The role of variants of homologous recombination repair genes in breast cancer susceptibility and DNA repair

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I hereby declare that no part of this thesis has previously been submitted for any degree or qualification of this, or any other University or Institute of learning

TO Maryam

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Summary

Apart from being the most common malignancy in women worldwide, breast cancer is also one of the most extensively investigated human cancers. The breast cancer susceptibility genes, *BRCA1* and *BRCA2* are responsible for less than 10% of breast cancer cases. However the genetic basis of the majority of breast cancer has not yet been identified.

Since an acquired genetic instability resulting from the defects in DNA repair is known to promote tumorigenesis, a proportion of inherited breast cancers might be attributable to mutations in the genes involved in these functions.

Homologous Recombination Repair (HRR) is an accurate and high fidelity repair mechanism, which is mainly responsible for repair of DNA double strand breaks (DSBs). There is considerable evidence to suggest that defects in DNA damage repair in particular in HRR contribute to breast cancer.

In the present study we tested the effect of single nucleotide polymorphisms of HRR genes on breast cancer susceptibility. We screened a series of 522 breast cancer patients and 900 healthy controls for a panel of four polymorphic HRR genes. The rare allele of XRCC2 R188H was found to be associated with increased risk of breast cancer (odds ratio: 1.32, CI: 0.98, 1.79). This allele was also shown to be more associated with lobular breast carcinoma (p=0.001). Furthermore there was preliminary evidence of interaction between XRCC2 R188H and XRCC3 T241M, and also between XRCC3 T241M and BRCA2 N372H alleles (both p=0.06).

Four novel naturally occurring sequence variants of the *XRCC3* DNA repair gene were also identified. We studied the effect of these sequence variants on the repair ability of the XRCC3 protein. A rare XRCC3 variant (D213N), was shown to completely ablate the protein function. The association of this variant with cancer susceptibility was studied in a group of 1524 patients with common cancer and 1577 healthy individuals. However, no

association between this variant and cancer susceptibility was found, indicating that *XRCC3* is unlikely to be a tumour suppressor gene.

The results from the present study support the hypothesis that normally occurring variation in DNA repair genes can influence DNA repair capacity and cancer susceptibility in the population.

Abbreviations

AT	Ataxia-telangiectasia
ATM	Ataxia-telangiectasia mutated
ATLD	Ataxia-telangiectasia Like Disorder
ATP	Adenosine Tri-Phosphate
ATR	Ataxia-telangiectasia and Rad3-related
BCC	Breast cancer cases
BDC	Blood donor controls
BER	Base excision repair
BLM	Bloom syndrome
BRCA1	Breast cancer 1 gene
BRCA2	Breast cancer 2 gene
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
CHAPS	3--[(3-Cholamidopropyl) dimethylammonio]-1 propanesulfonate
CHEK1	Cell cycle checkpoint kinase 1 gene
CHEK2	Cell cycle checkpoint kinase 2 gene
CHO	Chinese hamster ovary
CI	Confidence interval
CPT	Camptothecin
DDT	1,4-Dithio-DL-threitol
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
DSB	Double-strand break
EDTA	Ethylenediamine Tetra-Acetic acid
ERCC2	excision repair cross-complementing rodent repair deficiency, complementation group 2
FANC	Fanconi anaemia complementation group
g	Gradient
G1	Gap between mitosis and the onset of DNA replication
G2	Gap between DNA synthesis and the onset of mitosis
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
HR	Homologous Recombination
HRR	Homologous Recombination Repair
HRT	Hormone Replacement Therapy
IR	Ionizing Radiation
kb	kilobase
kDa	kilodalton

