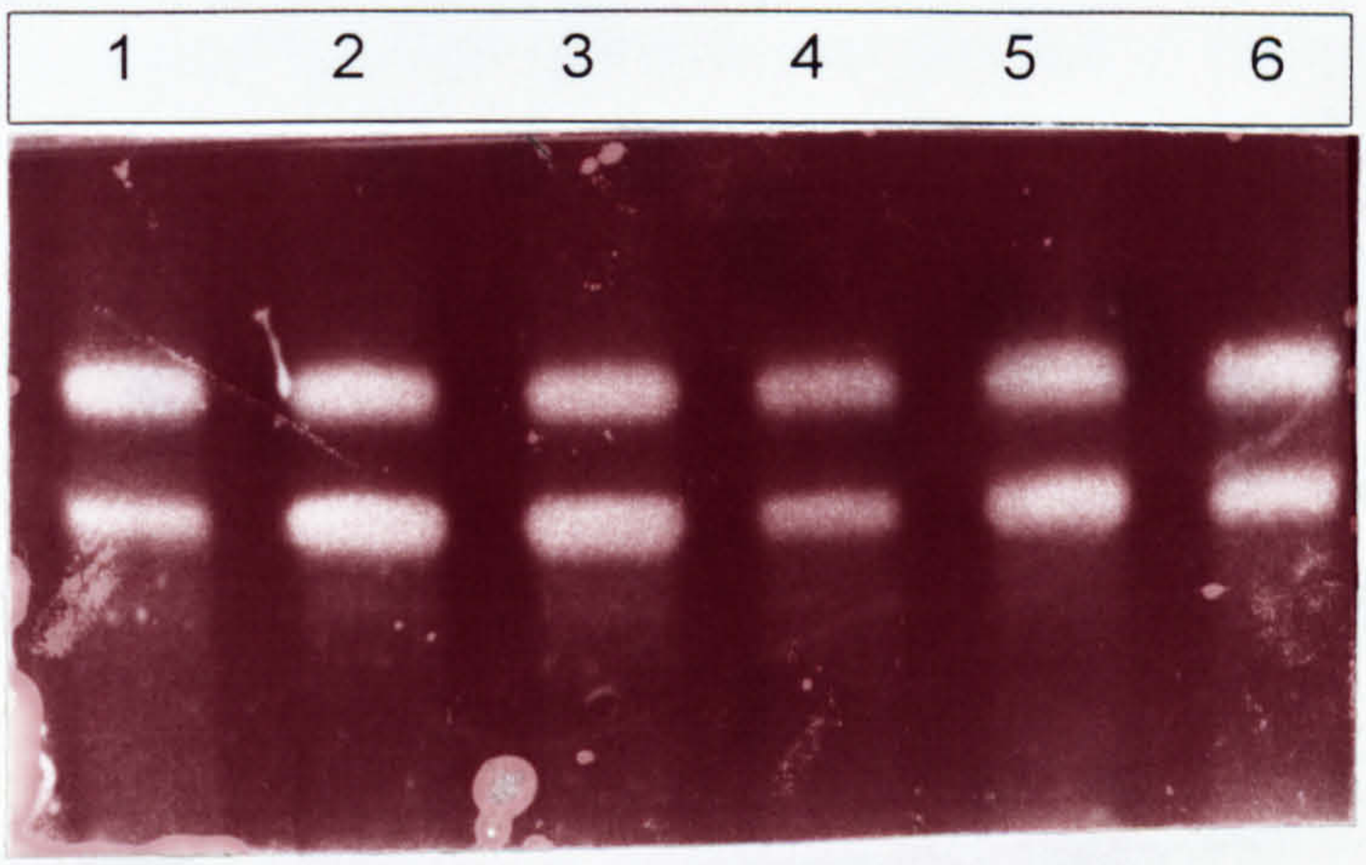
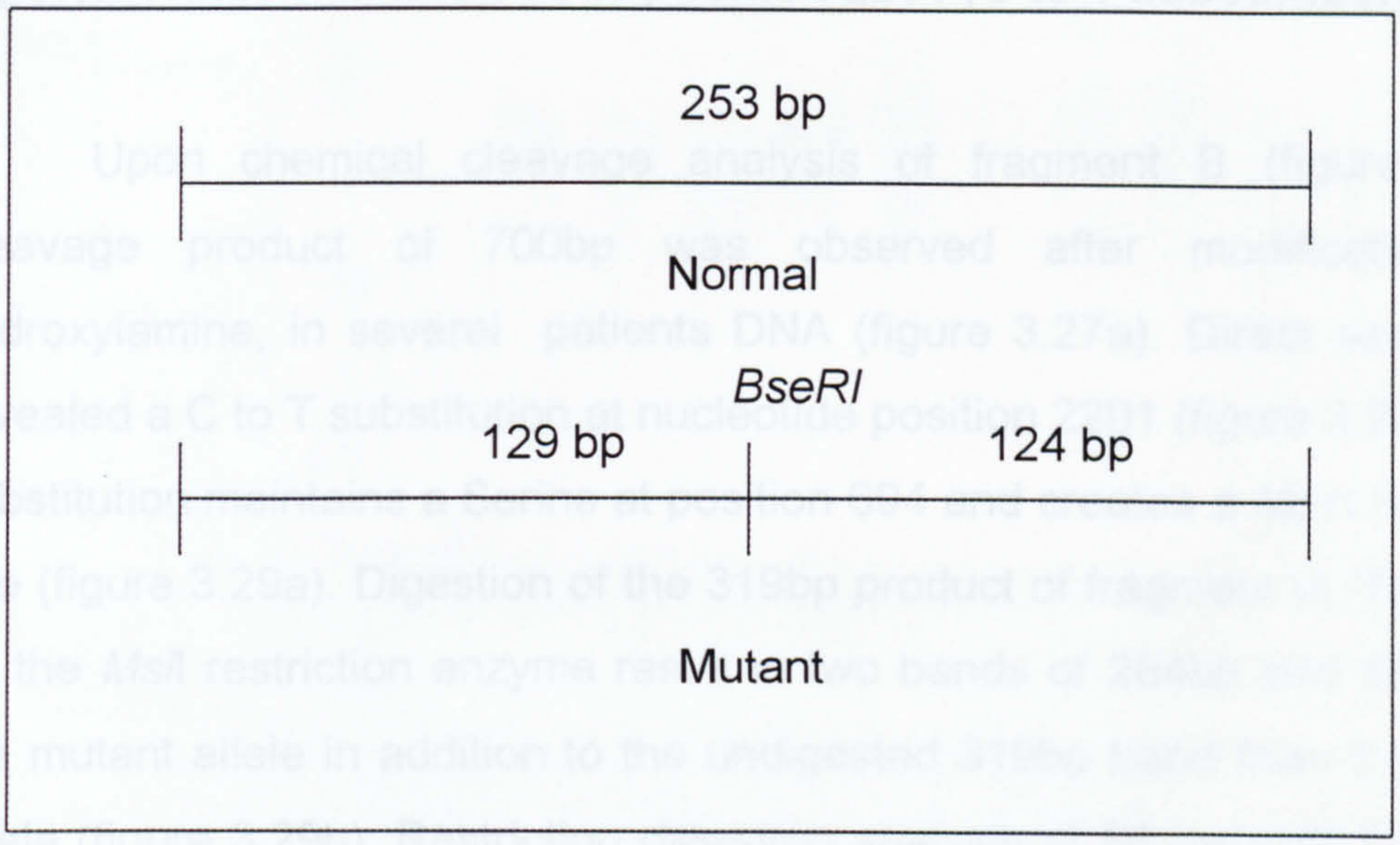
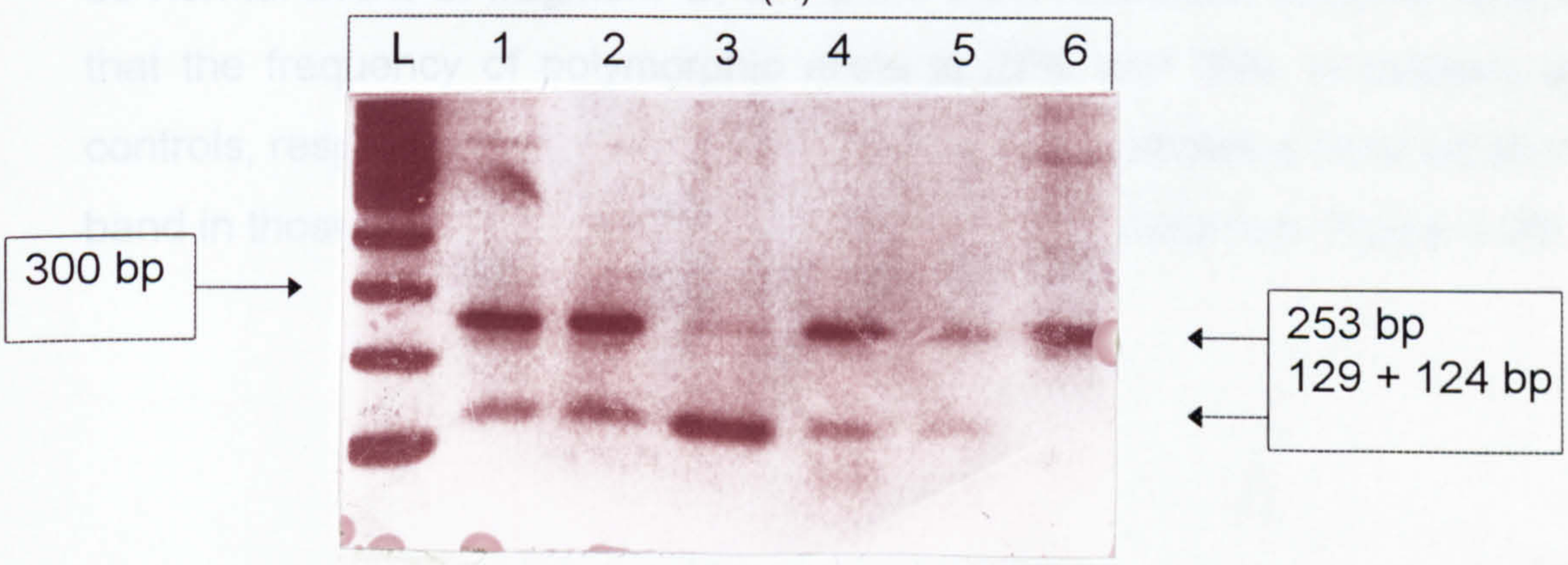


Figure 3.25b SSCP analysis of the *BRCA1* DNA fragment N.
Negative image of a silver stained MDE gel. Lanes 4 & 5 are positive for A to G substitution but show no band shift . Lanes 1-3 and 6 normal controls.



(a)



(b)

Figure 3.26 (a) Restriction map of *BseRI* to detect the *BRCA1* 3667 A to G mutation.

Restriction map of amplified DNA fragment N showing the restriction fragment sizes expected for the normal (253) and mutant (124 and 129) alleles upon digestion with *BseR*I restriction enzyme.

(b) Restriction analysis of the *BRCA1* 3667 A to G with *BseRI*.

Restriction enzyme digestion products in a 2% Sea Kem (GTG) agarose gel indicate the creation of a *BseRI* restriction site due to the mutation (3667 A to G). L= 100bp ladder with 100bp band indicated. Lanes 1, 2, 4 and 5 are heterozygous for the polymorphic allele G. Lane 3= homozygous for polymorphic allele G. Lane 6 = normal.

A polymorphism at nucleotide position 2201 (C to T substitution)

Upon chemical cleavage analysis of fragment B (figure 3.1) a cleavage product of 700bp was observed after modification with hydroxylamine, in several patients DNA (figure 3.27a). Direct sequencing revealed a C to T substitution at nucleotide position 2201 (figure 3.27b). This substitution maintains a Serine at position 694 and creates a *Ms1*I restriction site (figure 3.29a). Digestion of the 319bp product of fragment G (Table 2.2) by the *Ms*I restriction enzyme result in two bands of 264bp and 55bp from the mutant allele in addition to the undigested 319bp band from the normal allele (figure 3.29b). Restriction digestion analysis of 58 patients DNAs and 50 normal DNAs of fragment G, using the *Ms1*I restriction enzyme revealed that the frequency of polymorphic allele is 27% and 26% in patients and controls, respectively. SSCP analysis of fragment G shows a clear additional band in those who where heterozygous for this polymorphism (figure 3.28).

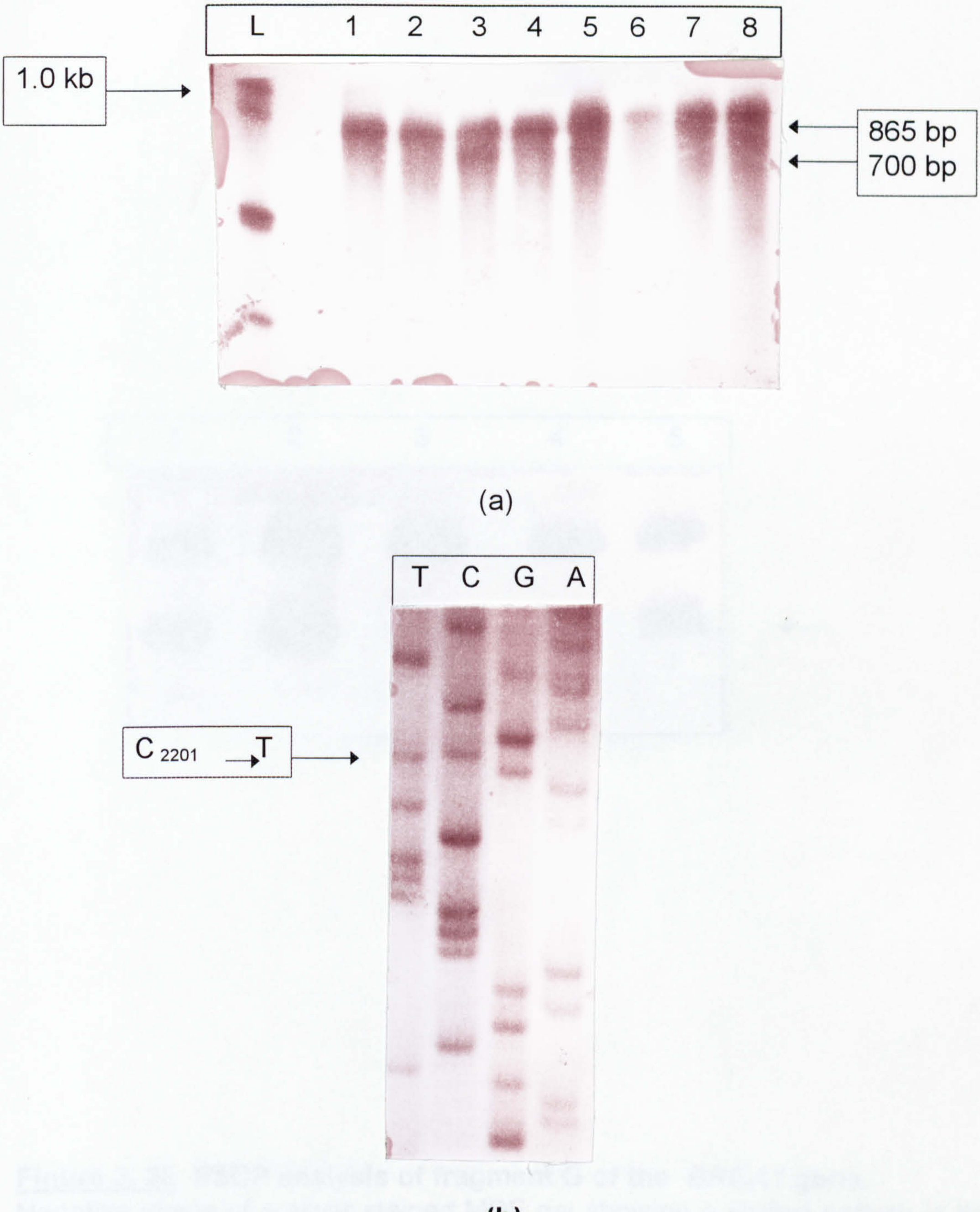


Figure 3.27 CCM and sequence analysis of the *BRCA1* DNA fragment B to detect the 2201 C to T mutation.

(a) Autoradiograph of the chemical cleavage products of DNA fragment B after hydroxylamine modification shows a cleavage product of 700bp in lane 3. The expected PCR product is 856bp. L= 1kb ladder with 1.0kb band indicated.

(b) Partial sequence analysis of DNA fragment B in the region of the 147bp chemical cleavage product revealed an C to T substitution at nucleotide position 2201 (indicated by an arrow).

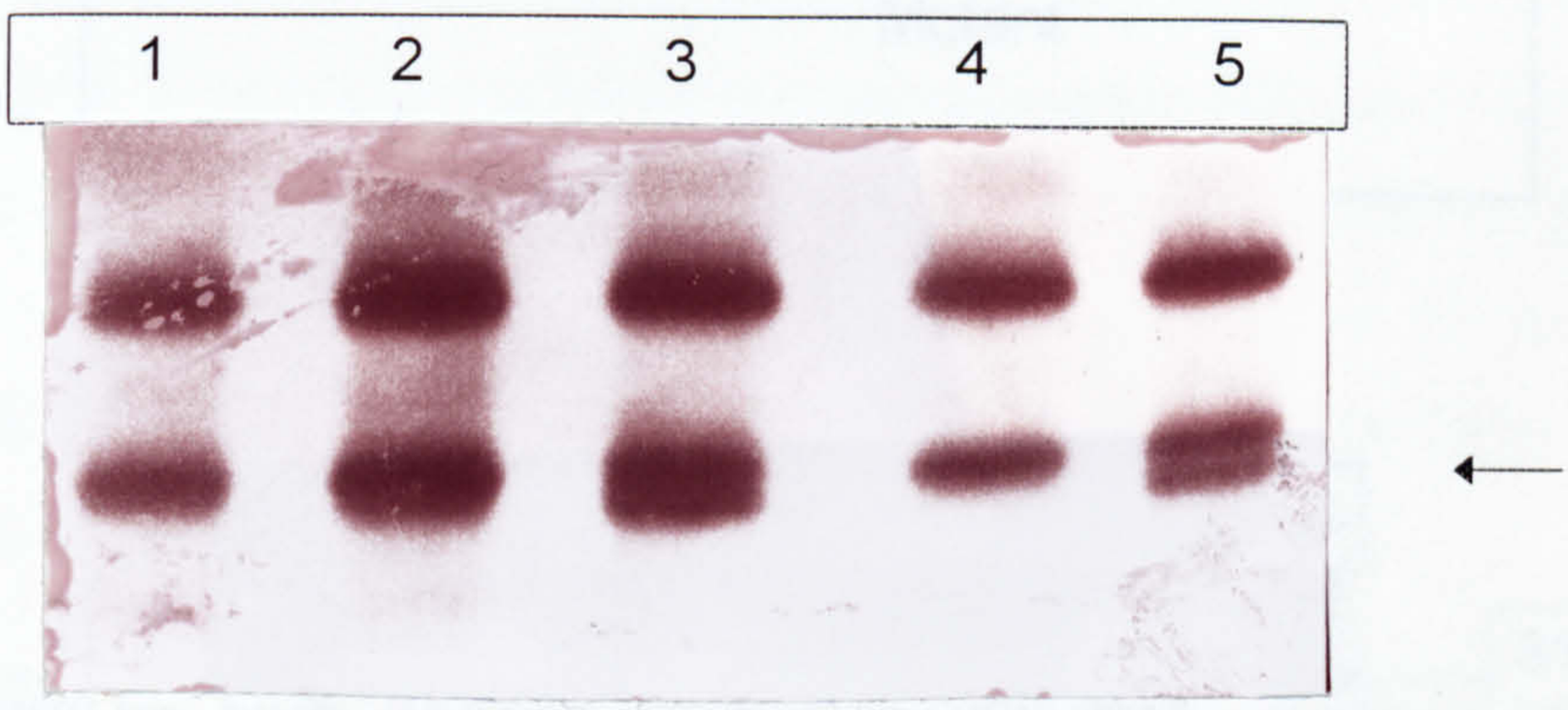
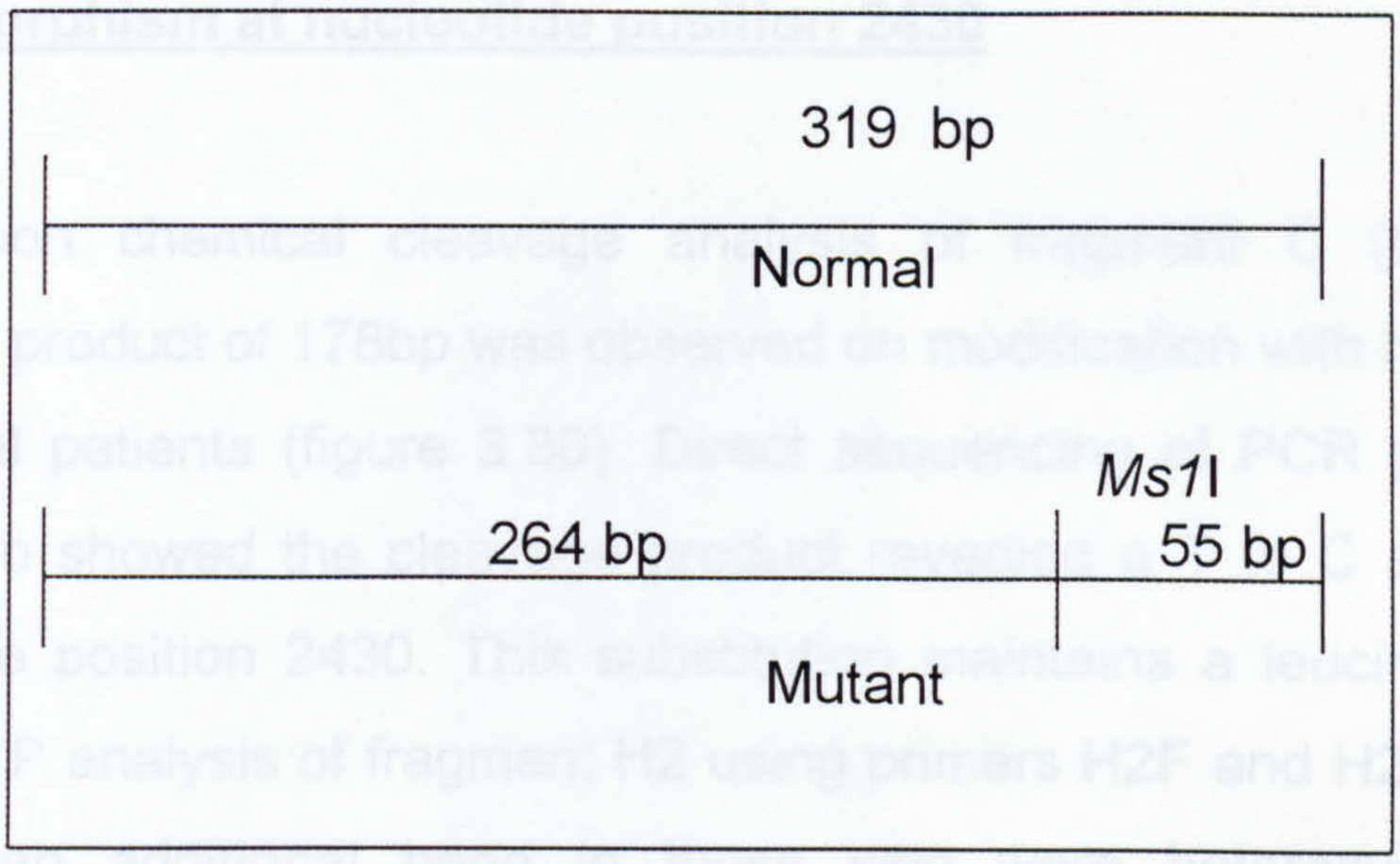
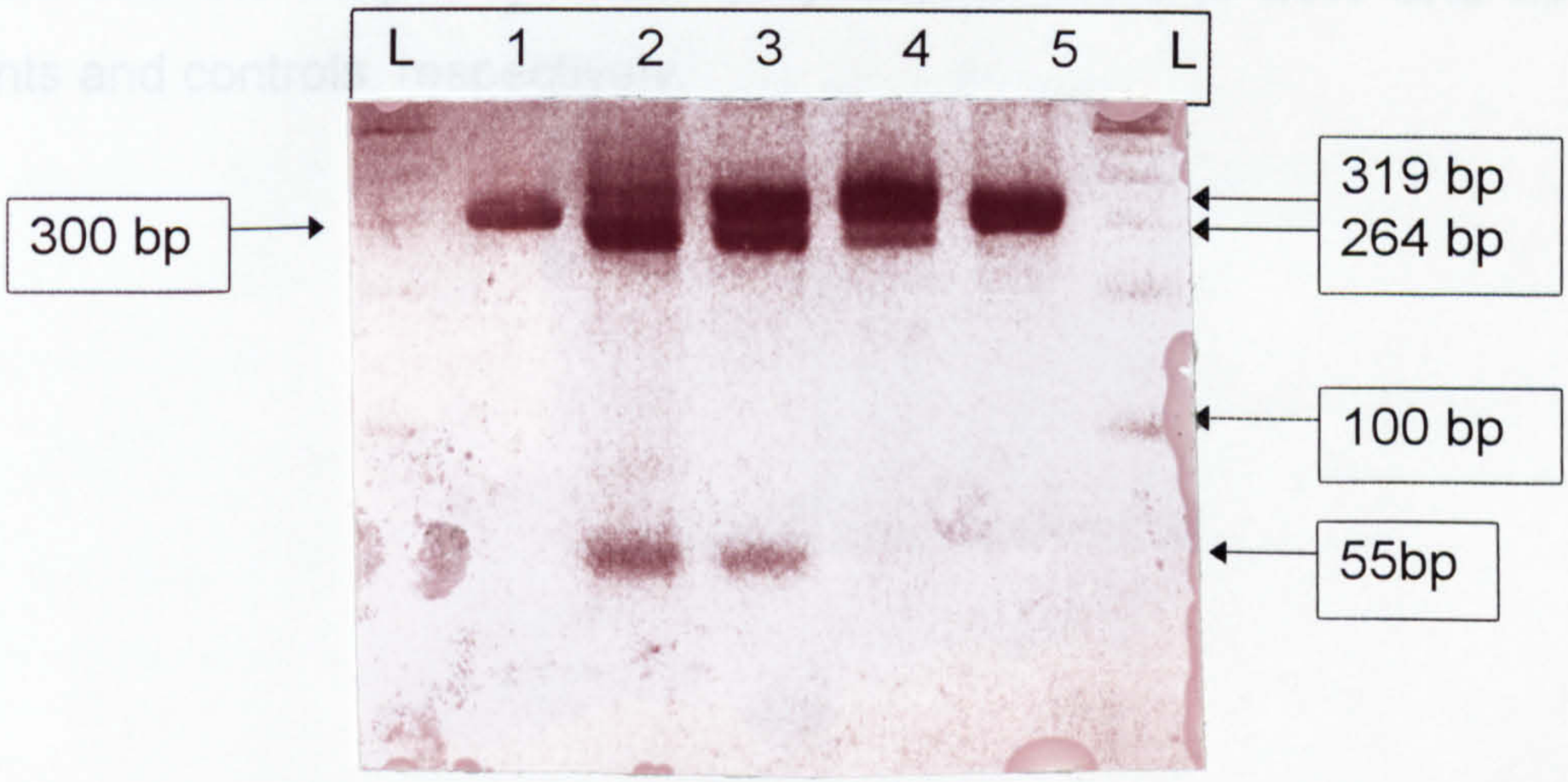


Figure 3. 28 SSCP analysis of fragment G of the *BRCA1* gene.
Negative image of a silver stained MDE gel showing a shifted pattern at the level of the single strands in lanes 3 and 5 due to the mutation, compared with the normal controls in lanes 1, 2 and 4.



(a)



(b)

Figure 3.29 (a) Restriction map of *Ms1*I to detect the 2201C to T mutation.

Restriction map of amplified DNA fragment G showing the restriction fragment sizes expected for the normal (319bp) and mutant (264 bp and 55bp) alleles, upon digestion with *Ms1*I.

(b) Restriction enzyme analysis of the 2201C to G mutation with M*s1*I.

Restriction enzyme digestion products on a 2% Sea Kem (GTG) agarose gel indicates the creation of a *Ms1*I restriction site due to the mutation (2201C to T). L = 100bp ladder with 100bp band indicated. Lane 1= undigested PCR product. Lane 2 = homozygous for polymorphic allele G. Lane 3= heterozygous for polymorphic allele G. Lanes 4 and 5 = normal.

A polymorphism at nucleotide position 2430

Upon chemical cleavage analysis of fragment C (figure 3.1) a cleavage product of 178bp was observed on modification with hydroxylamine in several patients (figure 3.30). Direct sequencing of PCR products from those who showed the cleavage product revealed a T to C substitution at nucleotide position 2430. This substitution maintains a leucine at position 771. SSCP analysis of fragment H2 using primers H2F and H2R (Table 2.2) showed an additional band in those who were heterozygous for the polymorphic allele. SSCP analysis of 58 patients and 50 normal control showed that the frequency of the polymorphic allele is 26% and 25% in patients and controls, respectively.

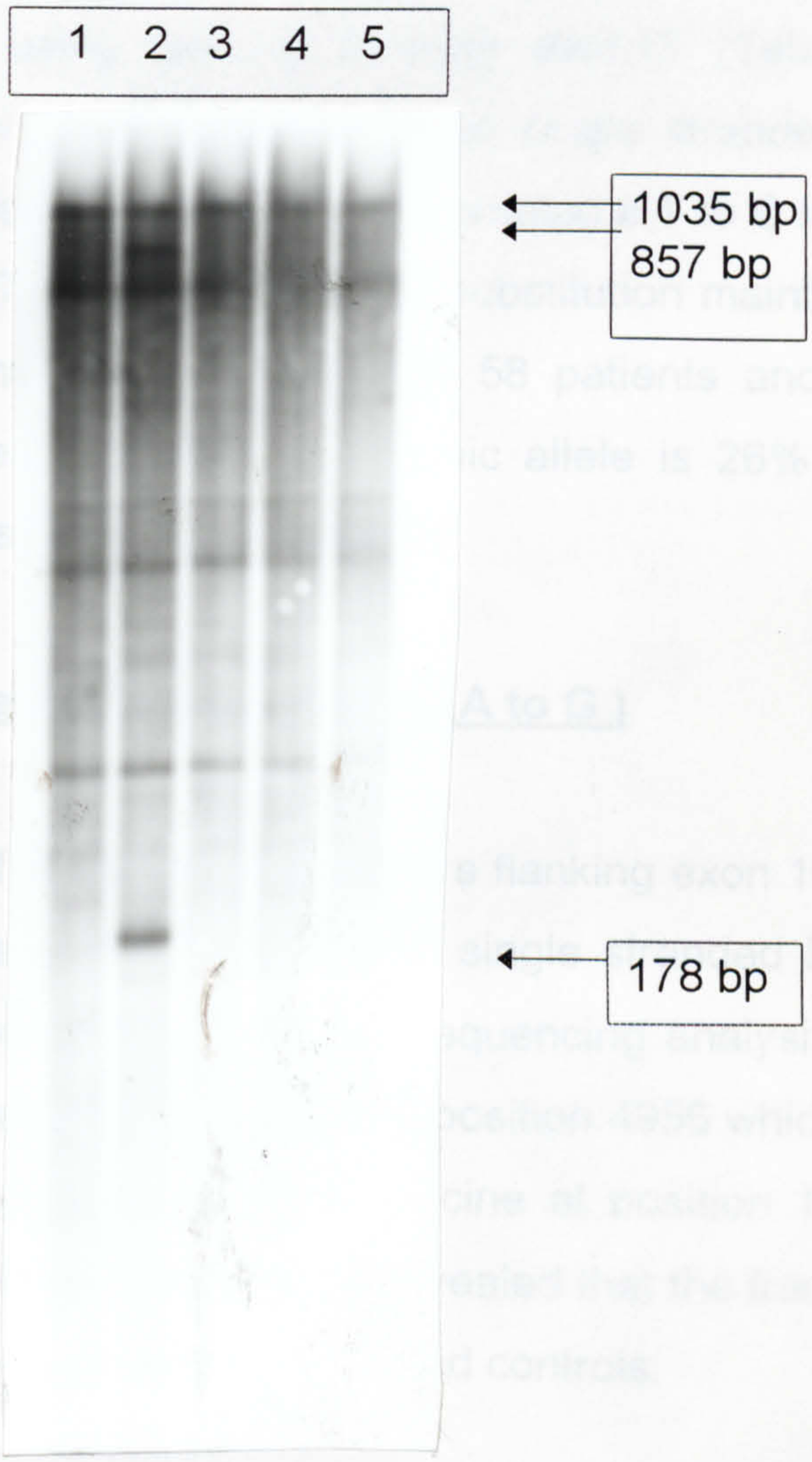


Figure 3.30 Chemical cleavage mismatch analysis showing the cleavage products due to the 2430 T to C mutation.
Autoradiograph of the chemical cleavage analysis of fragment C after hydroxylamine modification shows the cleavage products of 857 and 178bp in lane 2 (indicated by an arrow). The expected PCR product is 1035bp .

A polymorphism at nucleotide position 4427

SSCP analysis using primers flanking exon13 (Table 2.3) from several patients showed an additional band in single stranded DNA band (figure 3.31a). Direct sequencing of exon 13 revealed a T to C substitution at nucleotide position 4427 (figure 3.31b). This substitution maintains a serine at position 1436. Further SSCP analysis of 58 patients and 50 controls showed that the frequency of the polymorphic allele is 26% and 27% in patients and controls respectively.

Polymorphism at nucleotide position 4956 (A to G)

SSCP analysis of exon 16 using primers flanking exon 16 (Table 2.3) from several patients revealed an additional single stranded DNA band in some of the patients (figure 3.32a). Direct sequencing analysis of exon 16 revealed an A to G substitution at nucleotide position 4956 which causes the conversion of the amino acid serine to glycine at position 1613. Further SSCP analysis of 50 patients and controls revealed that the frequency of the polymorphic allele is ~24% in both patients and controls.

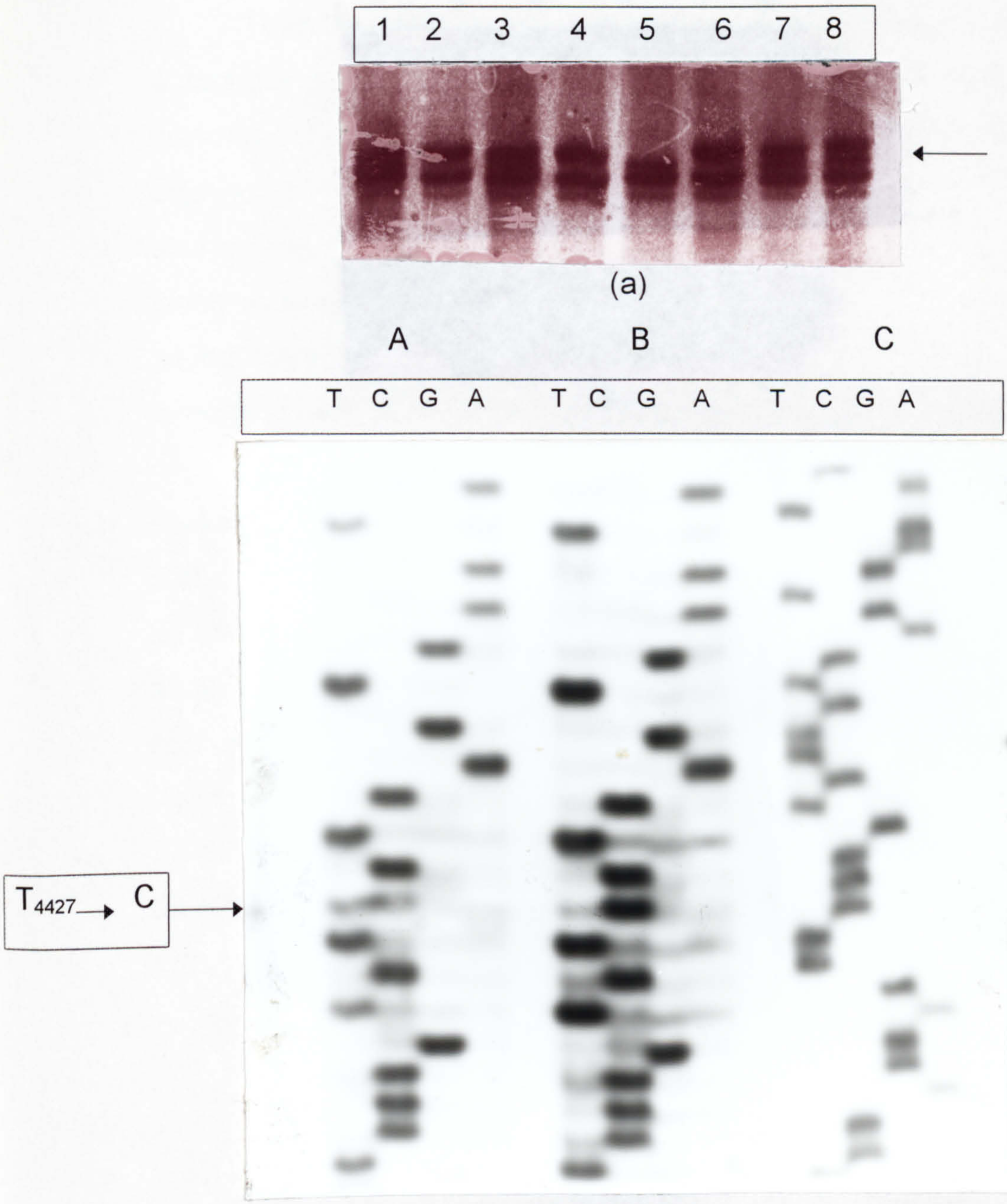


Figure 3.31 SSCP and sequence analysis of exon 13 showing the 4427 T to C mutation in the *BRCA1* gene.

(a) Negative image of a silver stained MDE gel showing a shifted pattern at the level of the single strands in lanes 3, 7 and 8 due to the mutation, compared with normal controls in lanes 2, 4 and 6. Lane 5 is homozygous for the mutant allele (indicated by an arrow). **(b)** Direct sequencing analysis of exon 13 revealed a T to C substitution at nucleotide position 4427 (indicated by an arrow). A = heterozygous for the mutant allele. B = homozygous for the mutant allele. C = normal

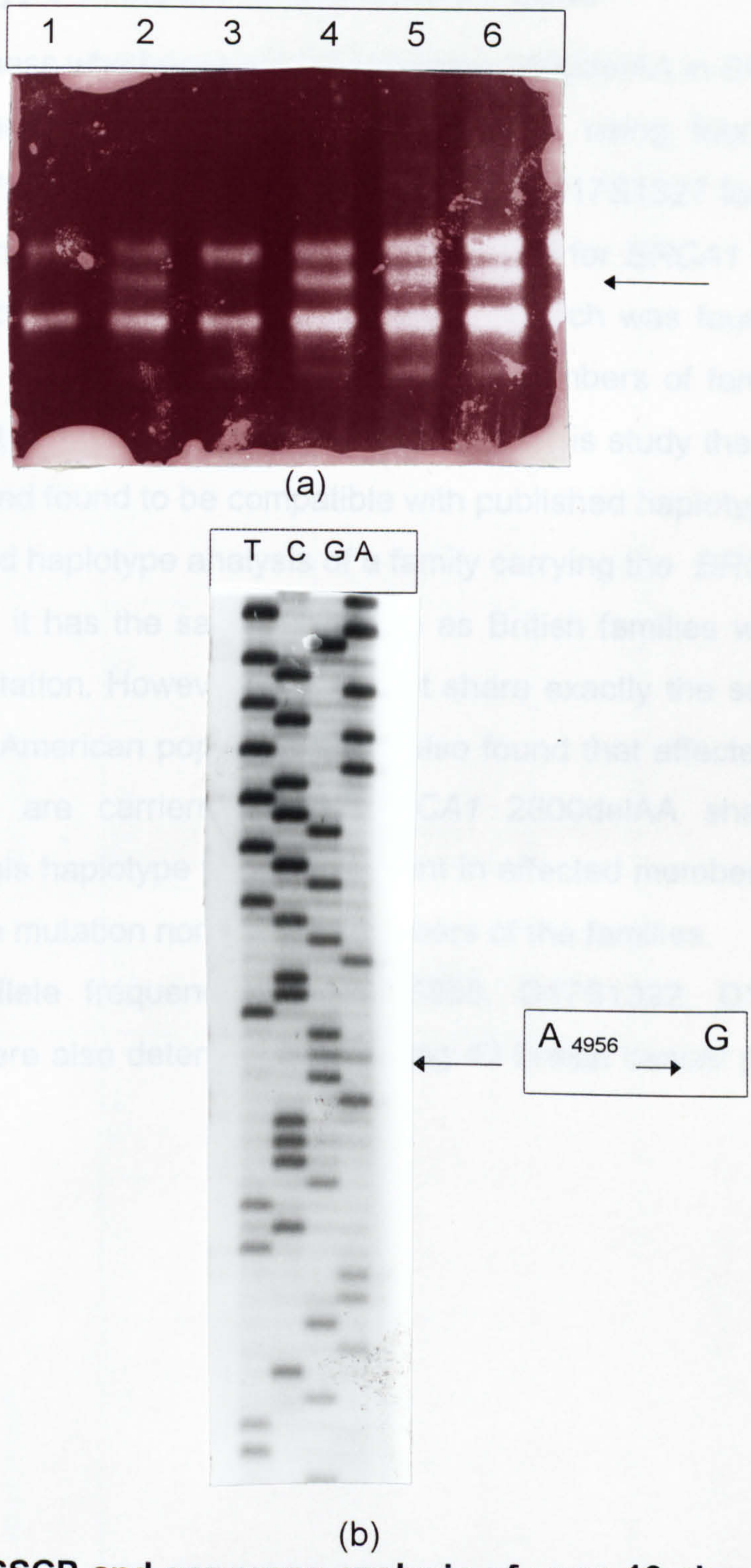


Figure 3.32 SSCP and sequence analysis of exon 16 showing the 4956 A to G mutation in the *BRCA1* gene.

(a) Negative image of a silver stained MDE gel showing a shifted band at the level of the single strands in lanes 2, 4, 5, and 6, compared to the level of the single strands in the normal controls in lanes 1 and 3.

(b) Direct sequencing analysis of exon 16 revealed an A to G substitution at nucleotide position 44956 (indicated by an arrow).

3.8 Haplotype analysis of the *BRCA1* gene

To assess whether or not the recurring 2800delAA in *BRCA1* is due to a founder mutations, A genotype was defined using four microsatellite markers, D17S855, D17S1322, D17S1323, and D17S1327 located within or adjacent to the *BRCA1* gene. Genotype analysis for *BRCA1* 5382insC was also constructed. This is a common mutation which was found only in one family in this study.Obtaining blood from the members of family in order to define haplotype was not possible therefore for this study the genotype was determined and found to be compatible with published haplotype.

Inferred haplotype analysis of a family carrying the *BRCA1* 5382insC, revealed that it has the same haplotype as British families which carry the 5382insC mutation. However they do not share exactly the same haplotype as the North American population. We also found that affected members of families who are carriers of the *BRCA1* 2800delAA share the same haplotype. This haplotype was not present in affected members who are not carriers of the mutation nor healthy members of the families.

The allele frequencies at D17S855, D17S1322, D17S1323, and D17S1327 were also determined by typing 40 breast cancer patients (Table 4.2).

		Affected member of family with mutation **Haplotype***				Affected member of family without mutation **Haplotype***				Unaffected member of family **Haplotype***			
Mutation	Family / Marker loci	17S855	17S322	17S323	17S327	17S855	17S322	17S323	17S327	17S855	17S822	17S323	17S327
	this study	D	E	F	N					D	E	F	M
5382insC	UK	D	E	F	N								
	Canada	D	E	F	O								
	Family 10	E	E	F	M	G	D	C	F	G	E	F	M
2800delAA													
	Family 35	E	E	F	M	F	E	F	M				

*: in this study the haplotype is inferred haplotype. (The genotype was determined and found to be compatible with published haplotype).

Table 3.2. Haplotype analysis in families with *BRCA1* mutations.

3.9 Strategy for the detection of germline mutations in the *BRCA2* gene.

Exon 11 of the *BRCA2* gene was first screened for mutations by subjecting the 4 overlapping amplified fragments A, B, C, and D (figure 3.33a) to the protein truncation test to detect and locate the presence of any changes leading to premature stop codons in the 40 patients (figures 3.33-36). Any truncated products were fully characterised by asymmetric PCR and direct sequencing. Every sequence change was checked by the GCG package "MAP" enzyme to see if it created or abolished a restriction enzyme site. Digestion of PCR products with an appropriate enzyme was then carried out to confirm the change in the sequence and also to screen the other members of a family and other patients also.

The rest of the *BRCA2*, exons 1-10 and 12-27 and associated exon-intron boundary sequences, were examined by PCR-SSCP analysis. When variant bands were detected direct sequencing analysis was carried out to characterise the mutation responsible.

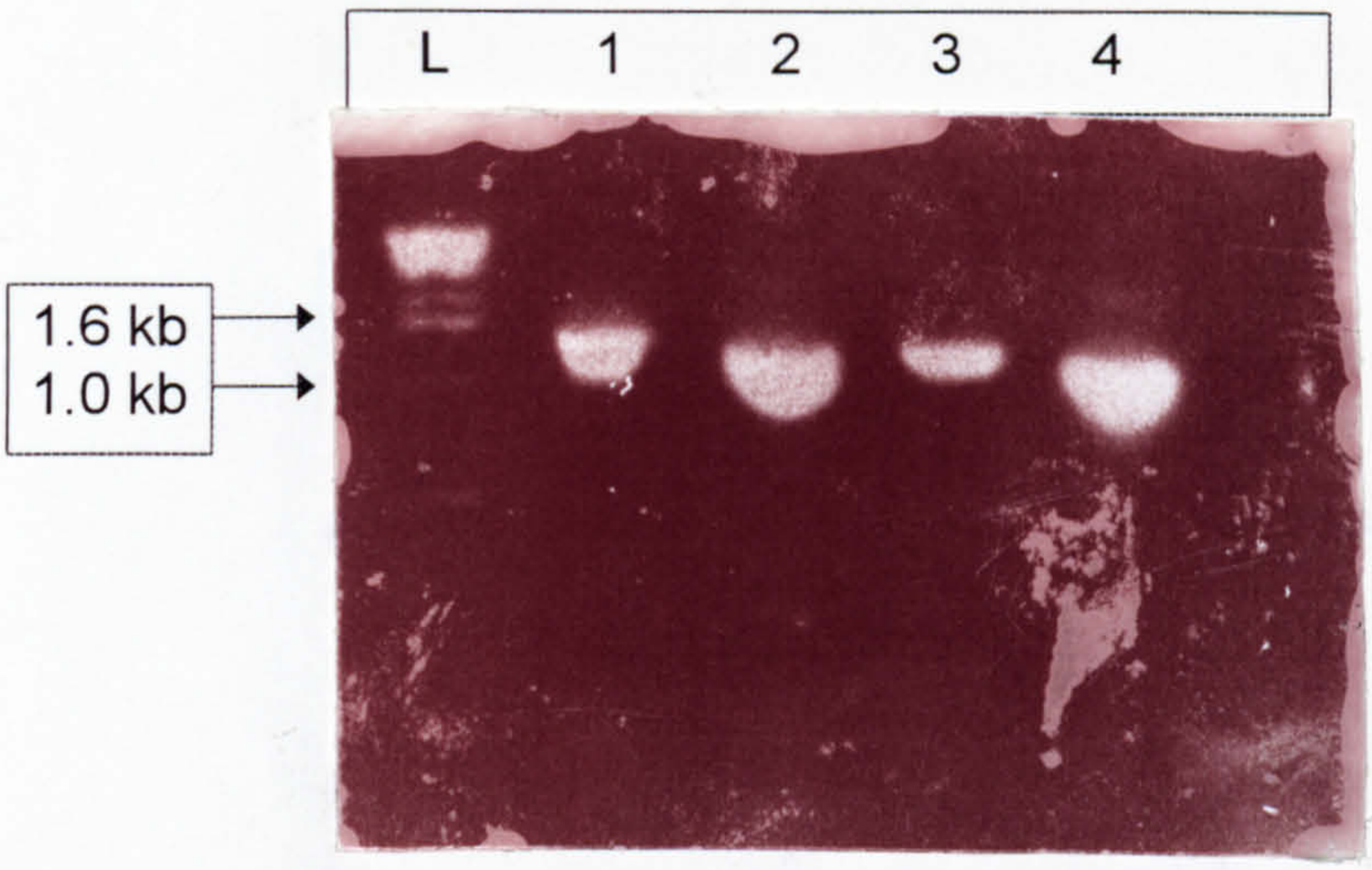


Figure 3.33a 1% agarose gel showing amplification products of four overlapping DNA fragments (A-D) of the *BRCA2* gene.