M	Mitosis
MMC	Mitomycin
MMR	Mismatch repair
MLH1	mutL (*E.coli*) homologue 1 gene
MRE11	Meiotic recombination protein 11 (*S.cerevisiae*) homologue
MSC	Mammography screening controls
MSH2	mutS (E.coli) homologue 2 gene
NBS	Nijmegen breakage syndrome
NBS1 (*nibrin, p95*)	Nijmegen breakage syndrome gene
NER	Nucleotide excision repair
NHEJ	Non-Homologous End-Joining
nm	Nano-meter
OR	Odds ratio
p	Short arm of the chromosome
PBS	Phosphate Buffered Saline
p53	Tumor protein 53
PCR	Polymerase chain reaction
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene difluoride
q	Long arm of the chromosome
RAD50	*S.cerevisiae* RAD50 homolog
RAD51	*S. cerevisiae* RAD51 homolog (RecA homolog, E. coli)
RET	Ret proto-oncogene
RNA	Ribonucleic acid
RPA	Replication protein A
RT-PCR	Reverse transcription-polymerase chain reaction
S	DNA synthesis part of the cell cycle
SDS	Sodium Dodecyl Sulphate
SNP	Single nucleotide polymorphism
SSB	Single strand breaks
SSCP	Single Strand Conformation Polymorphism
STK11	Serine/Threonine Kinase 11
TCR	Transcription coupled repair
TEMED	N,N,N',N'- Tetramethylethylenediamine
TP53	Gene for tumor protein 53
TSG	Tumor suppressor gene
UTR	Untranslated region
UV	Ultra violet
XRCC2	X-ray repair complementing defective repair in Chinese hamster cells 2
XRCC3	X-ray repair complementing defective repair in Chinese hamster cells 3
XRCC4	X-ray repair complementing defective repair in Chinese hamster cells 4

Contents

CHAPTER ONE

CHAPTER ONE- INTRODUCTION

List of tables and figures

Tables

Figures

1 Introduction

1.1 General concepts in cancer

One of the most important discoveries in cancer biology during recent decades has been that tumours arise as a result of cumulative genetic changes in somatic cells. The progression of a tumour from normal cell to cancer and finally metastasis is the result of the clonal expansion of cells. This uncontrolled expansion may be the result of as little as three to seven successive mutations in genes that control cellular proliferation and cell death (Miller, 1980, Weinberg, 1989). As the mutation rate is typically less than 10^{-6} per each cell division, the likelihood of a single cell accumulating the requisite number of independent mutations to inactivate the cellular defences against tumourigenesis appears to be very low (Loeb, 1991). However, there are two general mechanisms that make the series of cancer-promoting mutations more likely. Mutations enhancing the cell proliferation rate increase the size of the target population of cells in which the next mutation may occur, and mutations affecting the stability of the genome increase the overall mutation rate (Antonarakis, *et al.*, 2002, Strachan and Reed, 1999).

Pathogenic mutations usually occur in the genes controlling the cell cycle, apoptosis and genomic integrity. They can also influence proteins which are responsible for cell-to-cell contacts, or the factors needed for tumour expansion and invasion into nearby tissues. The changes involve alterations or mutations in specific oncogenes, and are generally divided into two major categories: those that activate proto-oncogenes, thus promoting cellular proliferation or inhibiting cell death; or those that inactivate tumour suppressor genes (TSG) whose normal function is to inhibit cell proliferation or promote cell death.

Non-mutational DNA alteration such as changes in DNA methylation or genomic imprinting may also alter gene expression and could, therefore, potentially be a mechanism that disrupts the function of tumour suppressor genes or increases the expression of proto-oncogenes. These are called epigenetic changes and defined as heritable alterations in gene function that are mediated by factors other than changes in the primary DNA sequence (Verma and Srivastava, 2002).

1.2 Proto-oncogenes and tumour suppressor genes

The normal activity of a proto-oncogene supports growth of cells, but a gain of function (e.g. amplification, point mutation, or transposition to an active chromosome domain) in a single allele may result in inappropriate or excessive activity (Park, 2002 and references therein). Tumour suppressor gene products, on the other hand, inhibit events leading towards cancerous behaviour. They are responsible for controlling the cell cycle progression and the induction of apoptosis and for maintaining genomic integrity by ensuring accurate replication, repair and segregation of the cell's DNA. Inactivation of both alleles of a TSG may leave some of these crucial regulatory functions uncontrolled, and thus provide the mutated cell with an overgrowth advantage (Fearon, 2002 and references therein).