L = 1kb ladder with 1.6kb band indicated. Lane 1 = fragment A (1469bp). Lane 2 = fragment B (1313bp). Lane 3 = fragment C (1300bp). Lane 4 = fragment D (1166bp).

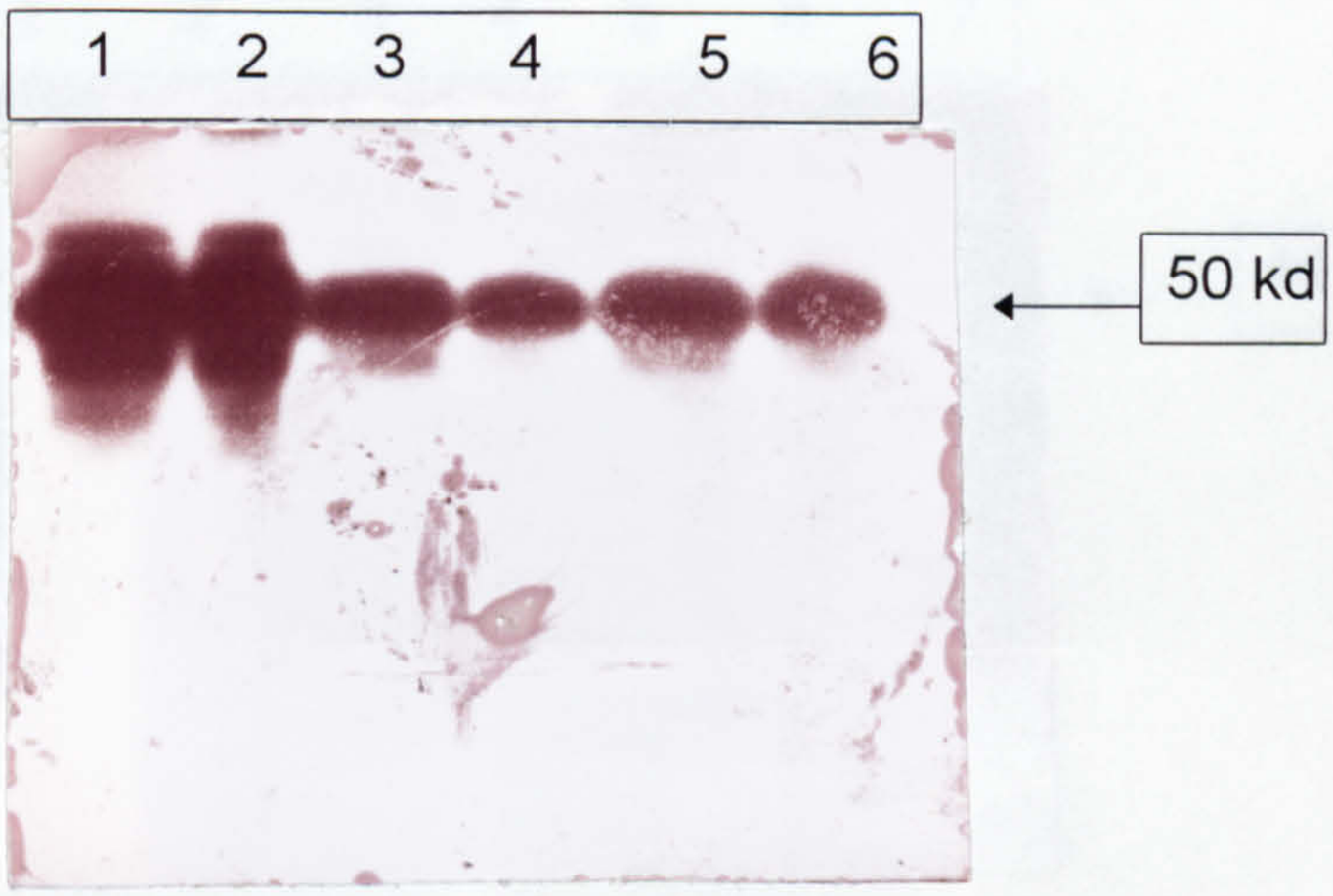


Figure 3.33b PTT analysis of fragment A of the *BRCA2* gene.
Transcription products separated in a 15% SDS polyacrylamide gel. The 50kd protein is indicated by an arrow. Lanes 1-5= Protein products from index cases.

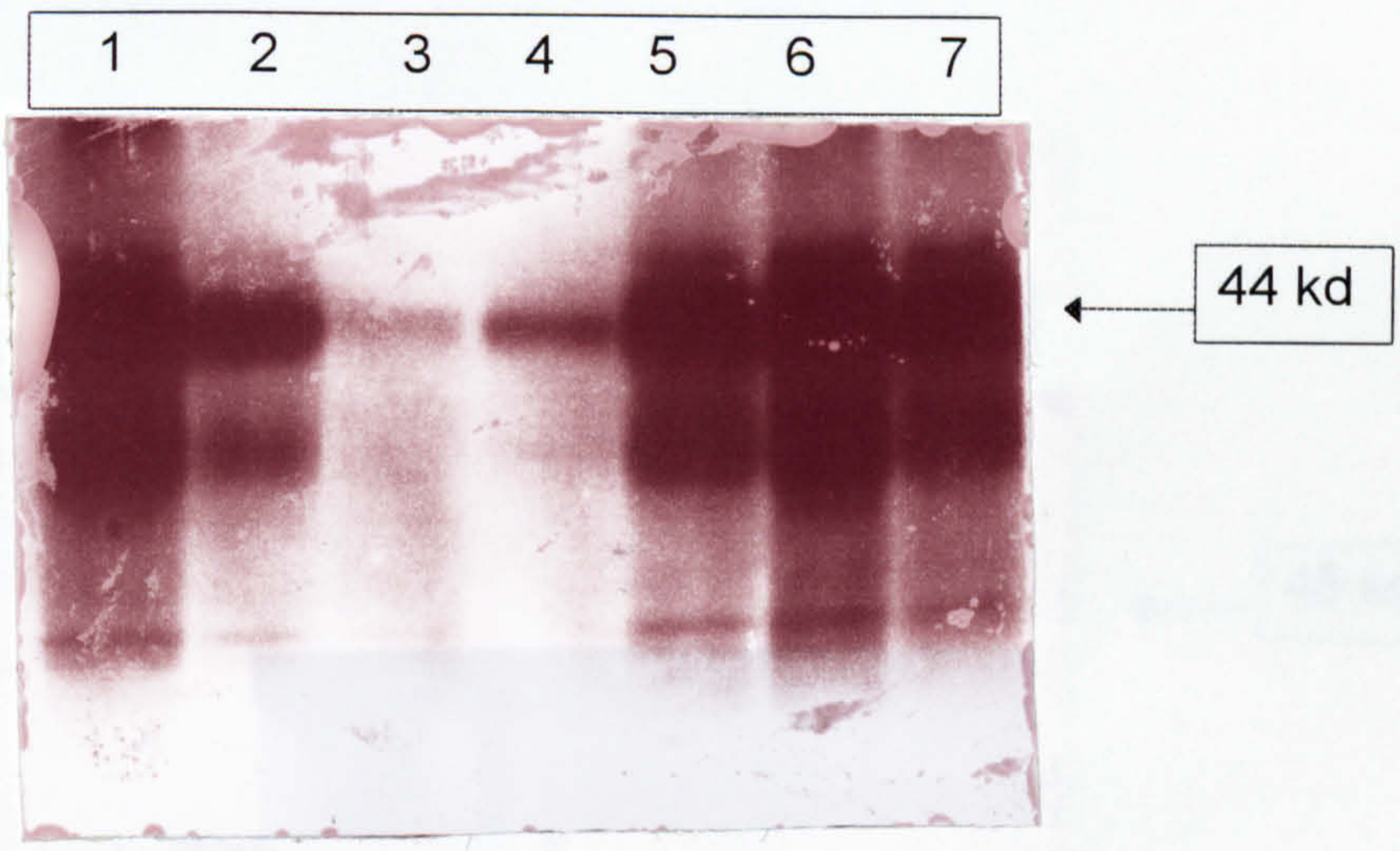


Figure 3.34 PTT analysis of fragment B of the *BRCA2* gene.
Transcription products separated in a 15% SDS polyacrylamide gel. The 44kd protein is indicated by an arrow. Lanes 1-7= Protein products from index cases.

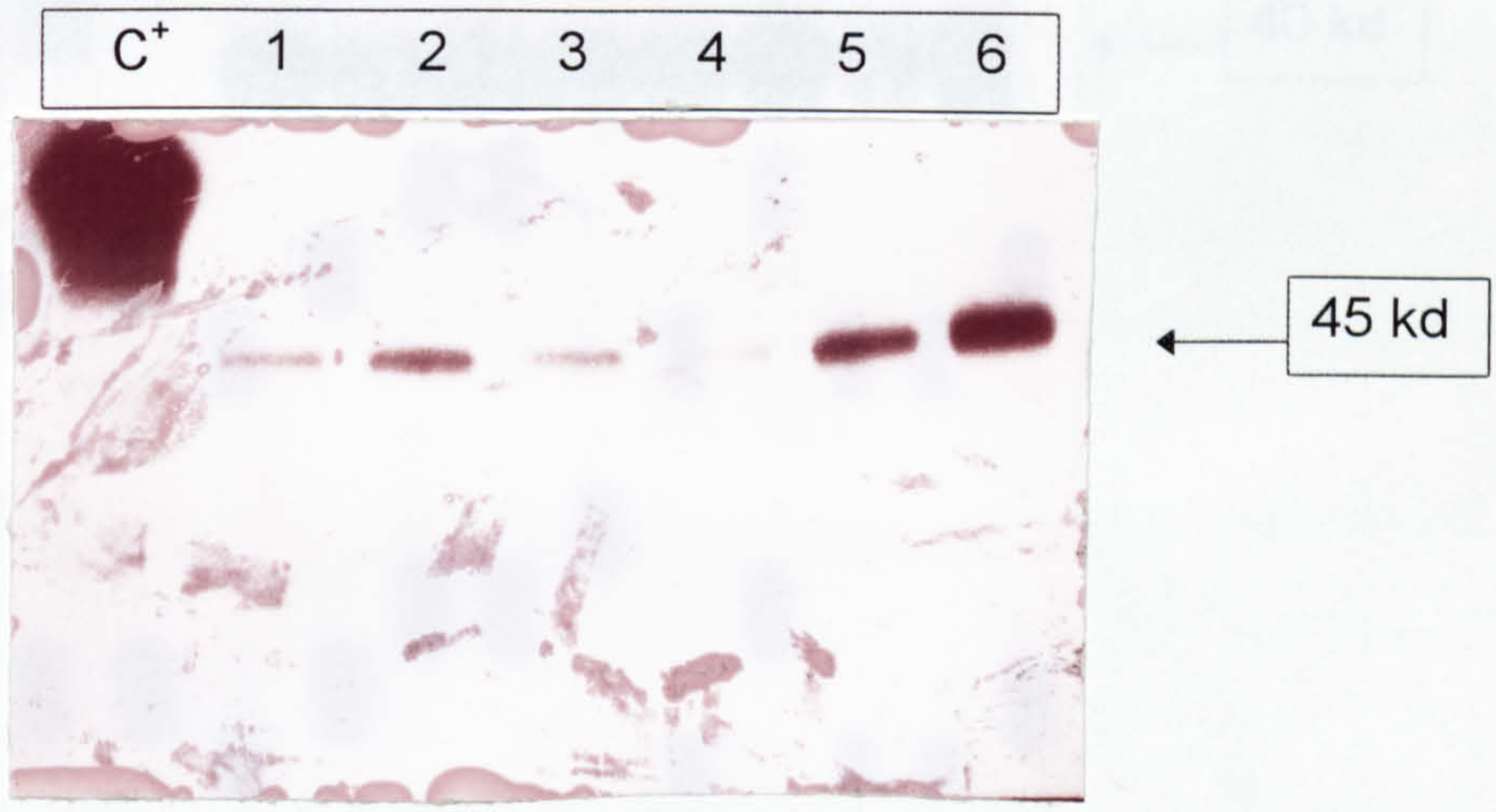


Figure 3.35 PTT analysis of fragment C of the *BRCA2* gene.
Transcription products separated in a 15% SDS polyacrylamide gel. The 45kd protein is indicated by an arrow. C+ = control positive using DNA supplied by manufacturer (Amersham). Lanes 1-6 = Protein products from index cases.

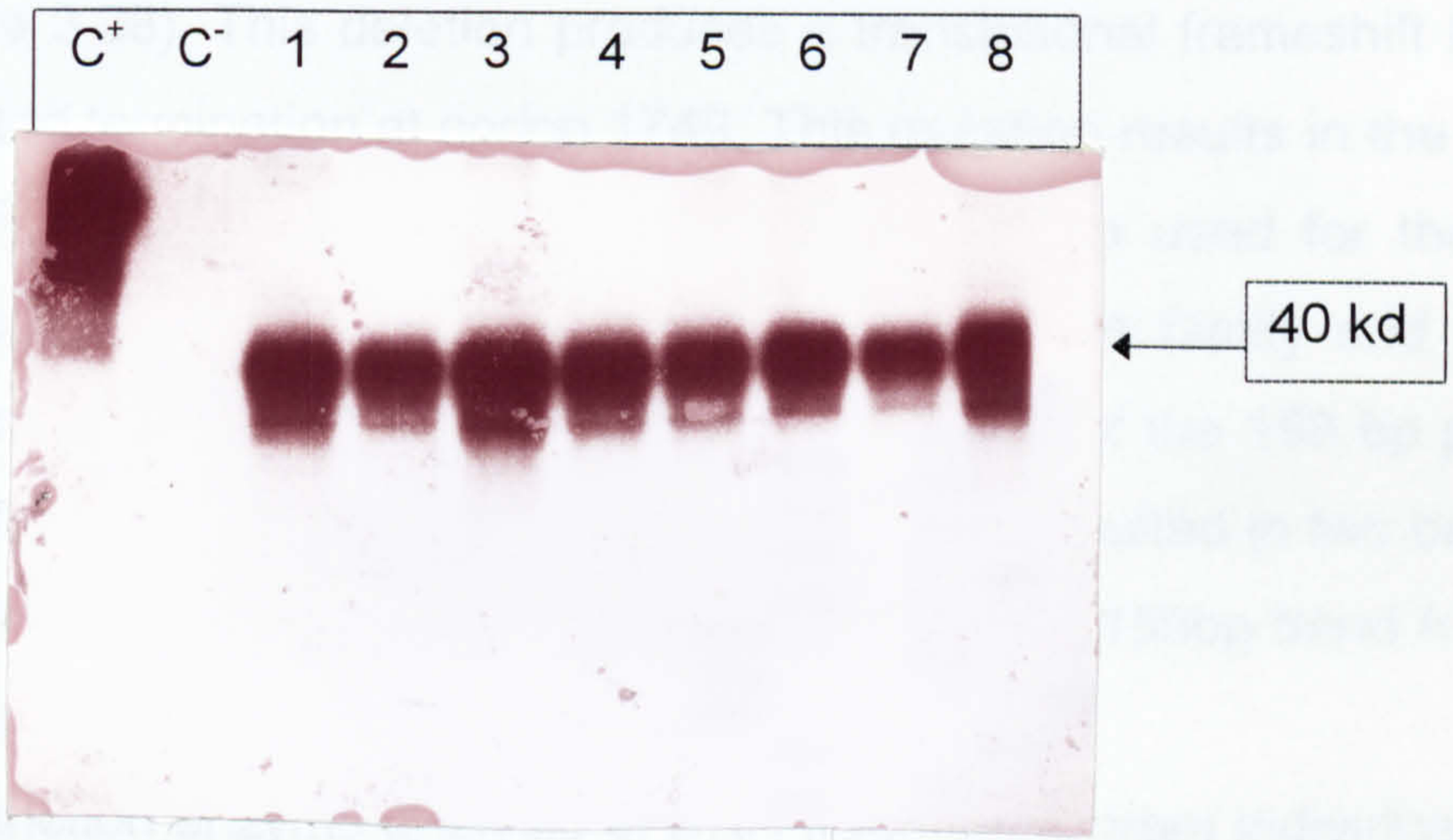


Figure 3.36 PTT analysis of fragment D of the *BRCA2* gene.
Transcription products separated in a 15% SDS polyacrylamide gel. The 40kd protein is indicated by an arrow. C+ = control positive using DNA supplied by manufacturer (Amersham). C^- = negative control. Lanes 1-8= Protein products from index cases.

3.10 Protein truncation test analysis of the *BRCA2 gene.*

PTT analysis of exon 11 of the *BRCA2* fragment C in the index cases of families 14, 18, and 33 (Appendixes 6-8) revealed truncated products at fragment C of exon 11 (figures 3.37 and 3.41) The truncated products from index cases of families 14 and 33 appeared to be of similar size suggesting that both families might contain identical mutations. Direct sequencing of the abnormality revealed a novel 7 bp deletion at nucleotide positions 5445-5451 (figure 3.38). This deletion produces a translational frameshift leading to a predicted termination at codon 1749. This mutation results in the loss of the restriction site for the enzyme *Mse*I which can be used for the rapid detection of this mutation in the other members of the family and also in other breast cancer patients (figure 3.39a). Digestion of the 159 bp product of fragment Q using primers QF and QR (Table 2.5) resulted in two bands of 90bp and 69bp from the normal allele, in addition to a 159bp band from the mutant allele (figure 3.39b).

Restriction enzyme analysis of DNA-fragment Q from individuals III.8, III.9, IV.1, and IV.2 (one affected and the other three normal) from family 14 (Appendix 6) revealed that this mutation is present in individuals III.8, III.9, IV.1, even though two of them are unaffected members of the family. This family has a family history OF prostate cancer. Unfortunately DNA from the affected male patient was not available. Further screening of 50 more patients revealed the presence of this mutation in family 31.

This mutation can also be easily detected by running the PCR product of fragment Q in a 3% metaphor agarose gel (flowgene) (figure 3.40).

Direct sequencing of the abnormality in individual III.1 from family 18 (Appendix 8) revealed an AA deletion at nucleotide positions 5573-5574 (figure 3.41b).This deletion produces a translational frameshift leading to a predicted termination at codon 1786.

SSCP analysis of exon 2 of the *BRCA2* gene from several patients revealed an additional single strand band in several patients. Direct sequence analysis revealed a G to A transitions at nucleotide position 203.

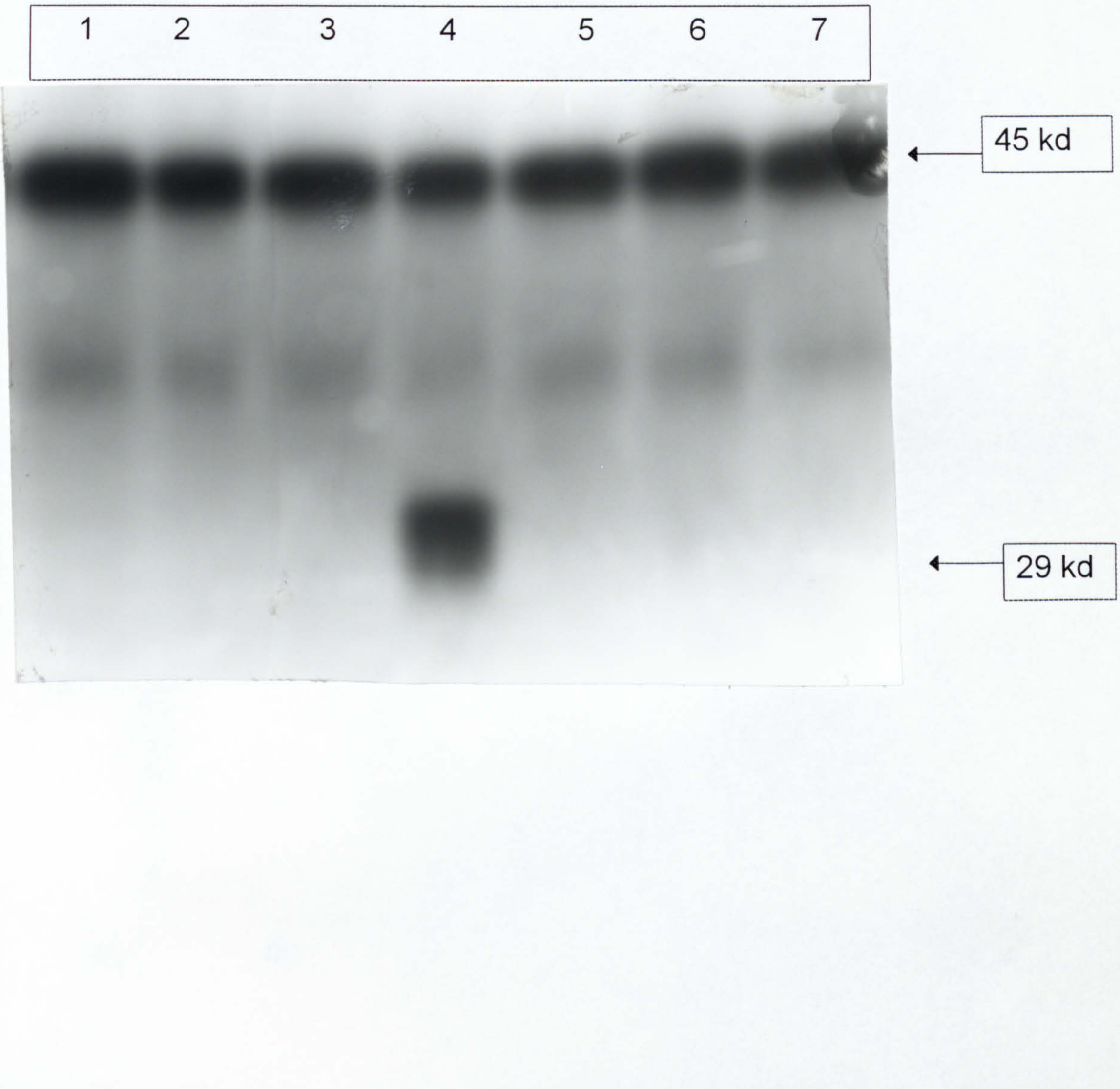
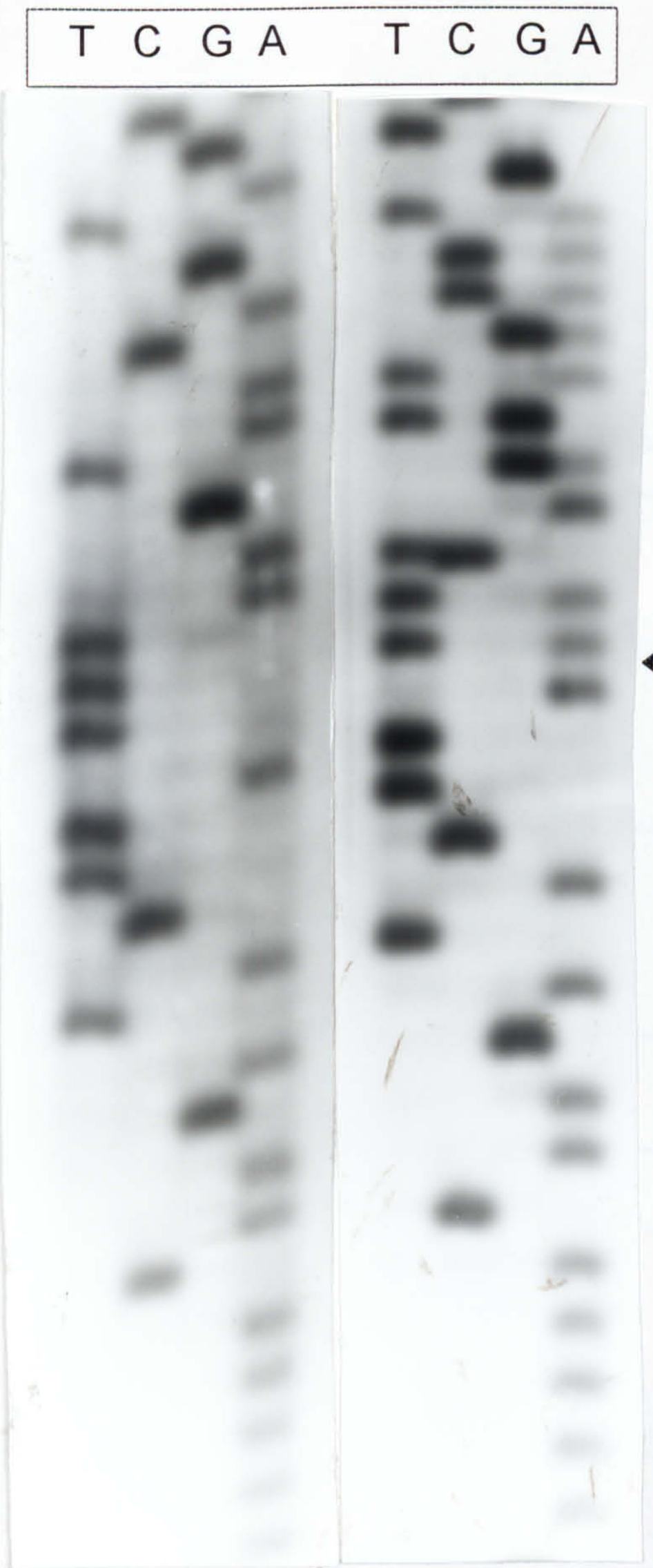


Figure 3.37 PTT analysis of fragment C of the *BRCA2* gene.

A truncated protein of 29 kd in addition to the normal 45 kd is shown in lane 4 (indicated by an arrows). Lanes 1-3 and 5-7 show a normal 45 kd protein product.



(a)

(b)

(c)

Figure 3.38 Partial sequence analysis of DNA fragment C showing the 5445del7bp mutation in the *BRCA2* gene.

Partial sequence of both sense and antisense strands showing a 7bp deletion at positions 5445-5451. A = normal allele (sense strand). B = mutant allele (sense strand). C = mutant allele (antisense strand).

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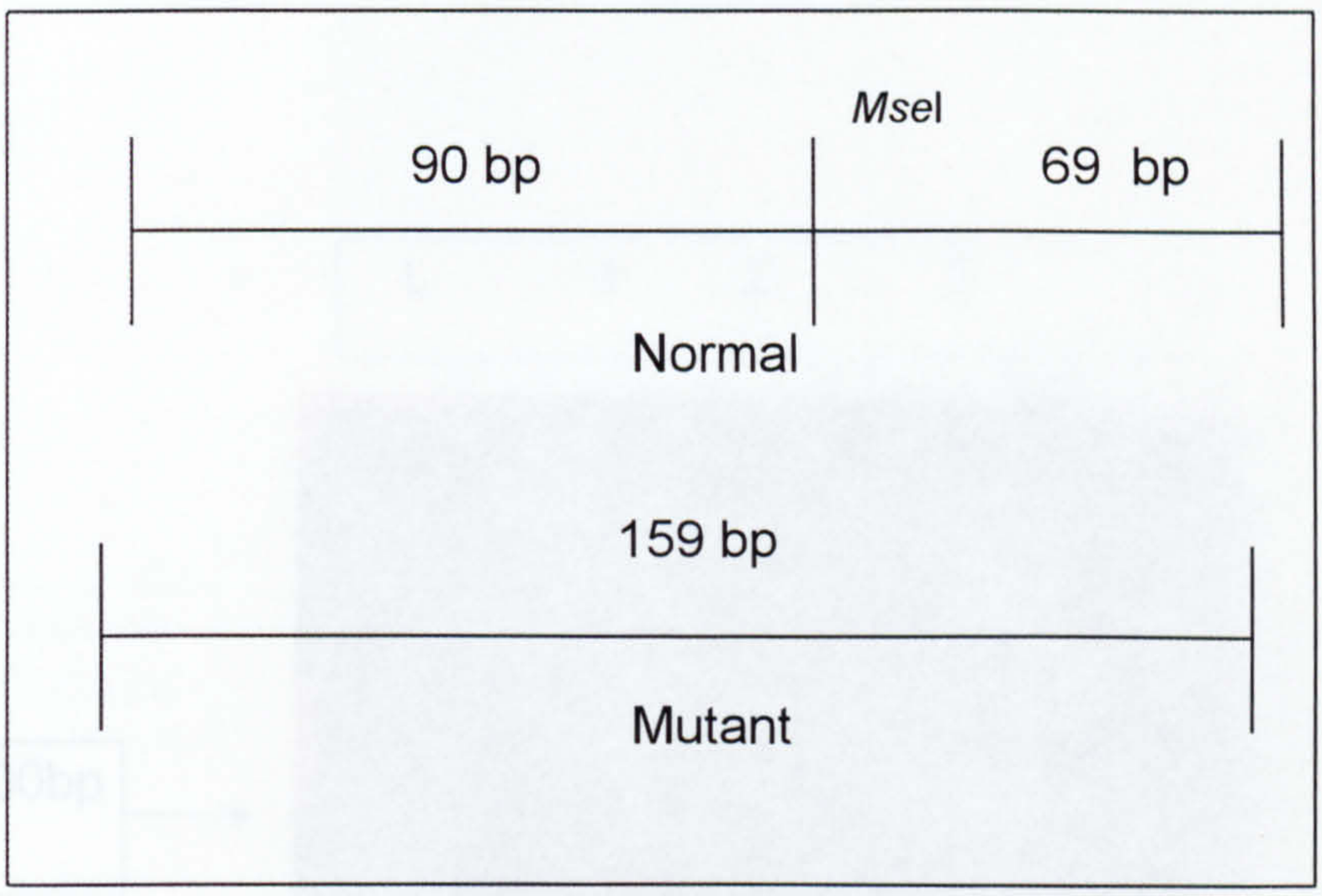


Figure 3.39 (a) map of Msel Restriction sites to detect 7 bp deletion at positions 5445-5451 .

Amplified DNA fragment Q (Table 2.5) with the expected sizes for the normal (90 and 69bp) and the mutant (159bp) alleles, upon digestion with *Msl*I.

(b) Restriction analysis of 7bp del$_{5445\text{-}551}$ with *Msel*.

Restriction enzyme digestion products in a 2% SeaKem (GTG) agarose gel (Flowgene) indicate the loss of a *Msel* restriction site due to the mutation in the index case of family No 14. L = 100bp ladder (Gibco BRL) with 100bp and 200bp bands indicated. Lane 1 = patient's undigested PCR products. Lane 2 = control digest. Lane 3 = patient's digest.

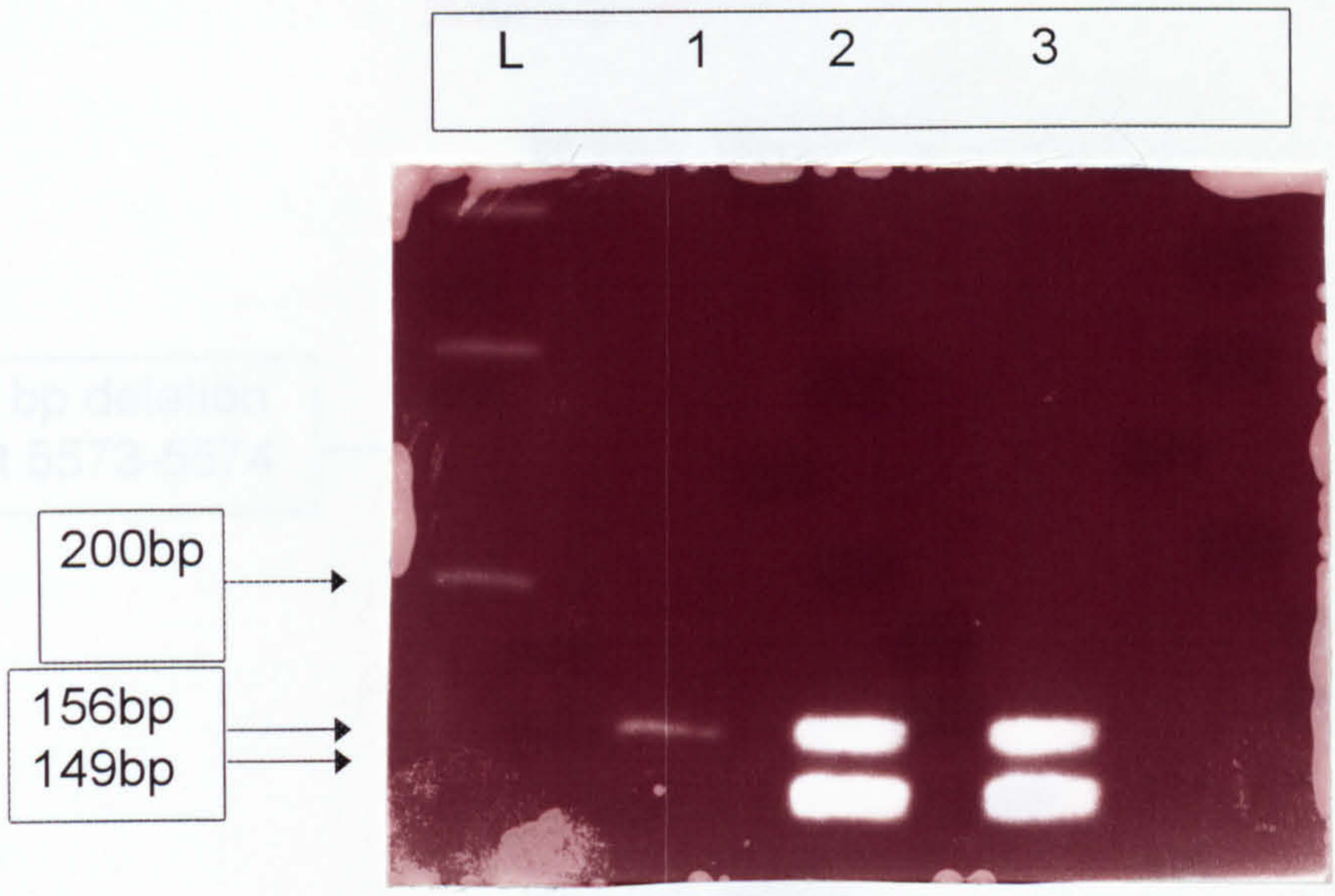


Figure 3.40 Agarose gel electrophoresis showing the PCR products of fragment Q in *BRCA2*.
Lanes 2 and 3 show the PCR products from carriers of the 7bp deletion at position 5445-5551. The normal and mutant alleles are indicated by arrows. lane 1= normal PCR product. L= 100bp ladder.

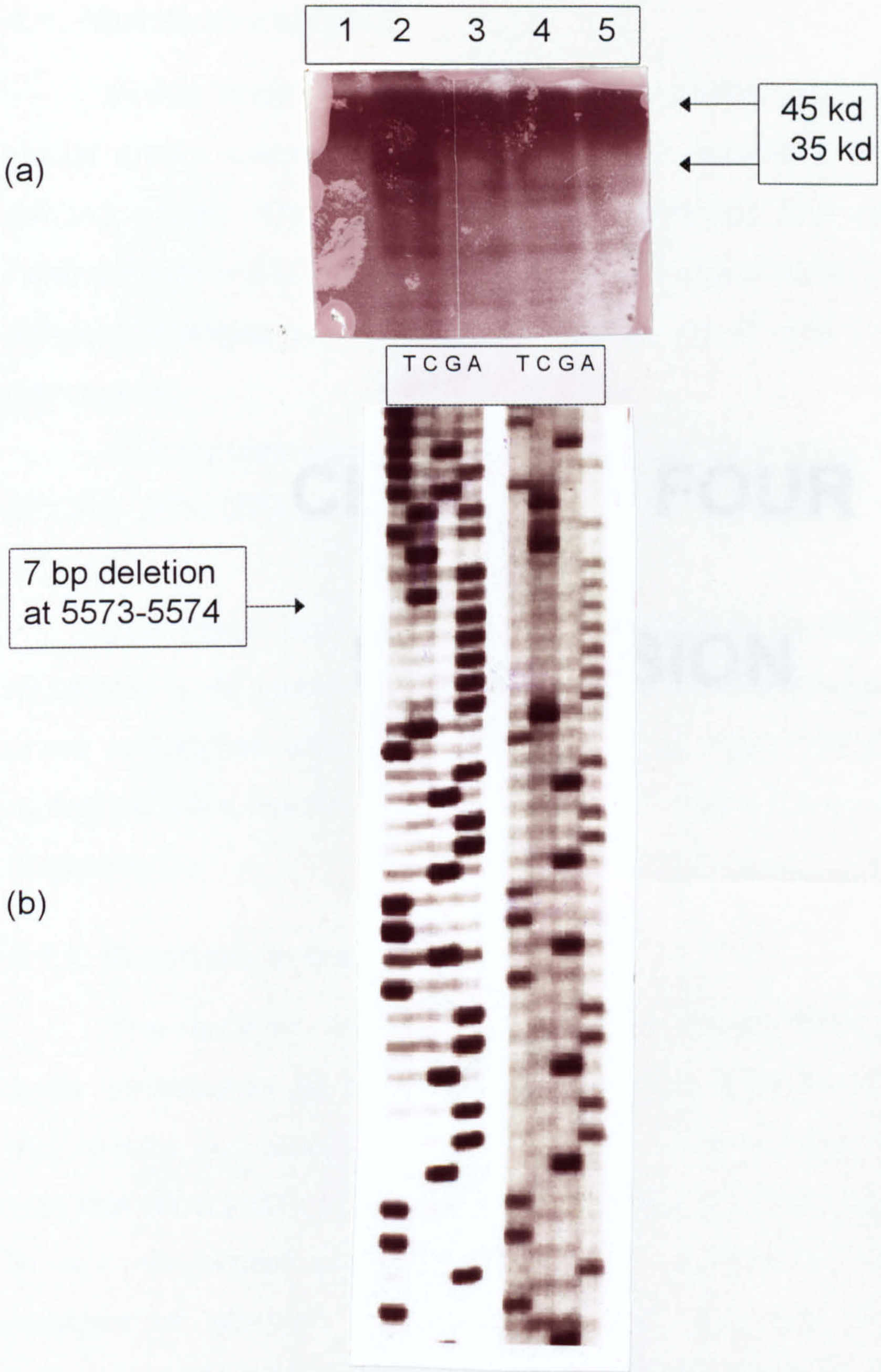


Figure 3.41 PTT and partial sequence analysis of fragment C Showing the 5573delAA in the BRCA2 gene.

(a) PTT analysis of fragment C in BRCA2 showing a truncated protein of 35kd in addition to the normal 45 kd products in lane 2 (indicated by arrows). Lanes 1 and 3-5 show the normal 45kd protein.

(b) Partial sequence analysis of DNA fragment C. Beyond the point marked by the arrow, the sequences of the normal and mutant alleles can be seen. N= normal sequence. M= mutant sequence.

CHAPTER FOUR

DISCUSSION

4.1. Mutation analysis

In this study we have screened 40 patients with a family history of breast and/or ovarian cancer for germ-line mutations in the *BRCA1* and *BRCA2* genes. The patients had 1-8 affected first or second degree relatives with onset between 23-70 years of age (Table 2.1a). A total of 16 different changes were detected; four are novel and five are potentially pathogenic.

Of 9 families with inherited pathogenic mutations in either *BRCA1* and *BRCA2*, 56% (5/9) were attributed to *BRCA1* and 44% were attributed to *BRCA2* .

The mean age of onset of breast cancer in families with *BRCA1* mutations is 40 years which is lower than the mean age of breast cancer onset in families with *BRCA2* (50 years of age). Those families whose mutations were not detected in these genes had a mean age of onset of 45 (Figure 4.1).

4.1.1. Mutations in the *BRCA1* gene

The mutation designated 2800delAA results from deletion of two of three consecutive As of the normal sequence GAAAC of codons 899 and 900 results in a stop codon at codon 901. This recurrent *BRCA1* mutation was first detected in an index case (individual III:1) from family 10 (Appendix 3) who developed breast cancer at the age of 32 years. Subsequent analysis of affected individuals II:2, II:3, II:4, and III:3 revealed that individuals II:2, II:3, and II:4 are carriers of the defective allele. Surprisingly, individual III:3 who developed bilateral breast cancer at the ages of 34 and 41 is not a carrier of the defective allele suggesting that she may either have the sporadic breast cancer or a mutation in another predisposing breast cancer gene which remains to be identified.

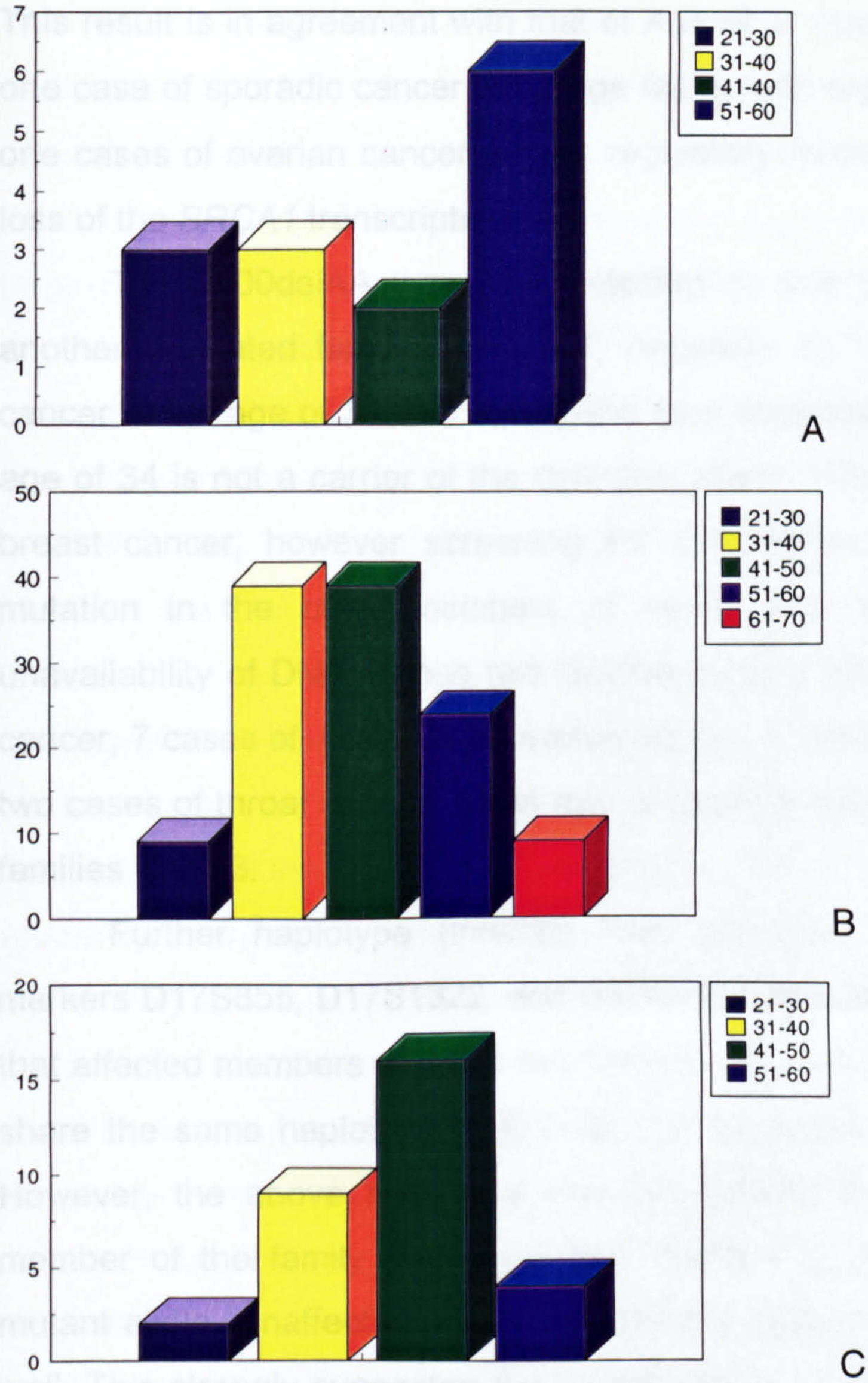


Figure 4.1 Distribution of age of onset of breast and/or ovarian cancer in families with and without mutations.

a: individuals from families with mutation in the *BRCA1* gene.

b: individuals from families with no mutations in the *BRCA1* and *BRCA2* genes.

c: individuals from families with mutations in the *BRCA2* gene.

This result is in agreement with that of Xue *et al.* (1997) who also reported one case of sporadic cancer in a large family with eight cases of breast and one cases of ovarian cancer with a regulatory mutation which leads to the loss of the *BRCA1* transcripts.

The 2800delAA was also detected in one other index case from another unrelated family (family 35, Appendix 4) who developed breast cancer at the age of 34. Her sister who also developed breast cancer at the age of 34 is not a carrier of the defective allele. This family has 7 cases of breast cancer, however screening for the presence or absence of this mutation in the other members of family was not possible due to unavailability of DNA. These two families have a total of 7 cases of breast cancer, 7 cases of breast and ovarian cancer, 1 case of ovarian cancer and two cases of throat cancer. Mean age of onset of breast cancer in these two families was 43.

Further haplotype (inferred from genotype) analysis of the four markers D17S855, D17S1322, and D17S1327 (Simard *et al.* 1994) revealed that affected members of these two families who are carrier for mutant allele share the same haplotype ,E E F M, for the above markers, respectively. However, the above haplotype was not present in any of the affected member of the family (individual III.3, family 10) who did not carry the mutant allele. Unaffected noncarriers did not share the above haplotype as well. This strongly suggested the cosegragation of the mutation with breast and ovarian cancer in these two families and also indicates a founder mutation rather than a de novo mutation.

This mutation initially was reported by Friedman *et al.* (1994) in one family from the USA with a family history of breast and ovarian cancer.

The 5382insC is the second frequent mutation in the *BRCA1* gene reported so far. This mutation was initially observed in northern and eastern European families (Simard *et al.* 1994; Friedman *et al.* 1995; Shattuck-Eidens *et al.* 1995). It has now been observed in Russian (Gayther *et al.*

1997), Hungarian (Ramus *et al.* 1997), and Ashkenazi Jewish (Tonin *et al.* 1996) families.