Tumour suppressor genes have been categorised into gatekeepers and caretakers. The "gatekeepers" refer to genes whose mutation or altered expression affect the normal controls of cell division, death or life span, promoting the uncontrolled proliferation of cancer cells while "caretakers" are genes whose mutation or disruption do not initiate a tumour directly but cause genome instability and increase the frequency of mutations in gatekeeper genes. Genes involved in DNA repair and genome integrity that are responsible for the fidelity of information transfer are classified in this latter group (Kinzler and Vogelstein, 1997).

The germline alterations related to inherited cancer susceptibility are frequently found in tumour suppressor genes, and only on a few occasions such as multiple endocrine neoplasia type 1, hereditary papillary renal carcinoma and familial melanoma (e.g. *RET*, *CDK4* and *MET*) have they been found in proto-oncogenes (Mulligan, *et al.*, 1993, Schmidt, *et al.*, 1997, Zuo, *et al.*, 1996). Conversely, cancer-associated somatic mutations are found in genes belonging to both categories.

1.3 The cell cycle

The cell cycle is an ordered set of events, culminating in cell growth and division into two daughter cells. Cells need to replicate their genome and double their cell mass. This requires precise replication and segregation of chromosomes and equal distribution of other cell components. The cell cycle consists of several phases (figure 1). In the first phase (G1) the cell grows and becomes larger. When it has reached a certain size it enters the (S) phase, in which DNA-synthesis takes place. The cell duplicates its hereditary material (DNA-replication) and a copy of each chromosome is formed. During the next phase (G2) the cell checks that DNA-replication is completed before the cell cycle proceeds to the next step. During (M) phase the pairs of chromosomes are separated (mitosis) and the cell divides into two daughter cells. After division, the cells resume G1 and the cell cycle is completed. In G1 checks of whether the previous cycle was error free take place before cells proceed to another division. For all living eukaryotic organisms it is essential that the different phases of the cell cycle are precisely coordinated. The phases must be followed in correct order, and one phase must be completed before the next phase can begin. Errors in this coordination may lead to chromosomal alterations, rearrangement or unequal distribution of genetic material between the two daughter cells. This type of chromosome alteration is often seen in cancer cells (Clurman and Roberts, 2002).

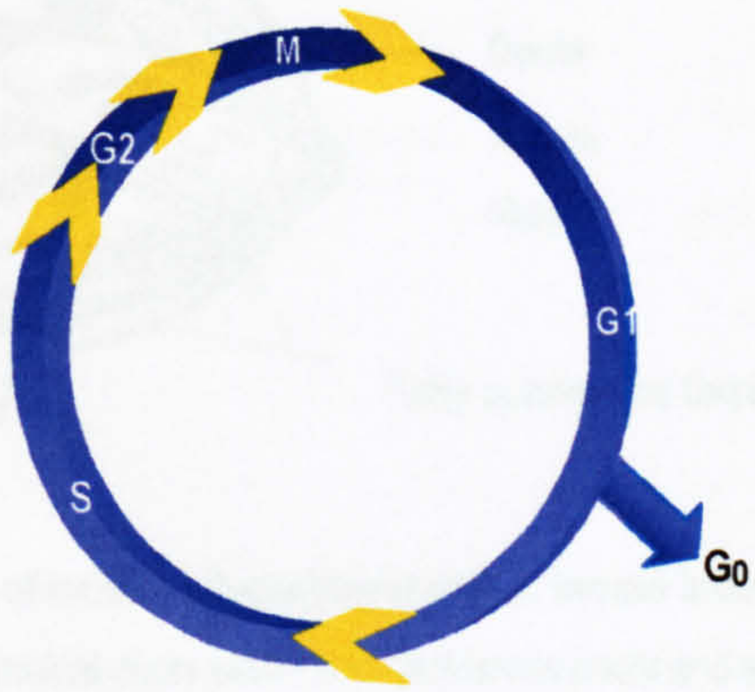


Figure 1: The cell cycle

In the next sections the breast cancer environmental and genetic risk factors are explained, we then discuss the homologous recombination repair (HRR) pathway of double strand breaks and the evidence linking DNA repair and breast cancer susceptibility.

1.4 Breast Cancer

1.4.1 Normal development of mammary glands

The ductal system of the adult human breast consists of 15-25 lactiferous ducts opening at the nipple (figure 2). Lobule formation in the female breast occurs within 1-2 years after the onset of the first menstrual period. Full differentiation of the mammary gland is a gradual process, which takes years to complete. The breast of nulliparous women contains more undifferentiated structures while in pre-menopausal parous women the predominant structure is the most differentiated form. Full lobular differentiation only occurs in parous women, especially in those experiencing full term pregnancy at a young age. After menopause the breast undergoes regression in both nulliparous and parous women manifested as an increase in the undifferentiated structures.

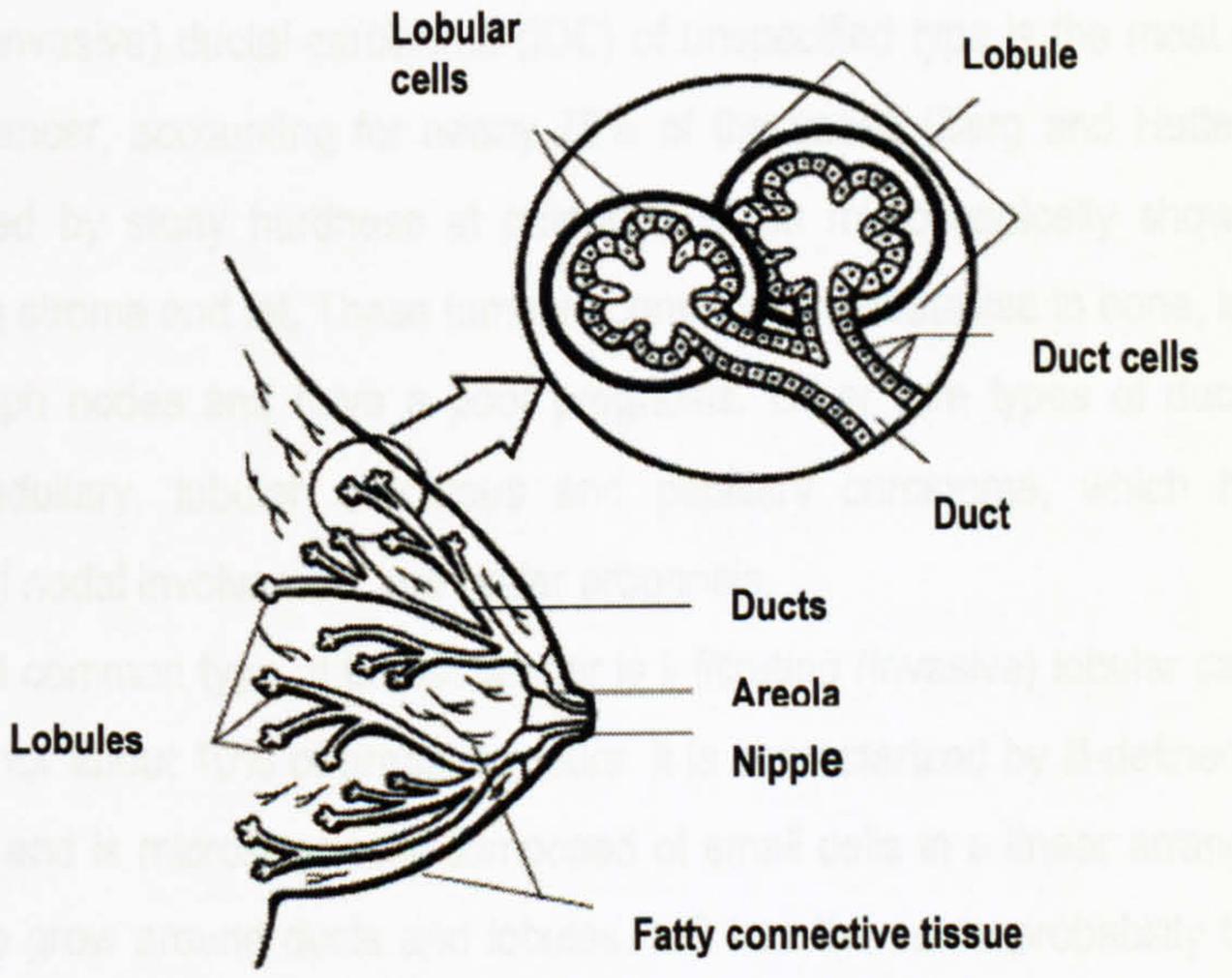


Figure 2: Schematic picture of terminal ductulobular unit of female breast.