This mutation was detected in individual III.1 from family 34 (Appendix 5). This family is remarkable because eight cases of cancer appeared in a large sibship, including three cases of breast cancer, three cases of breast and ovarian cancer and one case of brain cancer. Another case of unknown type was detected. The mean age of developing breast cancer among affected members was 42. Further genotype analysis of the four markers D17S855, D17S1322, D17S1323 and D17S1327 (Simard *et al.* 1994) revealed that affected members of the family share the same haplotype (inferred from genotype) , D E F N , as the British family (Gayther *et al.* 1995) with the same mutation which, however, is different from the common haplotype reported for this mutation , D E F O, (Neuhausen *et al.* 1996) for the above markers respectively, suggesting that the 5382insC may have arisen independently in the two populations. However, the above haplotype differs only in the allele associated with the marker D17S1327. Since the both alleles are extremely rare and differ by only 2bp, it is likely that the mutation in the British families has the same ancestral origin as in the Canadian families. However some doubt remains because, the 5382insC occurs in sequences containing four Cs and therefore slippage during DNA replication may account for the independent mutational events in different ethnic groups.

The 5382insC is located in exon 20 of *BRCA1*, and is more commonly associated with a low risk of ovarian cancer. However our data is in agreement with the results obtained by Struewing *et al.* (1995) and Gayther *et al.* (1997) who found this mutation in families with a history of both breast and ovarian cancer in the United Kingdom and Russia, respectively. However, it may be that there is a real variation, between different populations, in the breast and /or ovarian cancer risks associated with the 5382insC mutation and these risks are modified by genetic background (Phelan *et al.* 1996a) or by environmental factors.

The mother of our index case (Individual II:5 from Appendix 5) who is a carrier of the defective allele has three affected sisters who seem to have inherited the defective allele from their mother who developed a brain tumour at the age of 60. Their father died for an unknown reason, suggesting that there may be some relation between the cause of brain and breast cancer tumours.

The novel skipping of exons 5-7 was identified in two unrelated breast cancer patients from families 7 and 8 who developed breast cancers at the ages of 23 and 40 respectively. RNA from the other members of the family was not available to study the presence of this exon skipping in the other member of these two families. No alteration of sequence in the splice site of exons 5, 6 and 7 was identified. However, it is possible that DNA sequences, located distally to the region examined, are involved in the exon skipping. Another possibility is genomic rearrangement which remains to be proven. A recent report of the first genomic rearrangement identified within the *BRCA1* locus in a breast and ovarian cancer family, and which result in the removal of exon 17, underscores this point (Puget *et al.* 1997). The absence of these three exons was not observed in the cDNA from 38 index cases from the other families with breast and/or ovarian cancer or in twenty healthy individuals. It can thus be suggested the absence of exons 5-7 in these two families appears to be the direct cause of a predisposition to breast cancer. This is similar to the mutation lacking exon 3 in the cDNA of carriers reported by Friedman *et al.* (1994) where no genomic changes were identified. This kind of exon skipping has also been reported in adenosine aminohydrolase (ADA) deficiency and medium-chain acyl-coA dehydrogenase (MCAD) deficiency (Akenson *et. al.* 1987, Kelly et al 1990). It seems likely that a mutation(s) within introns 4-7 cause the deletion of exons 5-7 or that a mutation in a factor modulating mRNA processing may be the cause.

The deletion of exons 5-7 does not alter the transcriptional reading frame but the protein produced may be rapidly degraded. These exons are

also involved with the RING zinc finger domain which is important for normal function of the protein.

Alternative Splicing Generates Variant *BRCA1* Transcripts

The existence of multiple mRNA species from a single genetic locus, arising from alternative splicing and encoding distinct yet structurally related proteins, is an increasingly appreciated phenomenon. Often, the different proteins that derive from the same gene as a result of alternative splicing are functionally related, but in some cases they exhibit distinctly counteracting biological activities. Recently described examples include the Wilms' tumour susceptibility gene *WT1* and the gene for interleukin 4 and hepatocyte growth factor (Wang *et al.* 1995; Boise *et al.* 1993).

We have detected different alternatively spliced *BRCA1* transcripts with deletions of exons 2 to 10. All of these variant transcripts maintain the *BRCA1* open reading frame, supporting the theory that an open reading frame (ORF) scrutiny may be present in the process of pre-mRNA splicing (Dietz and Kendzior, 1994).

Using RNA to screen for the *BRCA1* gene mutations, we identified that a previously reported germline mutation is in fact a common, alternatively spliced variant in the population (Serova *et al.* 1996). Using RT-PCR and sequencing, the exon 5 deletion variant was present in the cDNA from 38 index cases from the breast cancer and thirty healthy individuals. The exon 5 deletion transcript was only present in lymphocyte RNA, no abnormalities were detected in genomic DNA flanking exon 5. These findings highlight further that caution should be exercised in providing genetic risk assessment on the basis of currently used germline analysis. Awareness of the alternative splice forms characterised in this report should facilitate analysis of *BRCA1* expression and function.

We also found alternative splicing results in exons 9-10 and 2-10 skipping which was also reported by Miki *et al.* (1994) and recently by Xue *et al. (1997)*. Further genomic DNA analysis of exon 9 revealed that the

deletion of one base pair at position -54of the 3′ acceptor site, found in 50% of patients, may be the reason for this alternative splice site.

Alternative splice sites have previously been demonstrated to play a significant role in modulating the biological functions of a number of proteins. One such example are the leptin receptor isoforms, and the abnormal splicing of one of these variants encodes a protein missing the region specifying a cytoplasmic location and is likely to be defective in signal transduction (Lee *et al.* 1996). Another example is the *FMR1* gene which is responsible for the fragile X mental retardation syndrome. The *FMR1* gene encodes a cytoplasmic protein with RNA-binding properties. Its complex alternative splicing leads to the different localisation of the *FMR1* protein. The splice variants that exclude exon 14 were shown to be nuclear instead of cytoplasmic, suggesting the presence of a cytoplasmic retention domain, encoded in exon 14, and of a nuclear association domain encoded within the first eight exons. However these appear to lack a typical localisation signal. (Sittler *et al.* 1996). Another example is splicing of exons that encode part of the zinc finger domain alter the DNA binding properties of *WT1* (Bickmore *et al.* 1992). More interestingly, this alternative splicing within the zinc finger domain was recently shown to influence the localisation of *WT1* within the nucleus, i.e. either with splicing factors or with DNA in transcription factor domains, suggesting that alternative splicing can generate protein with distinct functions.

Although the subcellular localisation of *BRCA1* protein is at present controversial (Chen *et al.* 1995; Chen *et al.* 1996; Jensen *et al.* 1996; Scully *et al.* 1996) and its biological function remains unknown, Thakur *et al.* (1996), recently, in an *in vivo* assay, found that the *BRCA1* protein encoded by full length cDNA was primarily located in the nucleus, whereas *BRCA1* encoded by a construct lacking exon 11 was mainly localised in the cytoplasm. Examining the mRNA sequence at exon 11 reveals the presence of two potential nuclear localisation signals, the deletion of these may prevent the translocation of the newly synthesised *BRCA1* to the nucleus.

We therefore believe that different *BRCA1* transcripts generated by alternative splicing, encoding protein with different motifs, may play a significant role in modulating the subcellular localisation and therefore the physiological function of *BRCA1* protein.

Important functional roles for *BRCA1*-derived proteins encoded by exon 11-lacking transcripts have been suggested by recently reported observations with *BRCA1* knockout mice and *BRCA1*-linked human tumours(Hakem *et al.* 1996, Gowen *et al.* 1996). Two laboratories have reported embryonic lethality in homozygous *BRCA1* knockout mice; one group targeted a portion of exon 11, whereas the other disrupted exons 5 and 6. Interestingly, their finding was different with regard to the stage of embryonic development. Homozygous Brca1$^{5-6}$ mutant mice die before day 7.5 of embryogenesis. Mutant embryo is poorly developed, with no evidence of mesoderm formation. In vivo, mutant embryos do not exhibit increased apoptosis but shows reduced cell proliferation. Homozygous Brca1 which lack part of exon 11 die in utero between 10 and 13 days of gestation, abnormalities are most evident in the neural tube. The alternative splicing of exon 5 which is part of the ring finger domain generates some interesting questions about the normal function of the *BRCA1* gene.

BRCA1 polymorphisms

Seven different polymorphisms were detected in *BRCA1*; six in the coding sequence and one in intron 8, reported here for the first time. The one base deletion which is 54 bp downstream of the exon/intron boundary of exon 9 possibly produces an increase of aberrant splicing that results in alternative splicing of exons 9-10. In order to assess whether *BRCA1* variants have any effect on predisposition to breast and ovarian cancer we examined the frequency of five polymorphisms in breast cancer and in the normal population (Table 4.1) . However, our data suggests that for each of the polymorphisms there is no genotype difference between the breast cancer patients and the control group.

Haplotype analysis of mutations in the BRCA1 gene

Of the three pathogenic mutations reported in the *BRCA1* gene two of them were recurrent and one of them has been reported elsewhere as a common mutation . To assess whether these recurrent alterations in *BRCA1* occurred de-novo or are founder mutations, the genotype was determine using four microsatellite markers located within or adjacent to the *BRCA1* gene and found to be compatible with published haplotype.

Inferred haplotype results support evidence from a previous study that the *BRCA1* British families which carry the 5382insC mutation have a common ancestor, however they do not share exactly the same haplotype as the North American population. Inferred haplotype analysis revealed that patients with recurrent mutation 2800delAA share the same haplotype suggesting that this is also a founder mutation.

The allele frequencies at D17S855, D17S1322, D17S1323, and D17S1327 in the breast cancer patients were determined by typing the 40 breast cancer patients included in the study. The obtained allele frequency was almost comparable to those reported in the GDB at locus D17S1322, D17S1323 and D17S1327; however, it shows differences at locus D17S855 (Table 4.2). To make the accurate comparison we need to compare the allele frequencies of the normal group from the Scottish population.

BRCA1 nucleotide position	Mutation	Affect on coding sequence	Allele frequencies: in breast cancer patients (in normal population)
intron 8(-54bp)	deletion of A	non-coding	
nt 2201	T/C	Ser / Ser	0.73 / 0.27 (0.74 / 0.26)
nt 2430	T/C	Leu / Leu	0.74 / 0.26 (0.75 / 0.25)
nt 3232	A/G	Glu /Gly	0.71 / 0.29 (0.68 /0.32)
nt 3667	A/G	Lys / Arg	0.70 /0.30 (0.69 /0.31)
nt 4427	T/C	Ser /Ser	0.64 / 0.36 (0.73 / 0./27)
nt 4956	A /G	Ser / G ly	0.76 /0.24 (0.76 / 0.24)

Table 4.1: The polymophisms and their relative frequencies in breast cancer patients and normal controls found in this study. The number of breast cancer patients and normal control were 58 and 50 respectively.

Locus	D17S855			D17S1322			D17S1323			D17S1327		
probe												
Allele	Size (bp)	freq (%) #	freq (%) *	size (bp)	freq (%) #	freq (%) *	size (bp)	freq (%) #	freq (%)	size (bp)	freq (n)#	freq (%) *
A	158	-		134	4		161	2	4	173		
B	156	5	-	131	3	6	159	4	5	171		
C	154	19	19	128	11	12	157	19	29	169		5
D	152	18	4.5	125	25	22	155	3	7	167	(1)	
E	150	15	33	122	51	58	153	4	1	165	(2)	4
F	148	12	4.5	119	4	2	151	68	54	163	(1)	12
G	146	26	37	116	1		149	-		161	(5)	4
H	144	5	2				147	-		159		
I	142	-					139	-		157		
J										155		
K										153		
L										135		
M										133	(6)	73
N										131		2
O										129		

Frequency reported by Anderson *et al.* and GDB.

* Frequency of the allele in breast cancer patients who were screened in this study

(n) For the D17 S1327 polymorphisms, the numbers of particular size were based on 15 control chromosomes which were studied by Simard *et al.* (1994).

Table 4.2. Definitions of allele with the size and frequencies used for inferred haplotype analysis of *BRCA1* patients and their frequencies among the breast cancer patients who were screened in this study.

4.1.2. Mutations in the BRCA2 *gene*

The novel 7 bp deletion at position 5445 was initially identified in individuals III.6 and III.9 from pedigree 14 who developed breast cancer at the ages of 60 and 47, respectively. Subsequent analysis of individuals III.8 and IV.1 by *Mse1* digestion revealed that they are carriers of the defective allele but still have not developed cancer at the ages of 54 and 27, respectively. Taking this into account genetic counselling may help them to delay breast cancer or totally prevent it. These three sisters (individuals III.6, III.8, and III,9) seem to have got the defective allele from their grandfather who got bladder cancer at the age of 90. They also have one aunt who developed ovarian cancer at the age of 64 and three uncles who developed prostate and lung cancers in old age . Unfortunately, the other affected members of the family were not available for further study. However, the mutation in *BRCA2* in a patient with prostate cancer has been reported by Hilmi Ozcelik *et al.* (1997) in Ashkenazi Jewish pancreatic patients.

By employing a *Mse1* digestion for the presence of this mutation in the other families it was shown that two other Scottish families have this mutation. The presence of the 7bp deletion in three different unrelated families in Scottish population suggests that this mutation is a recurrent mutation in the Scottish population. It produces a translational frameshift leading to a predicted termination at codon 1749.

The *BRCA2* 5573del AA, previously reported by Gayther *et al.* (1997) in two families with breast and ovarian cancer family history was identified in an individual who developed breast cancer at the age of 56 from a family with the history of breast and ovarian cancer. However, the DNA from the other members of the family was not available for further study.

The G to T transition at nucleotide position 203 in the *BRCA2* gene which is situated at 5′ untranslated region (UTR) of *BRCA2* gene was also identified in 30% of breast cancer patients.

There is a case of ovarian cancer in one of the families in which *BRCA2* 5445del7bp was found. The mutation was found in a region which

was previously reported by Gayther *et al.* (1997) to contain a cluster of ovarian cancer mutations.

The mean age for developing breast cancer in the patients who are carriers for *BRCA2* mutation is higher than those who are carriers for *BRCA1* gene mutations..

The fact that early-onset cases generally appeared in the younger generations, suggests that the modern lifestyle may influence mutation penetrance; in agreement with the previous report (Wooster *et al.* 1994). Although the incidence of ovarian cancer in *BRCA2* families seems to be considerably lower than *BRCA1* families, two families who each have a mutation in *BRCA2* have one case of ovarian cancer also.

The occurrence of other malignancies in *BRCA2* families also has been noted elsewhere (Phelan *et al.* 1996c; Thorlacius *et al.* 1996; Wooster *et al.* 1994) and in the present study, included cases of pancreatic, gastric, and gallbladder cancers, malignant melanoma, leukaemia, and prostate and cervical carcinomas, as well as other tumour types.

4.2. Spectrum of mutations in the *BRCA1* gene

So far, a total of 245 different mutations have been reported in the *BRCA1* gene in 813 different families, of which 157 (64%) were unique and 26 (14.5%) were reported only twice. These were entered into a database established by the breast cancer information core (Bic, http://WWW.nghri.nih.gov/intramural_lab/bic). A total of 780 (96%) of all mutations are small insertions, deletions, nonsense point mutations, splice variants, and regulatory mutations that result in truncation or absence of the *BRCA1* protein (figure 4.2). A total of 15 disease-associated missense mutations (6 unique) and 70 variants (36 unique) as yet unclassified as missense mutations or polymorphisms had been described (Bic). Forty-five independent benign polymorphisms have been described (Couch, F. J. 1996). The most common mutations were 185delAG and 5382 insC, which account for 151 and 73, respectively, of all mutations reported so far in 813

families. The mutations are spread over the entire length of the coding sequence and no hot-spots have been reported (Figure 4.3). The only possible evidence of clustering is in the zinc finger motif. There is no significant evidence between the position of the mutations within the gene and the ratio of breast to ovarian cancer incidence in the family. However, there is some data which suggests a transition in risk, such that mutations in the 3′ third of the gene are associated with a lower proportion of ovarian cancer. However mutations in the 3′ portion of *BRCA1* are less likely to lead to ovarian cancer than in the 5′ portion of the gene. However phenotypically severe mutations have been found in the extreme 5′ end of *BRCA1* and in the 3′ portion of the gene.

4.3. Spectrum of mutations in the *BRCA2* gene

A total of 102 different *BRCA2* mutations have been reported in the *BRCA2* gene in 160 different families, 87 (85.3%) of which were unique. These were entered into a database established by the breast cancer information core (Bic). A total of 71% of all mutations are small insertions, 14% deletions, 7% nonsense point mutations and 5% splice variants and regulatory mutations that result in truncation or absence of the *BRCA2* protein (figure 4.2). A large number of missense mutations or silent nucleotide substitutions also have been reported, of which none has been linked clearly to disease inheritance. The significance of these could be tested when a functional analysis is available.

The reported disease-associated mutations are distributed over the entire length of the coding sequence of the *BRCA2* gene and no hot-spot have been reported (figure 4.4). However, Mazoyer, S. et al. (1997) reported a polymorphic stop codon (Lys3326ter) that result in loss of the final 93 amino acids, including the putative granin domain. 58% of the reported mutations occurring in the large exons 10 and 11, comprise 60% of all the coding sequence of *BRCA2* gene. The most common mutations were

995del5bp and 6503delTT, which account for 14, 11 and 9 mutations, respectively, of all mutations reported so far in 160 families.

Frequencies of *BRCA1* and *BRCA2* mutations in high-risk families from all populations represented thus far in the literature are indicated in Table 4.3. As shown in Table 4.3 the proportion of high-risk families with breast or ovarian cancer attributable to *BRCA1* or *BRCA2* varies widely among populations. *BRCA1* mutations are by far the most common in Russia (occurring in 79% of breast and ovarian cancer patients), where most mutation carrying families have one of the two common alleles 5382insC or 4153delA. *BRCA1* mutations are the second next highest in Israel (occurring in 47% of high- risk families) and in Italy (29% of families). Between 20-25% of high risk families in Britain, Scandinavia, France, and Hungary have *BRCA1* mutations, and in each region, *BRCA1* mutations are subsequently more common than *BRCA2* mutations. Inherited *BRCA1* mutations explain <20% of high risk families in Holland, Belgium, Germany, Norway, and in Japan. The United states and Canada where high risk families are exclusively migrants, are intermediate.

In all regions other than Iceland, the frequency of *BRCA1* mutations is 1.5-2.0 fold higher than the frequency of *BRCA2* mutations. *BRCA2* mutations are more frequent than *BRCA1* mutations only in Iceland, which is unique in that one mutation explains almost virtually all inherited breast and ovarian cancer (Thorlacius *et al.* 1996).

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). The combined data from USA studies suggest that *BRCA2* is responsible for 19% of familial male breast cancer (Couch *et al.* 1996; Friedman *et al.* 1997) but for a considerably lower fraction of male breast cancer in the general population (Couch *et al.* 1996; Friedman *et al.* 1997).

Ovarian cancer occurred only in the two families with mutations in *BRCA2* "Ovarian cancer clustering region" (Gayther *et al.* 1997).

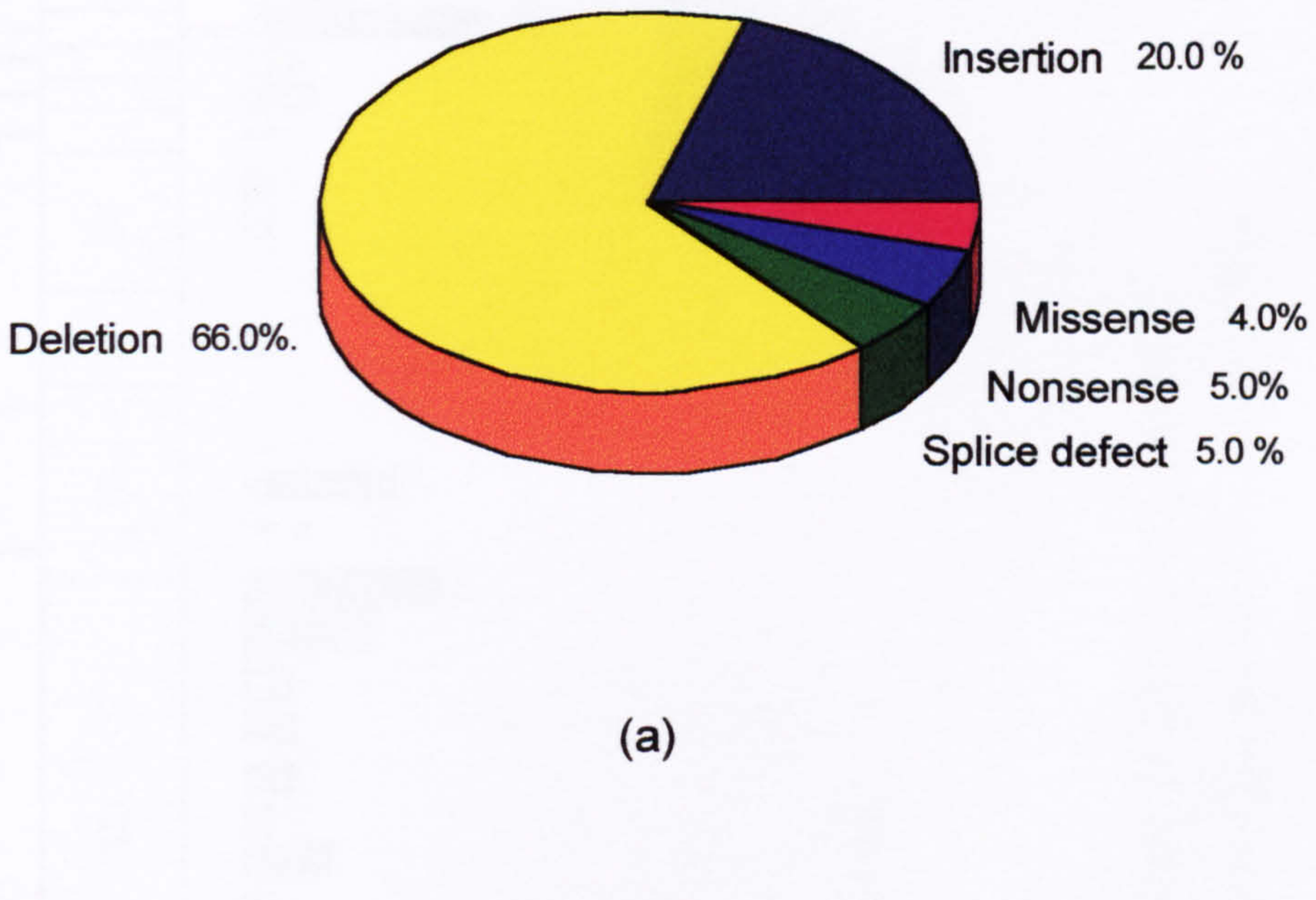
The lower prevalence of *BRCA2* mutations in families and patients could be due to fewer mutations, to lower penetrance, and/or to later age at onset of *BRCA2* breast cancer. Survival analysis adjusted for multiple ascertainment of high-risk families indicates that the lifetime risk of breast cancer associated with *BRCA1* and *BRCA2* are approximately equal but that the age of onset is later among *BRCA2*-mutation carriers (Schubert *et al.* 1997). The difference in *BRCA1*- versus *BRCA2*- mutation frequency probably represents a true difference in the relative contribution of these two genes to the hereditary breast/ovarian cancer burden, rather than ascertainment bias (Friedman *et al.* 1994; Friedman *et al.* 1995; Serova *et al.* 1997; Schubert *et al.* 1997).