Each lobular unit opens into terminal ducts which form lactiferous ducts and subsequently open at the nipple.

In breast cancer, mammary cells proliferate abnormally. The diagnosis of breast cancer is based on histological examination of breast tissue. Breast cancer may arise in the intermediate-sized ducts or terminal ducts and lobules. The cancer may either be invasive (infiltrating ductal carcinoma, infiltrating lobular carcinoma) or *in situ*.

1.4.2 In situ breast carcinoma

In situ breast carcinoma (CIS) is a transitional phase in the evolution of invasive malignancy from normal breast tissue. There are two histological forms of CIS. Ductal carcinoma in situ (DCIS), also known as intraductal carcinoma or non-invasive ductal carcinoma, does not invade the surrounding stroma. Lobular carcinoma in situ (LCIS) is characterized by clusters of analplastic small cells of high nuclear grade that lie within the lobules (Frykberg, 1999). LCIS is considered as a pre-malignant stage with a substantially higher risk of subsequent development of carcinoma (Allred, *et al.*, 2001).

1.4.3 Invasive breast carcinoma

Infiltrating (invasive) ductal carcinoma (IDC) of unspecified type is the most common type of breast cancer, accounting for nearly 75% of the cases (Berg and Hutter, 1995). It is characterized by stony hardness at palpation which microscopically shows invasion to surrounding stroma and fat. These tumours commonly metastasise to bone, lung, liver, and axillary lymph nodes and have a poor prognosis. Other rare types of ductal carcinoma include medullary, tubular, mucinous and papillary carcinoma, which have a lower incidence of nodal involvement and better prognosis.

The second common type of breast cancer is infiltrating (invasive) lobular carcinoma (ILC) accounting for about 10% of breast tumours. It is characterized by ill-defined thickening in the breast, and is microscopically composed of small cells in a linear arrangement with a tendency to grow around ducts and lobules. ILC has the same probability to metastasise as IDC though invading preferentially to meninges, serosal surfaces, and retroperitoneum. Most undifferentiated structure in the breast has been found to be the site of origin of ductal carcinomas. Although differentiated lobular structures have been found to give rise to tumours but they are less aggressive (Russo, *et al.*, 2000).

1.4.4 Staging

Tumour staging is used to assess the extent of the breast cancer. In the tumour node metastasis (TNM) staging system, the extent of the disease is determined based on clinical

and pathological assessment of the tumour, local lymph nodes and presence of distant metastasis. The status of tumour can be sub-classified according to the size of tumour from T_{is} (carcinoma in situ) to T_4 where tumour size is more than 5 cm and has invaded the chest wall or skin. Lymph node involvement is also sub-classified from N_0 (no regional lymph node involvement) to N_3 (Ipsilateral internal mammary node involvement). Absence of metastasis is classified as M_0 while distant metastasis is M_1 (Sainsbury, *et al.*, 2000). Based on this classification breast cancer is staged from I to IV, where stage I is a small locally invasive tumour without lymph node involvement, stage II is a small or medium-sized tumour with or without nodal metastases, stage III cancer is a locally advanced cancer, usually with axillary node metastases, and stage IV cancer has already metastasised to distant sites (Anderson, *et al.*, 1996).

1.4.5 Incidence

Breast cancer is the most common cancer in women worldwide. Over one million new cases occur each year worldwide, comprising about 25 per cent of all female malignancies (Ferlay, *et al.*, 2001). This proportion is higher in women in western, developed countries. The highest age-adjusted incidence rate is reported for North America, being 86.3 per 100 000 women per year, while the lowest rate, reported in China, is only 11.8 (Parkin, 1998). In England and Wales, 33100 new breast cancer cases were registered in 1997 which account for almost 30 per cent of all cancers in women (National Statistics UK, 2000). One in 9 women will develop breast cancer at some time in their life in the UK. Breast cancer in men is rare with around 250 cases each year in the UK (National Statistics UK, 2000). In England and Wales, 11,574 women and 80 men died from breast cancer in 2001 (National Statistics UK, 2002), and in the UK, where the mortality is highest in the world, 17 per cent of all female cancer deaths was due to breast cancer by year 2000 (General Register Office for Northern Ireland, 2001, General Register Office for Scotland, 2001, National Statistics UK, 2001).