4.4. Recurring mutations

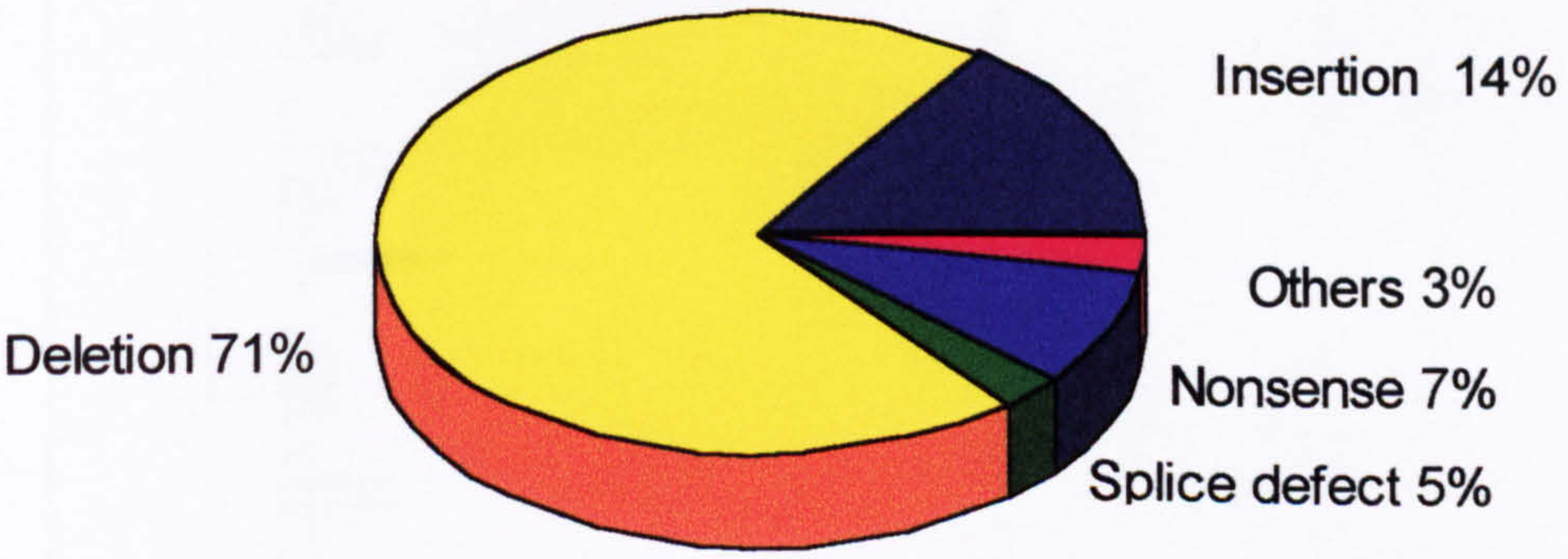
There are several recurring mutations within *BRCA1* and *BRCA2* genes. Some of them are common in different populations but some are limited just to specific populations. For example the most common allele in Russia, 5382insC, is also the most common among Europeans as a whole and has migrated far from the Baltic area where it probably originated. In contrast, the second most common allele in Russia, 4153delA, has not yet been observed outside Russia (Gayther *et al.* 1997).

Two recurrent mutations, *BRCA1* 185delAG and *BRCA2* 6174del T, among Ashkenazi Jews appear in the general population with about 1% frequency each; and account for 147 (48%) of 310 Ashkenazi high-risk breast and/or ovarian cancer families. A third mutation, *BRCA1* 5382insC, also occurs at a population frequency of 0.11% (Roe *et al.* 1996; Tonin et al 1996). Although the significant proportion of breast and ovarian cancer in Ashkenazi Jews is attributed to these three recurring mutations (Abeliovich *et al.* 1997), 163 (52%) of 310 Ashkenazi high-risk breast and/or ovarian cancer families do not carry these (Tonin *et al.* 1996; Abeliovich *et al.* 1997; Levy-Lahad *et al.* 1997). Whether these families carry other novel *BRCA1 or BRCA2* alleles or have mutations in other, as-yet-unidentified susceptibility

loci, or are at high risk for nongenetic reasons, remains to be determined. The proportion of ancient mutations in *BRCA1* and *BRCA2* which have been identified in multiple families varies widely among populations, ranging from non repeated mutations in Italy to nearly all hereditary breast and ovarian cancers being attributed to one or a few mutations in Iceland and Israel. These differences represent historical influences of migration, population structure, and geographical or cultural isolation.



(a)



(b)

Figure 4.2: Percentage of occurrence of different types of mutations in the *BRCA1* and *BRCA2* genes.
a: The pie chart shows the frequency of the different types of mutations encountered in the *BRCA1* gene. Deletions form the majority of mutations reported so far in the *BRCA1* gene.
b: The pie chart shows the frequency of the different types of mutations in the *BRCA2* gene. Deletions form the majority of mutations reported so far in the *BRCA2* gene. A large number of missense mutations or silent nucleotide substitutions also have been reported , of which none has been linked clearly to disease inheritance.

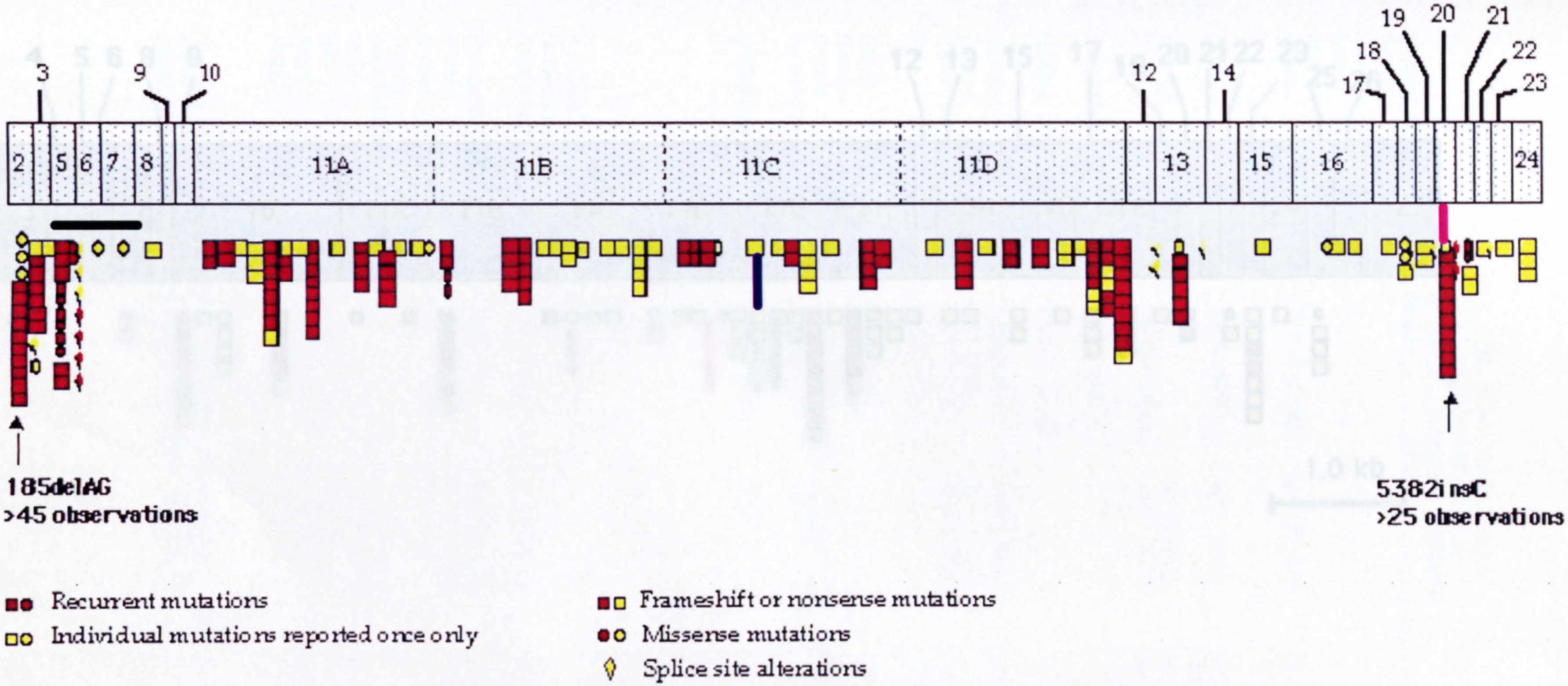


Figure 4.3 Distribution of published mutations in the *BRCA1* gene (http:www.nghri.gov/intramural_lab/bic).

: Exon 5-7 skipping (Found in two families in this study). : 2800delAA (Found in two families in this study).

: 5382insC (Found in one family in this study).

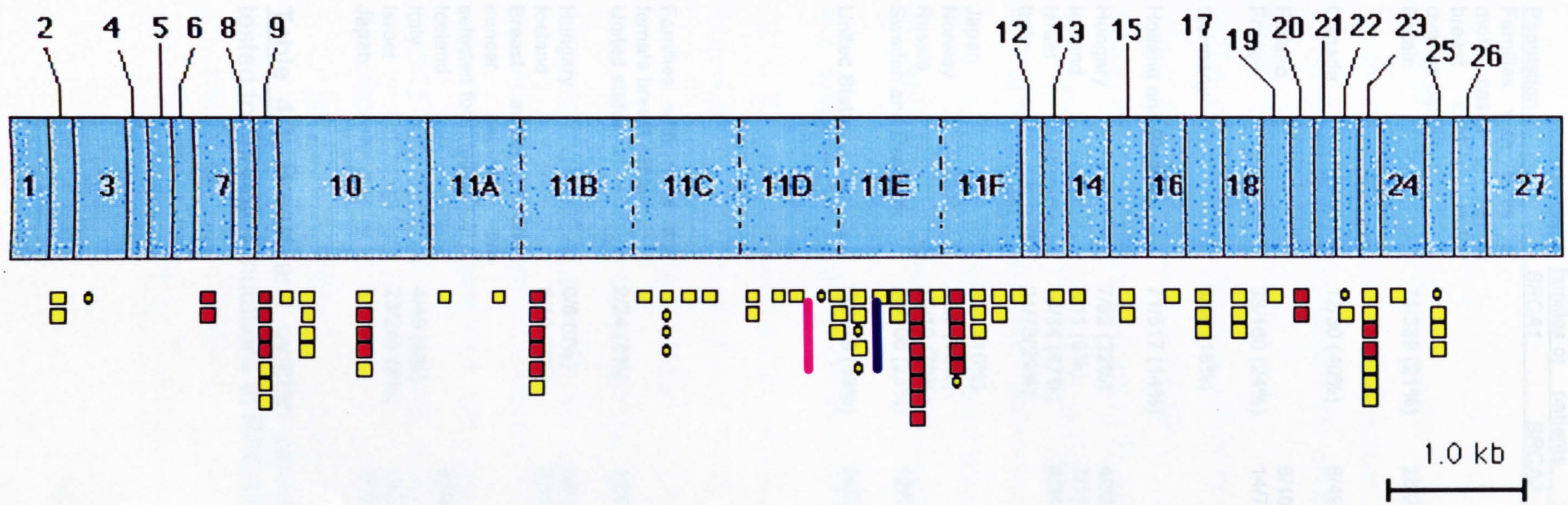


Figure 4.4 Distribution of published mutations in the *BRCA2* gene (http:// www.nghri.gov/intramural_lab/bic) (definition for each kind of mutation is the same as figure 4.3).

: 5445del7bp (Found in three families in this study). : 5573delAA (found in one family in this study).

Population	No. of families or patients with mutations/No. of families or patients screened *BRCA1*	*BRCA2*	Reference(s)
Families with three or more cases of female breast cancer and/or ovarian cancer			
Britain	71/339 (21%)	25/290 (9%)	Xu *et al.* 1997; Gayther *et al.* 1996; Gayther *et al.* 1997)
Canada	12/30 (40%)	8/49 (16%)	(Simard *et al.* 1994b; Phelan *et al.* 1996c)
Finland		8/100 (8%)	(Vehmanen *et al.* 1997)
France	38/160 (24%)	14/77 (18%)	(Serova-Sinilnikova *et al.* 1997); (Stoppa-Lyonnet *et al.* 1997)
Germany	9/49 (18%)		(Jandrig *et al.* 1996); (Hammann *et al.* 1997)
Holland and Belgium	71/517 (14%)		(Hogervorest *et al.* 1995) , (Peelen *et al.* 1997)
Hungary	7/32 (22%)	4/32 (13%)	(Ramus *et al.* 1997)
Iceland	1/11 (9%)	7/11 (64%)	(Thorlacius *et al.* 1996)
Israel	16/34 (47%)	8/34 (24%)	(Levy-Lahad *et al.* 1997)
Italy	21/73(29%)		(Caligo *et al.* 1997),(Montagna *et al.* 1996).(De Benedetti *et al.* 1996)
Japan	2/20 (10%)		(Inoue *et al.* 1995)
Norway	3/25 (12%)		(Andersen *et al.* 1996)
Russia	15/19 (79%)		(Gayther *et al.* 1997)
Sweden and Denmark	24/106 (23%)	12/106 (11%)	(Johannson *et al.* 1996); (Hakansson *et al.* 1997)
United States	69/179 (39%)	24/94 (25%)	(Castilla *et al.* 1994); (Friedman *et al.* 1994; Friedman *et al.* 1995); (Struewing *et al.* 1995); (Arena *et al.* 1996); (Serova *et al.* 1996; Serova *et al.* 1997); (Couch *et al.* 1996); (Tavtigian *et al.* 1996);(Gao *et al.* 1997); (Schubert *et al.* 1997)
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)
Hungary	0/6 (0%)	2/6 (33%)	(Ramus *et al.* 1997)
Iceland	0/10 (0%)	9/10 (90%)	(Thorlacius *et al.* 1997)
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 at.*1996)
Israel	23/243 (9%)	14/243 (6%)	(Abeliovich *et al.* 1997)
Japan	8/179	2/103	(Matsushima *et al.* 1995); (Katagiri *et al.* 1996); (Miki *et al.* 1996)

Table 4.3: Breast and ovarian cancer families from various populations tested for inherited mutations in *BRCA1* and *BRCA2* .

4.5 Difficulties of mutation analysis in the *BRCA1* and *BRCA2* genes.

Mutation analysis in *BRCA1* and *BRCA2* is complicated due to:

1-Large size of the genes; The *BRCA1* and *BRCA2* extend to 100 kb and 70 kb on chromosomes 17 and 13 respectively. *BRCA1* produces transcripts of 5.7 kb and *BRCA2* transcripts of almost 12 kb. They also have 24 and 27 exons respectively.

2-The presence of different splice sites at the 5′ and 3′ ends of *BRCA1* makes it difficult to work on mRNA.

3-Most of the mutations reported in both genes are deletions or insertions, this may sometimes complicate the analysis of complex sequences.

4- The presence of a large number of polymorphisms; During the process of mutation screening the polymorphisms may give us a false positive result whose confirmation by sequencing is time consuming.

4.6. Diagnostic and clinical relevance.

The method selected for mutation analysis is highly influenced by the mutational spectrum within the gene. *BRCA1* and *BRCA2* mutations are evenly spread across the entire coding region. As a result, high sensitivity will be attained only by screening the entire coding region of 5,592bp and 11,000 bp of *BRCA1* and *BRCA2*, respectively. The size of the *BRCA* genes, the wide array of mutations already described, and the presence of multiple polymorphisms suggest that the detection of carriers of mutations in the *BRCA1* and *BRCA2* genes will continue to be a problem for diagnostic laboratories.

BRCA1 and *BRCA2* mutations have not been identified in several families with good evidence of linkage to *BRCA1* and *BRCA2* genes (Castilla *et al.* 1994; Friedman *et al.* 1994; Simard *et al.* 1994; Miki *et al.* 1994: Xu *et al.*, 1997). The entire coding region of the *BRCA1* gene in

samples from these families has been screened by either direct sequencing or SSCP, suggesting that the mutations may be present in introns or other regulatory regions of the gene. The frequency of these presumed regulatory mutations which are located in regions not being currently analysed will affect the detection efficiencies of all diagnostic techniques. Furthermore, as shown in our study, mutations may be missed if a sporadic case of cancer in a family is mistakenly tested for the presence of a mutation. When possible, every effort should be made to avoid screening a sporadic case, perhaps necessitating screening two affected individuals from each family. Regardless, it is important to note that failure to identify a mutation in an at risk individual is a result which provides no clinically significant information. Current mutation detection techniques, are not 100% reliable. In addition, testing at the DNA level may miss regulatory and splicing mutations, and finally, other breast cancer susceptibility genes may be involved. Since most of the mutations described thus far are frameshift or nonsense mutations, a protein assay may be an effective screening test.

Many mutation detection techniques are currently being used to identify *BRCA1* and *BRCA2* genes. A diagnosis of increased susceptibility to breast and ovarian cancer may be considered upon identification of a frameshift or nonsense mutation in the *BRCA1* and *BRCA2* coding sequences of patients DNA samples. Approximately 90% of all women with such BRCA mutations will suffer breast cancer during their life time. In other words, an individual with a *BRCA* mutation has a 90% chance of developing cancer. However, the identification of most missense mutations is associated with a level of uncertainly, which makes clinical use of this information problematic. Unfortunately, there will be ambiguity concerning *BRCA1* and *BRCA2* missense mutations until a functional assay can be developed. However, some mutations may be present at high frequency in a particular population as a result of founder effects. Two such examples are the *BRCA1* 185AGdel and *BRCA2* 6174Tdel which have been found almost exclusively in breast and/or ovarian cancer families of Ashkenazi Jewish origin who

the same haplotype (Simard *et al.*, 1994: Struewing *et al.*, 1995: Neuhausen *et al.*, 1996).

4.7. Conclusions and future work

The strategy of using DNA as a template for PCR amplification and use of PTT and SSCP as the main screening methods, has proved to be a useful screening strategy for detection of mutations within the *BRCA1* and *BRCA2* genes. The initial strategy for amplification of the *BRCA1* gene was using both mRNA and DNA as template for PCR amplification. The advantage of using mRNA as a template is that it allows the amplification of large areas of the coding sequence of the *BRCA1* gene. However, due to the presence of alternative splice sites within the *BRCA1* mRNA, extra bands appear in the gel after electrophoresis, which made the interpretation of results difficult.

The screening methods used in this study included chemical cleavage mismatch analysis (CCM), single strand conformational analysis (SSCP), and the protein truncation test (PTT). Initially CCM analysis was used to screen the *BRCA1* gene using either DNA or RNA as template for PCR. Although CCM is able to screen relatively large fragments with an efficiency of up to 95%, the presence of many polymorphisms within the *BRCA1* gene makes PTT a more effective method. By taking the above into account and considering that most of the mutations within the *BRCA1* and *BRCA2* genes result in a stop codon, PTT was applied to screen for the mutations in exon 11 of the *BRCA2* gene and exon 11 of the *BRCA1* gene. SSCP was used to screen the rest of the *BRCA1* and *BRCA2* exons. Although some regulatory, missence mutations and larg deletions may be missed, these techniques allow the detection of ~95% of point mutations, small deletins and insertions within the *BRCA1* and *BRCA2* genes.

In summary, the present study of 40 breast cancer families from the West of Scotland provides evidence for the involvement of the *BRCA1* and *BRCA2* genes in approximately 22.5% of the cases. The frequency is

comparable with the previous report from the UK (25%). However, it is low compared to the reported frequencies from other populations in the USA, Israel and Russia. A recent study of 263 women with breast cancer by Couch et al. (1997) revealed that the presence of *BRCA1* mutations only in 16% of women with a family history of breast cancer and only 7% of women from family with a history of breast cancer not ovarian cancer had *BRCA1* mutation.

Our inferred haplotype results support evidence from a previous study that *BRCA1* British families which carry the mutation 5382insC have a common ancestor; however, they do not share exactly the same haplotype as the North American population. We also found that the recurrent mutation, 2800delAA, shares the same haplotype suggesting that this is a founder mutation rather than occurring de novo.

Only one of the two putative hot-spot mutations, 5382insC, which constitute ~30% of all mutations reported previously, was reported once in our study and not more than six times in other reports within the UK. This may reflect the difference in the geographical origins of our families. 185delAG and 5382insC are known founder mutations in Ashkenazi Jewish and are therefore relatively uncommon in the United Kingdom.

Considering all of the published mutations so far, the frequency in *BRCA1* is 1.5-2.0 times higher than in *BRCA2* in most areas other than Iceland. The percentage of *BRCA1* and *BRCA2* mutations found in this study are almost the same (56% for *BRCA1* and 44% for *BRCA2*) This may reflect the difference in the geographical origins of our families. The reasons for low mutation frequencies in the Scottish population may be that:

1) Some mutated cases may have escaped detection, because of technical insufficiencies of the method used or the presence of mutations in uncharacterised regions of the genes.

2) Mutant *BRCA1* and *BRCA2* alleles bearing duplications, translocation or large deletions which affect the target sequence for the PCR primers are

unlikely to have been detected using our PCR based analysis and we did not examine the regulatory regions of the gene.

3)Testing of the possible sporadic cases in predisposed families also may give rise to false-negative results, and a chance occurrence of familial breast cancer aggregations will result in dilution of material. We were only able to analyse a single individual from each family, and it is possible that some of these may by chance have been phenocopies.

4) Linkage analysis of the families in the present study with chromosomes 17q21-12 and 13q12-13 markers might have excluded the *BRCA1* and *BRCA2* genes in some of them. Unfortunately, obtaining blood samples from the relatives of our patients, to permit such an analysis, was found to be difficult.

5) Finally, it is likely that at least one more major gene for inherited breast cancer remains to be found. In reported studies ~ 30% of high- risk families have no detected mutations in either *BRCA1* or *BRCA2*. These include 3 of 4 Hungarian families with at least six cases of breast or ovarian cancer (Ramus *et al.* 1997); 2 of 6 male breast cancer families and 15 of 23 female breast cancer families in the series of midwestern American families analysed by the international agency for research on cancer (Serova *et al.* 1997); 4 of 25 Swedish families with both breast cancer and at least two cases of ovarian cancer (Hakansson *et al.* 1997); 9 of 48 American families with at least four cases of breast and/or ovarian cancer (Schubert *et al.* 1997; Hakansson *et al.* 1997). This material represents a starting point for the further characterisation of hereditary-breast cancer genes.

However, it is probably premature to exclude both genes for all families in whom we were not able to find mutations in *BRCA1* and *BRCA2* genes. Not one or even two, screening methods can detect all mutations, and no portion of the gene should be dismissed as being invariably free of mutations. A recent report of the first genomic rearrangement identified within the *BRCA1* locus in breast and ovarian cancer families underscores this point (Puget *et al.* 1997).

However, these cryptic mutations are worth the search, both for the sake of the families concerned and because mutational mechanisms other than small deletions or insertions or single base changes may reflect more closely the behaviour of *BRCA1* and *BRCA2* in sporadic breast cancer.

Future work:

Since some of the families with breast cancer mutations in *BRCA1* and *BRCA2* have other kinds of cancer also, the role of the *BRCA1* and *BRCA2* proteins in these other cancers is being studied by mutation analysis of the particular tumour types. The functions of the *BRCA1* and *BRCA2* proteins have yet to be determined. Future prospects therefore include the discovery of the role of *BRCA1* and *BRCA2* in cell function and the biological effects of each mutation and alternative mRNA splicing. The development of functional assays will help , greatly, the identification of mutations which will lead to cancer.

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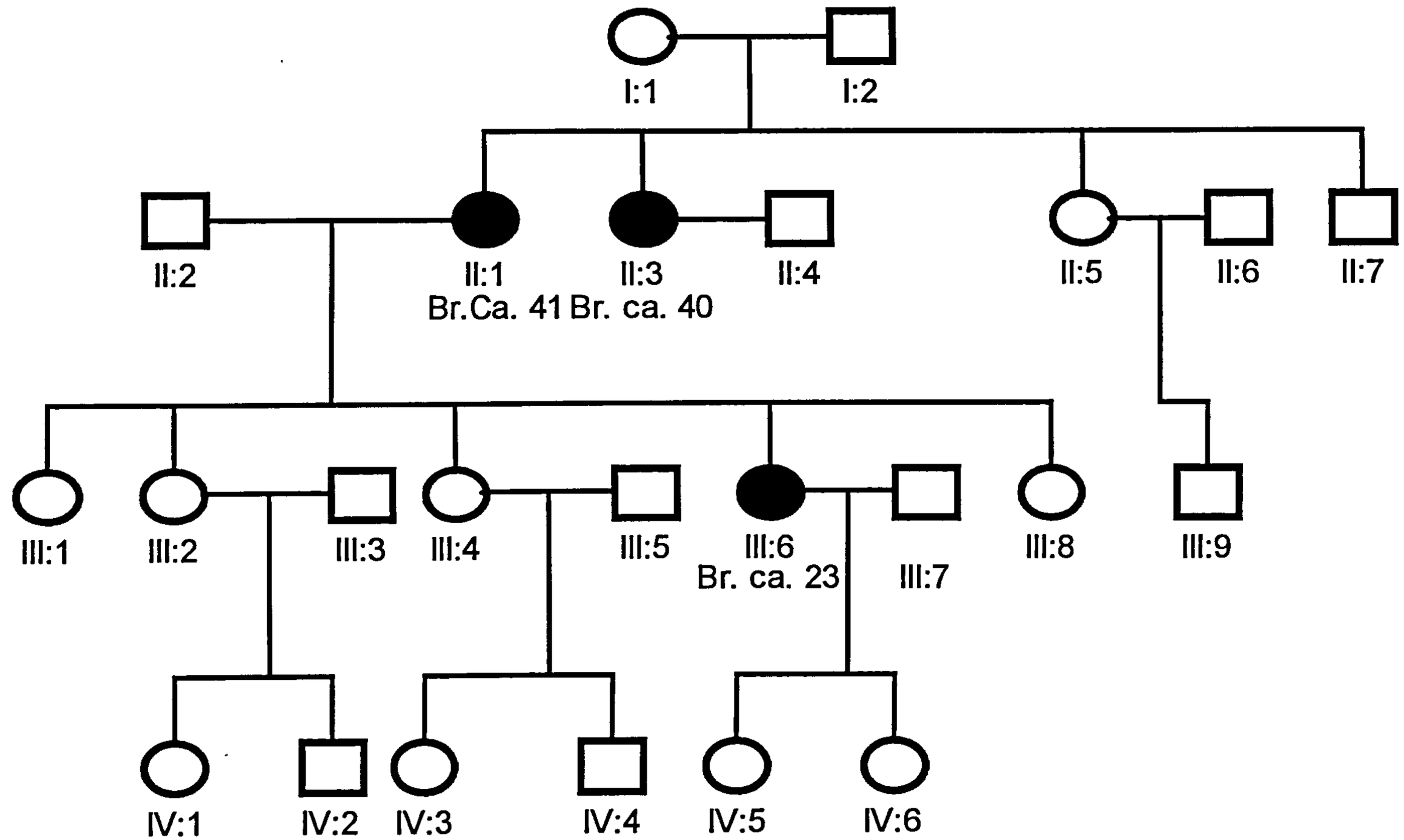
APPENDICES



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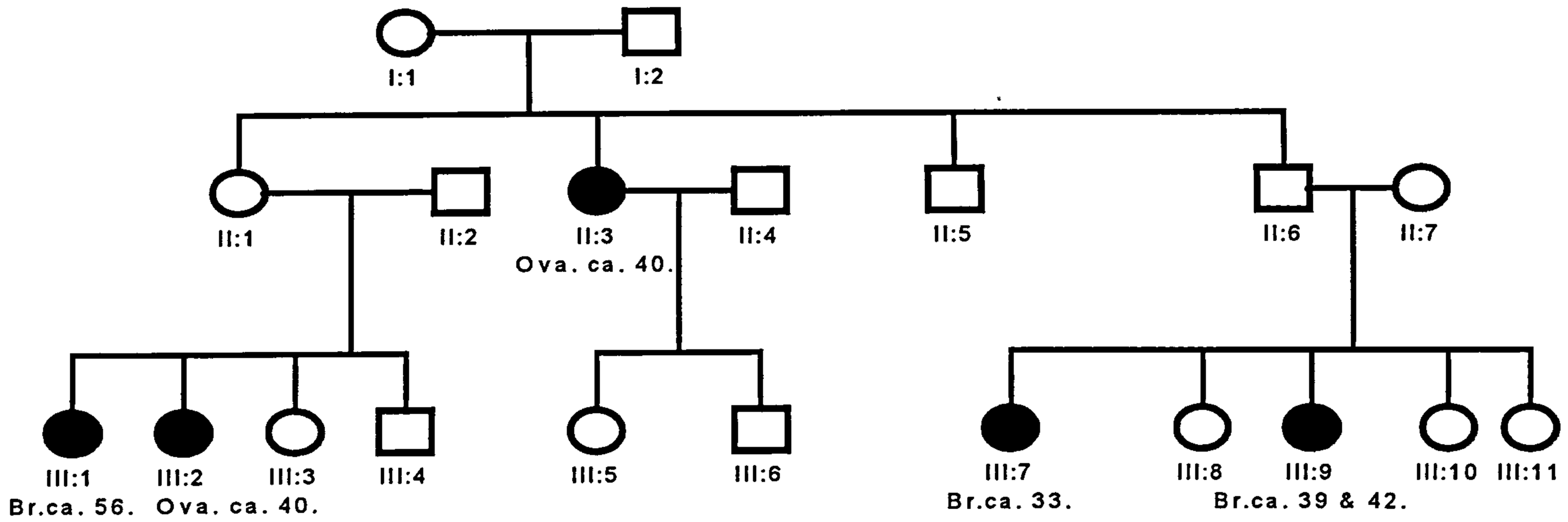

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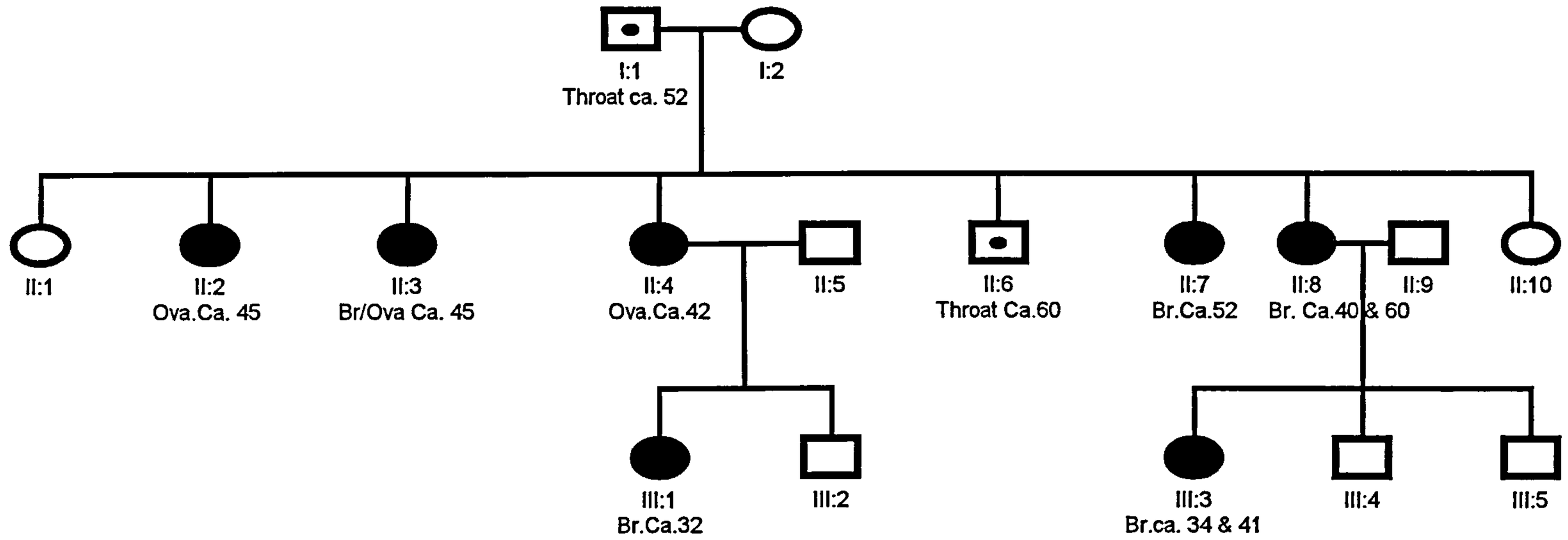

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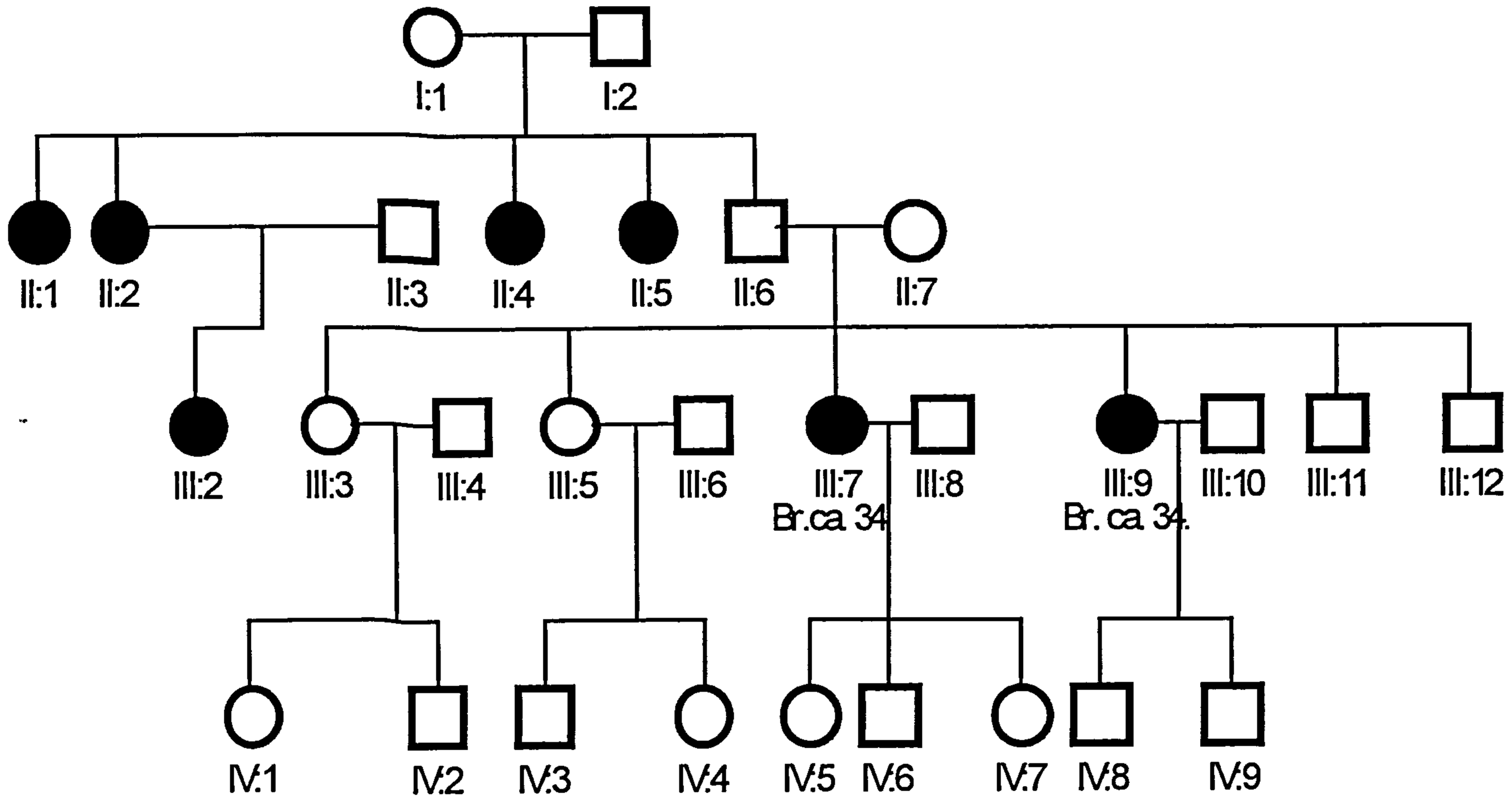

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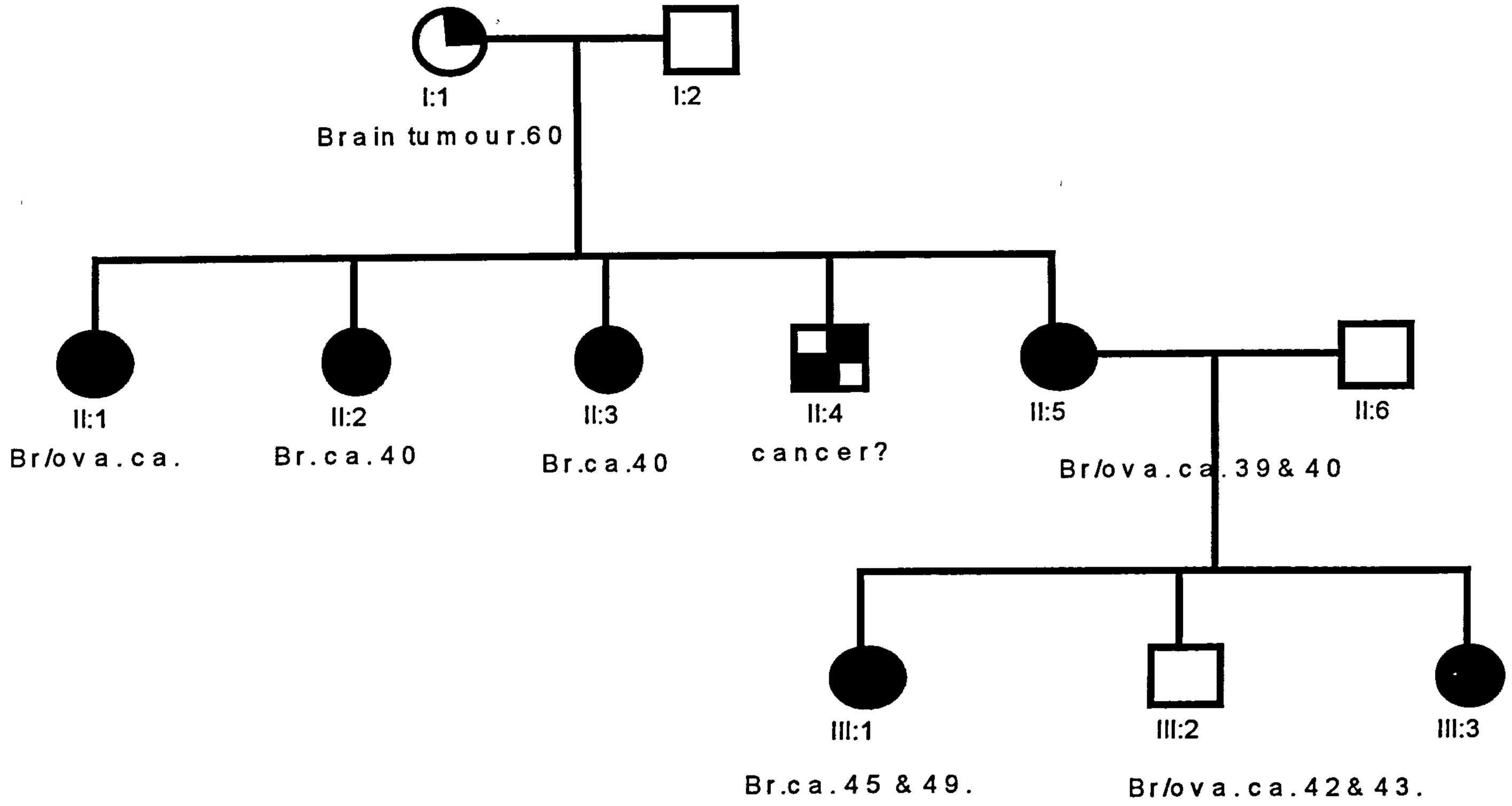

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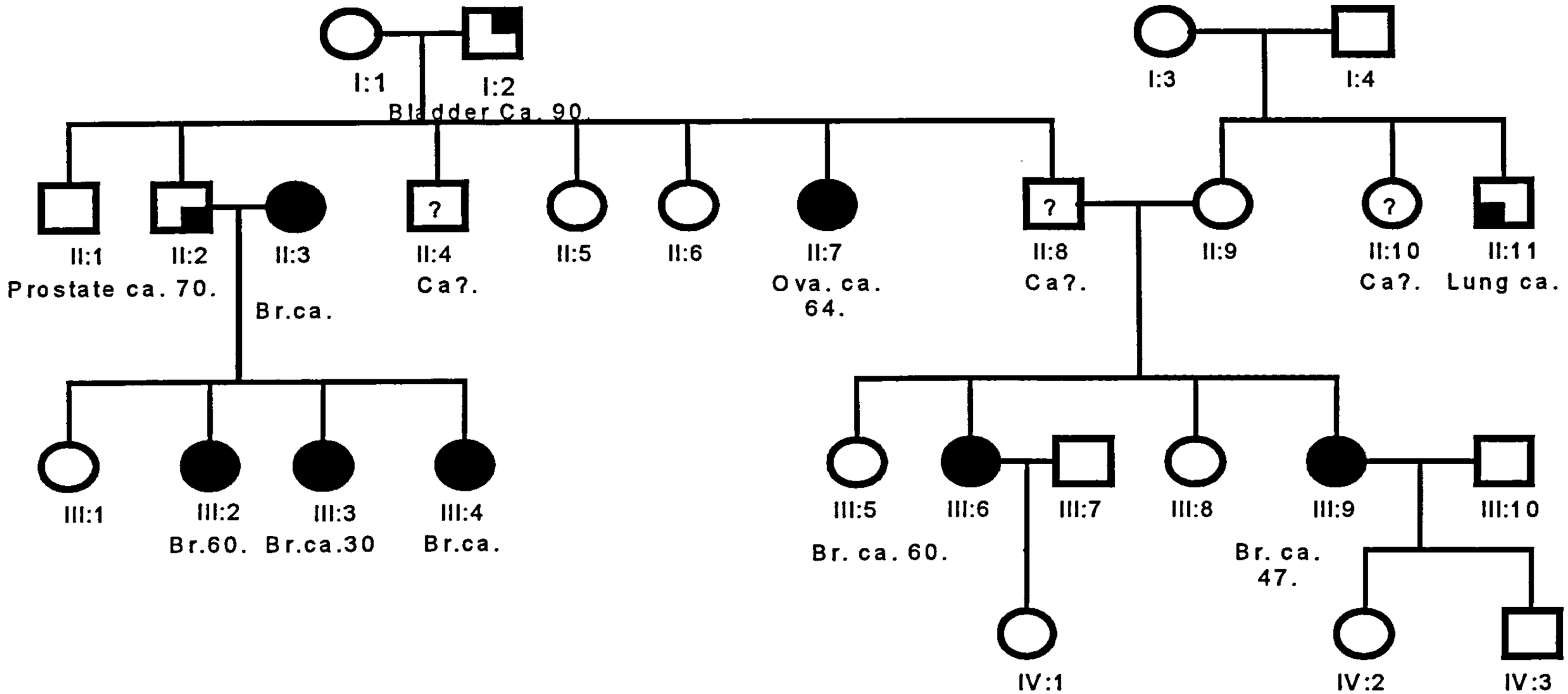
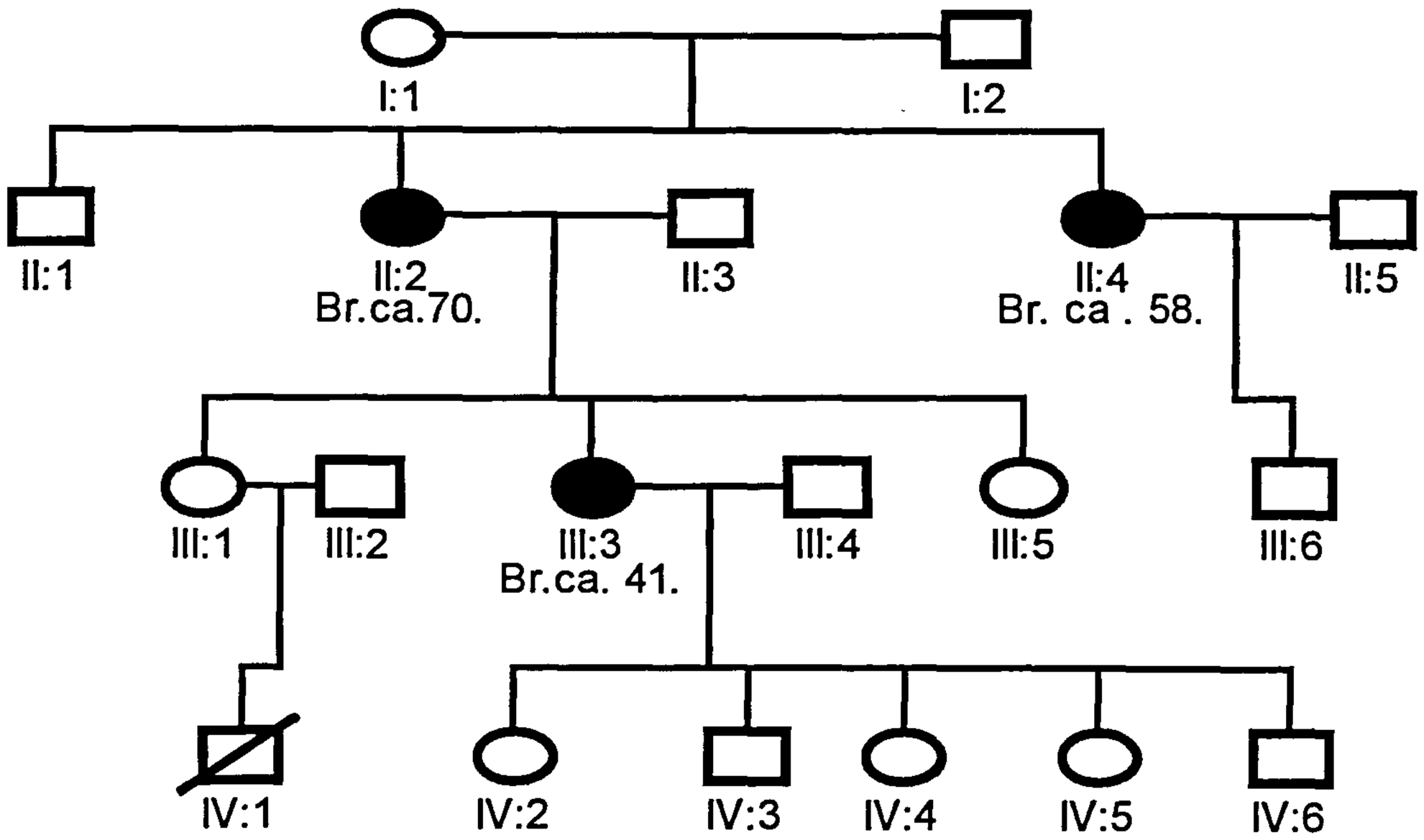


APPENDIX 6 Family 14



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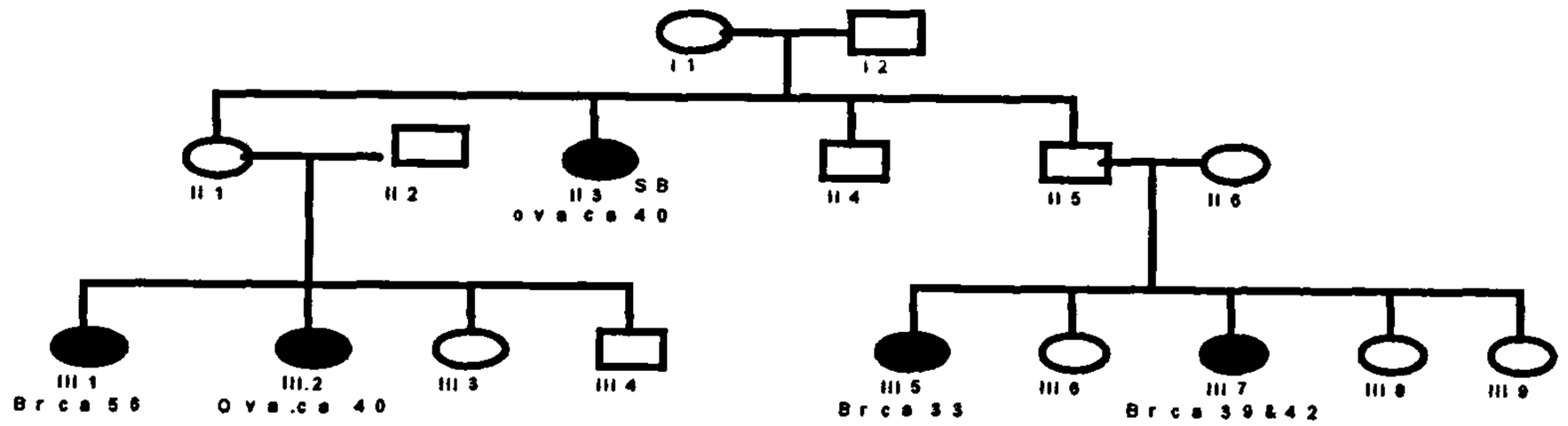

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LONDON 1995

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STICKLER SYNDROME: CORRELATION BETWEEN VITREORETINAL PHENOTYPES AND LINKAGE TO COL 2A1

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Sticker syndrome is characterised by ocular, articular, facial, auditory and oral features. Two thirds of families are linked to COL 2A1, the gene encoding type 2 procollagen. Two sub-groups of Stickler's syndrome have been described based upon the presence ("Type 1") or absence ("Type 2") of congenital vitreous anomoly.
In an initial study, 69 patients from 20 unrelated Stickler's families were classified as Type 1 and 28 patients from four families as Type 2. Type 1 families showed complete linkage to the COL 2Al locus (Zmax=12. 33 at θ=0). In two of the four Type 2 families which were informative, linkage to COL 2Al was excluded.
In a second study, patients from six Stickler's families on the Great Ormond Street genetic database were re-examined by Ophthalmologists unaware of pedigree structures or of previous molecular studies. Four families were reclassified as Type 1 and one as Type 2. One family had normal vitreous (and lacked some of the features typical of Stickler's) possibly representing a separate disorder. Type 1 families showed complete linkage to COL 2A1(Zmax=2.4 at θ=0) Linkage to COL 2A1 was excluded in the Type 2 family.
Data from both centres combined give a Zmax of 14.7 at θ=0 for linkage of Type 1 Stickler's to COL 2A1.