The high rates of breast cancer incidence and mortality in industrialised, Western nations and lower rates for less industrialised and Asian nations are considered to mirror differences in factors important in the aetiology of the disease such as parity and hormonal, environmental and dietary exposures.

1.4.6 Classification

The majority of breast cancer patients can be classified as sporadic cases, in which the individual is the only one in her family with breast cancer for two generations (Claus, *et al.*, 1991, Newman, *et al.*, 1988). On the contrary, familial cases imply to individuals that have one or more first-degree relatives with breast cancer (Lynch, *et al.*, 1994). Familial breast cancer is different from hereditary breast cancer in which family history suggests a highly penetrant major gene for breast cancer, characterized by early age of onset, high incidence of bilateral disease and association with other tumours (Claus, *et al.*, 1991, Kelsey and Gammon, 1991, Newman, *et al.*, 1998).

1.4.7 Risk Factors

Breast cancer is a complex, multifactorial disease involving interaction between genetic and non-genetic (environmental) factors for its development. Although the aetiology of breast cancer is not fully understood, several inherent and other factors can indicate an increased risk (Table 1).

Factor	Increased/Decreased Risk
Female gender	100 fold compared to males
Age	Double every 10 years until menopause
Diet	High intake of unsaturated fat increases the risk High intake of soy products decreases the risk
Obesity	Postmenopausal obesity (BMI >35 kg/m^2) doubles the risk
Alcohol consumption	Daily use of alcohol increases the risk
Family history of breast cancer	A first degree relative with breast cancer before the age of 50 doubles the risk
Cancer in other breast	3 to 5-fold cancer risk in contra lateral breast
Previous atypical hyperplasia	4 to 5-fold risk
Endometrial and ovarian cancer	1.5 to 2-fold risk
Age at menarche	Age below 12 years increases the risk
Age at menopause	Late age increases the risk
Nulliparity	Increases the risk
Age at first full-term pregnancy	High age at first delivery increases the risk
Lactation	Lactation over 24 months decreases the risk
Oral contraceptives	Current use of OCPs increases the risk, the risk disappears 10 years after cessation of OCPs
Menopausal hormone use (HRT)	Over 5 years of use increases the risk, Excess risk disappears 5 years after cessation of oestrogen. Adding progestin to oestrogen still increases the risk
Geographical location	Living in developed country increases risk
Exposure to ionising radiation	Abnormal exposure in female after age 10 increases risk by 3 fold

Table 1: Environmental factors and their effect on risk of breast cancer.

OCP: oral contraceptive pills; HRT: hormone replacement therapy; BMI: body mass index. Adopted from (Broeders and Verbeek, 1997, Colditz and Rosner, 2000, Collaborative Group on Hormonal Factors in Breast Cancer, 1996, 1997, Lopez-Otin and Diamandis, 1998, Mettlin, 1992).

1.4.7.1 Age

The incidence of breast cancer, as that of almost any cancer, increases with advancing age. Eighty per cent of cases occur in post-menopausal women. Breast cancer is extremely rare in women in their teens or early twenties and uncommon in women under 35. After this age the risk begins to increase, rising sharply after the menopause. The estimated risk of developing it for each of the other age groups has been calculated as follows (Cancer research UK, 2001):