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CAG REPEAT LENGTH ASSESSMENT FOR HUNTINGTON'S CHOREA USING A PCR ASSAY WHICH DOES NOT INCLUDE THE VARIABLE (CCG)n REPEAT: PREDICTIVE TESTING, PRENATAL DIAGNOSIS AND 'PREMUTATION'

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Assessment of CAG repeat lengths of the Huntington's Chorea gene originally involved PCR assays which also included the downstream polymorphic (CCG)n repeat.
We present data accumulated during the first year of Huntington's Chorea mutation analysis in the West Midlands, using a more accurate PCR assay, which does not include the polymorphic (CCG)n repeat. Normal/disease alleles were sized for a total of 195 individuals; 125 diagnoses were confirmed, 59 predictive tests, 1 prenatal diagnosis and also the assessment of the parental CAG repeat lengths in a family with a 'new mutation' were performed.
Out of a total of eight cases referred for testing no expansion of the CAG repeat was observed. However, from the information available, none of these cases appeared to combine both a typical clinical presentation and positive family history of Chorea.
Using this method, only 6/390 (1.5%) of alleles fell into the equivocal range (31-39 repeats inclusive) and one of these was the unaffected father of an isolated case. His CAG repeat length was observed to be 35 repeats lying at the boundary between the ranges for normal and affected alleles.
This data supports earlier reports that there is little overlap between the normal and affected CAG size ranges when the polymorphic (CCG)n repeat is excluded from the PCR assay.

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SEQUENCE ANALYSIS OF THE GLRA1 GENE IN SPORADIC AND FAMILIAL HYPEREKPLEXIA

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Hyperekplexia or startle disease is a rare autosomal dominant condition presenting in the neonatal period and characterised by an exaggerated startle response to unexpected stimuli. Genetic linkage studies revealed the presence of a locus on chromosome 5q (1) and subsequently mutations were detected in the α1 subunit of the human glycine receptor which maps to this region (2). Nine probands with this condition have been ascertained from the UK and Greece (5 familial and 4 sporadic).Investigation of the nature of GLRA1 mutations in this group of patients has been carried out by fluorescent automated sequencing of genomic PCR products. The previously published mutations occur exclusively in exon 6 of the GLRA1 gene. Two of our families have been found possess the most common mutation (Arg271Glu) but no other exon 6 mutations have been detected. Other exons are now under investigation. In particular, a novel mutation is anticipated in one large chromosome 5-linked pedigree in which the hyperekplexia phenotype is associated with spastic paraparesis (3). Detection of such a mutation in this family may therefore have interesting implications for genotype/phenotype correlations.
References: (1) Ryan et al. Annal. Neurol. 31: 663-668 (1992).
(2) Shiang et al. Nature Genet. 5: 351-358 (1993).
(3) Baxter et al. British Paediatric Neurology Association Abstract, Birmingham UK (1994).

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A NOVEL 7 bp DELETION IN AN IRANIAN BETA THALASSAEMIA CARRIER

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Beta thalassaemia is a disorder characterized by decreased or absent Beta globin chain synthesis. To date more than 150 different kinds of mutations have been found in the beta globin gene with frameshift mutations being the most common. In this study haematological examinations and DNA extractions were performed using standard methods. DNA samples from Patients were amplified by PCR then screened for sequence alterations by means of Single Strand Conformational Polymorphism (SSCP) analysis. Mutations were fully characterised by direct sequencing of PCR products . Here we describe a novel 7bp deletion involving codons 22, 23 and 24 of the Beta globin gene (previously found in the Kurdish population) in an Iranian Beta thalassaemia carrier. The deletion shifts the normal reading frame of Beta globin mRNA causing premature chain termination.

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IDENTIFICATION OF A FRAME SHIFT MUTATION IN THE FBNL GENE IN A MARFAN SYNDROME FAMILY

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A study is being carried out to compare the efficiency of various mutation screening strategies in patients with Marfan syndrome. In one family, a frame shift mutation has been identified in exon 4 of the FBNL gene, located at chromosome 15q15-21, using heteroduplex analysis of PCR amplified genomic DNA and subsequent sequencing. The same mutation was not detected when similar techniques were used on CDNA prepared from skin fibroblasts from the same patients. The protein truncation test has been used to verify the presence of the mutant message. The phenotypic effect of this mutation on the distribution and structure of fibrillin 15 has been studied by immunohistochemistry and metabolic labelling.

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APC GENE MUTATIONS IN FAMILIAL ADENOMATOUS POLYPOSTS (FAP) DETECTED USING A COUPLED TRANSCRIPTION/TRANSLATION TECHNIQUE

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Familial adenomatous polyposis (FAP) is an autosomal dominantly inherited condition predisposing affected individuals to early colon cancer due to the presence of hundreds of adenomatous polygas in. the colon and rectum. FAP has an incidence of 1 in 8000 in the UK population. Presymptomatic genetic screening in families in which FAP occurs, facilitates targeting of effective endoscopic and surgical management of 'at risk' patients.
Genetic screening may be performed by linkage analysis but more recently, mutation analysis has become established as the method of choice. We have used a coupled transcription/translation technique to detect mutations in FAP families ascertained through the Northern Regional Polyposis Registry. We have been successful in detecting APC mutations in seven out of 27 unrelated FAP patients which had previously escaped detection by heteroduplex analysis.
Comparisons between these two techniques will be made in relation to their applicability in the diagnostic laboratory.

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SCREENING THE BRCA1 GENE

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In October 1994, a candidate gene for BRCA1, which is responsible for the inherited predisposition to Breast and ovarian cancer in some families, was isolated by positional cloning. The isolation of the BRCAL gene, will allow predictive testing in relatives of patients in whom germline BRCAL mutation have been identified. In this study we have investigated 40 breast cancer patients with 1 to 6 affected first or second degree relatives and onset ages of between 23 and 48 years.
This screening study was based on chemical cleavage mismatch (CCM)analysis of approximately two third of the coding sequence of the BRCAL gene from these patients and utilised both the CDNA and large exon 11. CCM searching of exon 11 from 40 patients, revealed an abnormal pattern in three . Characterisation of these abnormalities by DNA sequencing revealed two polymorphisms in two patients; the third mutation is in the process of being fully characterised. PCR analysis of the CDNA spanning exons 2-10 showed in one patient the presence of two transcripts, one normal and one truncated. DNA sequencing of the short transcript revealed the absence of four exons. Experiments to determine the nature of this observation are in progress.

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MOLECULAR ANALYSIS OF HEREDITARY MOTOR AND SENSORY NEUROVATHY (HMSN)

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HMSN (Charcot Marie Tooth disease) describes a heterogeneous group of inherited disorders that cause progressive degeneration of the peripheral nerves. The major form, CMT1A, maps to 17p11.2 and has been shown to be due to a 1.5Mb duplication comprising the PMP22 gene (or to a point mutation within the gene). A minor form, CMTLB, is due to defects in the P0 gene on chromosome 1q21-q23, and the X-linked dominant form (CMTX1) to defects within the connexin 32 gene on Xq13-q21.
A total of 64 families/individuals with a Charcot Marie Tooth phenotype have been referred. Six of the families had a family history suggestive of X-linked inheritance and connexin 32 screening detected mutations segregating with the affected status. The remaining families were screened for the CMT1A duplication with pVAW409R3a. Thirteen were found to have the duplication, and 17 were uninformative. The non-duplicated samples were screened for mutations in the connexin 32, PMP22 and P0 genes by SSCP analysis and/or sequencing. To date, 6 connexin 32, 1 PMP22 and 1 P0 mutations have been detected. The uninformative samples are currently being screened with additional informative markers from the 17p11.2 region.

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Abstracts and Programme

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14.027
A novel 7-bp deletion in the BRCA2 gene in a family with a history of breast cancer

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Breast cancer is one of the most common female malignancies in the western world, affecting one in ten women during their life time. Five to ten percent of breast cancers may have a genetic component and can be attributed to dominant susceptibility genes. The BRCA1 and BRCA2 genes are equally responsible and account for around 90-95% of inherited breast cancers. BRCA2 contains 27 exons and has a mRNA transcript of 10,926bp,which encodes a protein of 3,418 amino acids. Mutations in this gene are found in families with a high incidence of breast cancer and are also associated with male breast cancer. In this study we have investigated 25 breast cancer patients with between 1 and 6 affected first or second degree relatives and with onset between 23-55 years of age. This screening study was based on the protein truncation test(PTT) which detects mutations leading to premature termination of protein synthesis. PTT analysis of exon 11(accounts for more than half of the coding sequence)from 25 patients revealed a truncated protein in two related patients with a family history of both male and female breast cancer. Characterization of the abnormality by DNA sequencing revealed a novel 7bp deletion at nucleotide position 5447.This deletion produces a translation frameshift leading to a predicted premature termination at codon 1749.

14.028
Three novel mutations and two normal variations in the hMSH2 gene in Scottish families with colorectal carcinoma.

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Hereditary nonpolyposis colorectal cancer(HNPCC)is dominantly inherited and accounts for about 6% of all familial colon cancers. Five different mismatch repair genes are believed to be associated with this disorder and have been shown to cause microsatellite instability when mutated. Recently, in a relatively large study of 74 HNPCC kindreds with 92% genomic instability, 70% of the mutations were found in the five human mismatch repair genes. It seems therefore, that other genes are involved in this disease. In this study sixty-one specimens from 55 Scottish families, including patients with colorectal carcinoma and their relatives, were investigated. Five of these families fulfill the Amsterdam criteria for HNPCC. Of the 61 colorectal patients studied, 4 had a splice site mutation in intron 15 and three had deletions of nucleotides 1577 to 1811. One of them also had a deletion of exon 1 through to exon 7. Two of the patients had a T to C transition at position -6 in intron 12. Subsequent investigations showed that 15% of patients and 16% of the normal population also have this T to C mutation. We also found in two unrelated individuals a G to A transition in exon 6 at codon 322, which changes glycine to asparatic acid. The published mutation spectrum for the hMSH2 gene indicates that there is no mutational hot spot and that approximately 75% of the mutations identified produce premature stop codons. Of the 40 known mutations, in this gene, only two have been reported in different families. Thus, the novel mutations described above underscore the heterogeneous nature of the disease.

THALASSAEMIA

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PROGRAMME & ABSTRACTS

Molecular characterization of eight β– thalassaemia mutations in the south of Iran.G.Ghaffari (1) W.G.Lanyon (1) M.Haghshenas (2) J.M.Connor (1). (1) Department Of Medical Genetics, Duncan Guthrie Institute, Yorkhill Hospital, Yorkhill, Glasgow, G3-8SJ.(2)Namazi Hospital, Shiraz Medical School, Shiraz, Iran.

In the course of the study of β-thalassaemia (β-thal) mutations among ethnic Iranians from the South of Iran, DNA samples from 20 unrelated patients or carriers were examined. The presence of β-globin gene mutations was detected by means of the polymerase chain reaction (PCR), single strand conformational polymorphism (SSCP) analysis and direct sequencing. The amplification refractory mutation system (ARMS) was also used.
Eight different mutations were identified.
1- a G to T substitution at IVS-1 position 1.
2- a G to A substitution at IVS-1 position 110.
3- a novel 14 bp duplication of codons 56-60.
4- a 7 bp deletion involving codons 22, 23 and 24.
5- a 7 bp duplication involving codons 37, 38 and 39.
6- a G to C transition at position 5 in IVS-1.
7- a 25 bp deletion in IVS-1 including the 3 acceptor splice site.
8- a C to T transition in codon 39.
Mutations 1 and 2 account for 40% of samples.The 7 bp deletion and duplication were previously found in the Kurdish and Turkish populations. Although the number of chromosomes studied is small, the eight different mutation discovered illustrates the diversity of the ethnic groups in the south of Iran. The lack of common mutations in this population therefore presents difficulties for the provision of carrier risks and prenatal diagnosis.

An approach for the prevention of thalassaemia in Pakistan.

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Thalassaemia is the commonest inherited disorder in Pakistan. The overall carrier rate for β-thalassaemia is approximately 5%. It is estimated that each year more than 3500 new cases of thalassaemia major are born in Pakistan. The facilities for management of these patients are grossly insufficient. Prevention of thalassaemia, therefor, forms an essential part of handling this problem.

We have worked on a pilot project of developing a suitable and cost effective approach for prevention of thalassaemia in Pakistan. We have found that targeted carrier screening i.e. screening of the close family members of a thalassaemic child, can be very effective in identifying the carriers. Advantage is taken of the fact that most of the marriages take place between close relatives. Our experience of screening several large families of 50-150 individuals has shown that the carrier rate within each family varies between 20-50%. It has been observed that in addition to counselling individual carriers it is also important to counsel the family elders who mostly hold the responsibility of arranging marriages. We have also worked on molecular basis of thalassaemia in all ethnic groups of Pakistan and this work has provided a basis for carrying out prenatal diagnosis of thalassaemia. We introduced prenatal diagnosis of thalassaemia in Pakistan a couple of years ago. So far over 200 diagnosis have been done. The facility is rapidly gaining popularity amongst the thalassaemic families. Over 90% of the women who had been diagnosed to carry an affected fetus also opted for termination of pregnancy.

In the light of our experience we are in the process of formulating a strategy for application at the National level. This experience will also be useful for achieving control over new births of thalassaemia in other developing countries where thalassaemia is a common problem.

SERUM PREVALENCE OF HAV INFECTION IN POLYTRANSFUSED THALASSEMIC PATIENTS.

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Chronic liver disease in polytranfused thalassemie patients is an important cause of morbility and mortality;the genesis is multiform:haemosiderosis and virus infections resulting from repeated transfusions may play an important role.
Since HAV infection could make greatly worse the prognosis,in order to evaluate the opportunity of the HAV vaccination in polytransfused thalassemic patients with chronic liver disease,we studied the prevalence of HAV infection by detection of anti HAV antibodies (IgG)(enzyme immunoassay competitive tecnique) in 75 polytransfused thalassemic patients (36 males, 39 females, 67 affected with thalassemia major,7 affected with thalassemia intermedia and 1 patient with thalassodrepanocitosis,aged 21.1±9.6, mean ± SD,range 4.9-61.4).Fourty-two patients (56%) were HCV positive with persistent increase of transaminasis.
Eight patients were HAV antibodies positive (10.6%); the incidence was 2.7% in patients aged 1 to 19 years,11.4% in patients aged 20 years-39 years and 100% over 40 years of age.No differences were observed between sexes.Five out of eight HAV positive patients (mean age 35 years,range 17-61 years) were positive for HCV antibodies.
In conclusion,in order to reduce further the incidence of liver infections in polytransfused thalassemic patients,we recommend an active immunization for HAV.

Beta Thalassemia Major: pulmonary involvment: V. Rametta(1) F. De Michele(2) S. Buffardi(1) P. Cinque(1) A. Viola(1) L. Pagano(1) F. Iodice(2) C. De Rosa(1). (1) Sezione Microcitemia "A.Mastrobuoni"(2) Fisiopatologia Respiratoria.Ospedale "Antonio Cardarelli" Naples, Italy.
Severe hemolytic anemia and ineffective erythropoiesis from infancy are the main characteristics of homozygous beta-thalassemia. This abnormal hemoglobin syntesis leads to impaired oxygen delivery to the issues, ineffective erythropoiesis, and iron overload. To maintain adequate hemoglobin concentrations and prevent the development of the adverse effects associated with this disorder, patients receive hypertransfusion therapy and chelation with desferrioxamine (DFO).These transfusions lead to the accumulation of iron in the body and organ damage.Recently, abnormal pulmonary function tests were described in thalassemia patients.Unfortunately, there is no consensus about the exact nature of the pulmonary dysfunction. Restrictive defect, obstructive disease are the most common, but the relationship between these findings and iron deposition as the effect of the transfusion and the use of desferrioxamina remain unclear. The purpose of this study is to assess the predominant pulmonary function abnormality in patients with beta-thalassemia major.From July 94 to October 96 131 patients with homozigous beta thalassemia were enrolled in the study. The group consisted of consecutive 63 male and 68 female, age ranging from 12 to 39 years.At the time of study all patients were on a transfusion protocol designed to maintain a pre trasfusion hemoglobin level of 10 ± 0.5 gr/Dl.The patients received chelation therapy with DFO, which was administered in a 8-h subcutaneus infusion given 5 to 7 time weekly. Spirometry and arterial blood gases (radial artery puncture) were performed in all patients.Hypoxemia (oxygen saturation of less 95%) was observed in 91 patients (69%); 30 patients (33%) had permanent value of less 80%.A reduction in the total lung capacity (TLC)was found in 101 patients (77%); modeste or severe reduction in TLC was observed in 73 patients (72%). Expiratory flow rates were decreased below predicted value in 74 patients (57%), with 9 patients had pure obstructive disease. In conclusion restrictive disease is the predominant abnormality in pulmonary function in homozigous beta thalassemia with a mixed restrictive pattern in a small number of patients.

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14.06
Comparison of enzyme mismatch cleavage and chemical cleavage of mismatch on a defined set of heteroduplexes

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In this work we present a comparison of two mutation detection techniques, both with potential for widespread application, and an evaluation of their respective advantages and disadvantages. The two techniques, chemical cleavage of mismatch (CCM) and enzyme mismatch cleavage (EMC), have the advantages over some conformation based methods of being able to detect and localise mutations in relatively large fragments of DNA (> 1Kb). We have constructed clones that enable us to create heteroduplexes within 500bp, 1Kb and 1.5Kb fragments and assessed each of the methods over a range of criteria. Both methods were able to detect and localise all four types of single base pair mismatches and insertion/deletions of 1-5bp. CCM using hydroxylamine was efficient over the entire range of fragment sizes tested and potassium permanganate with tetraethylammonium chloride was efficient up to 1Kb. Whilst EMC is efficient at detection of insertion/deletions in a broad size range of fragments and has the advantage over CCM of not using hazardous chemicals, in our hands it has not been sufficiently robust for diagnostic use.

14.07
BRCA1 and BRCA2 mutations associated with Breast and Breast-Ovarian patients in the West of Scotland.

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Genetic susceptibility has been estimated to contribute to 5-10% of all breast cancer cases and can be attributed to at least two dominant susceptibility genes. Germ-line mutations in BRCA1 and BRCA2 gene are though to account for ~90% of all inherited breast cancers. In this study we have screened 40 breast and/or ovarian cancer patients for germ-line mutations in the BRCA1 and BRCA2 genes. the patients had between one and five affected first or second degree relatives with onset between 23-55 years of age. Mutation analysis was performed by a combination of the protein truncation test, single strand conformational polymorphism analysis, chemical cleavage mistmach analysis and direct DNA sequencing on any variants identified by the above techniques. A total of 9 families (22%) exhibited 5 different mutations in BRCA1 and BRCA2, of which three were present in BRCA1 and two in BRCA2. They are: 1- The skipping of exons 5-7 in BRCA1 in two unrelated families 2- a deletion of AA 2800 in BRCA1 in two unrelated families 3- an insertion of C in the BRCA1 gene 4- a 7bp BRCA2 deletion at position 5445 5- a AA BRCA2 deletion at position 5573 Haplotype analysis of recurrent mutations suggests a common founder for each of these mutations. The relatively low frequency of BRCA1 and BRCA2 mutations in the present study could be explained by insufficient family history for breast cancer, insufficient screening coverage of the gene, or an uncharacterised BRCA gene.

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14.09
mRNA and genomic mutations in mismatch repair genes.

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Mutation analysis of mismatch repair genes has revealed that at least four genes, namely hMSH2, hMLH1, hPMS1 and hPMS2 are involved in the non-polyposis form of colorectal cancer. It is believed that hMSH2 and hMLH1 have the major share in the pathology of hereditary non-polyposis colorectal carcinoma (HNPCC). In this study, we screened 47 colorectal cancer families in whom RNA and DNA samples were available and 37 additional families from whom only DNA was available for investigation. A combination of RNA and DNA based methods was used and detected the 11 germline mutations described below: (I) a G to C trnasversion at position +5 in intron 15, resulting in exon 15 skipping (ii) a mRNA deletion of exons 2-6, inclusive(iii) a mRNA deletion of exons 2-8, inclusive (iv) an out-of-frame mRNA deletion of codons 9-50 (v) an in-frame mRNA deletion of codons 228-340 (vi) an out-of-frame mRNA deletion of codons 493- 603 (vii) an out-of-frame deletion of codons 743-871(viii) a C to G transversion at position +9 in intron one (ix) a G to A transition at codon 322 (x) a T to C transition at position -6 in intron 12 (xi) an A to G transition at position -19 in intron 14. The pathology of these mutations will be discussed. 9315831d@udcf.gla.ac.uk

14.11
Detection of CMT1A duplication and HNPP deletions with reduced dependence on dosage analysis

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One hundred and thirty-two CMT1A and 41 HNPP referrals were originally screened using a set of four CA repeat markers that span the commonly duplicated/deleted region, an approach validated by dosage analysis using EcoRI/Southern hybridisation with CMT1A-REP probe pNEA102. Forty-eight (36%) CMT1A diagnoses were confirmed. Twenty-four (69%) of 35 unrelated CMT1A patients with the common duplication were detected by a 3-allele microsatellite pattern at one or more loci. Eight patients, including 6 referred for HNPP and 2 for CMT1A, were homozygous or hemizygous for all four microsatellite loci. Retrospective analysis of the same unrelated CMT1A patients using EcoRI/SacI/Southern hybridisation with the probe pNEA102 confirmed the presence of a junction fragment in 26 (74%) of these patients. A junction fragment was also detected in 7 of the 8 (88%) HNPP patients with a deletion. Similarly, by combining microsatellite and junction fragment analysis, 31 (89%) of duplications were detected by a 3-allele microsatellite pattern and/or a unique junction fragment. This approach substantially reduces reliance on variable dosage analysis for duplication/deletion detection.