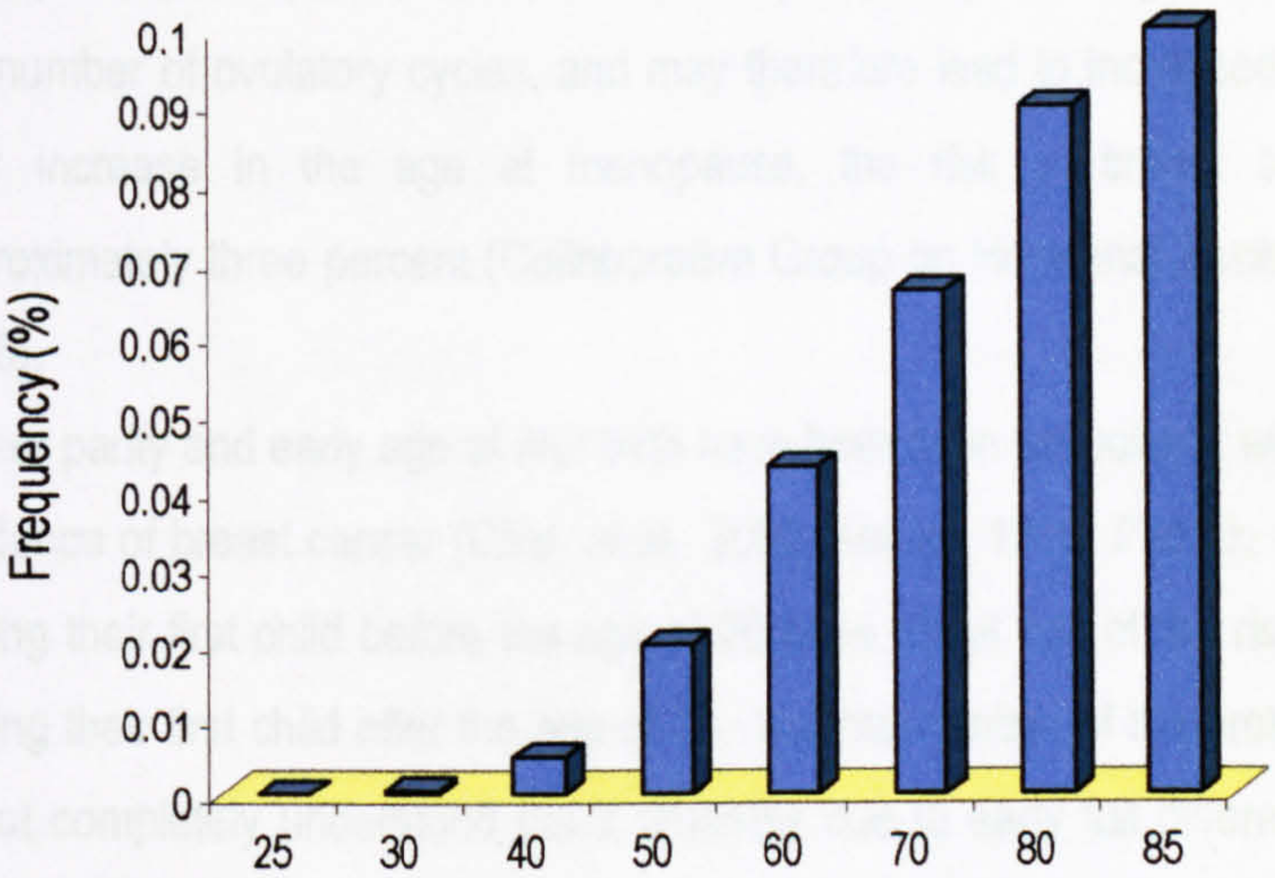


Figure 3: Frequency of breast cancer among women in the UK.

Adopted from Cancer research UK, breast cancer fact sheet, 2001.

1.4.7.2 Reproductive factors

Recent studies have revealed the relationship between endogenous hormone levels and breast cancer risk (Dorgan, *et al.*, 1997, Kabuto, *et al.*, 2000, Key and Verkasalo, 1999). The serum levels of at least oestrone, oestradiol, oestriol, androstenedione, testosterone, dehydroepiandrostenone, progesterone, sex-hormone-binding globulin, and prolactin have been considered as important in this context (Bernstein and Ross, 1993). Lifetime exposure to endogenous sex hormones is determined by several variables including timing

of menarche, age at first full term pregnancy, number of pregnancies, and age at menopause. These variables have therefore all been studied in relation to breast cancer risk (Feigelson and Henderson, 1996).

The increased breast cancer risk associated with early age at menarche (<12 years) (Apter, *et al.*, 1989, Bernstein and Ross, 1993, Henderson, *et al.*, 1985, Kelsey, *et al.*, 1993), could probably be due to prolonged exposure of breast epithelium to estrogens and higher oestrogen levels for several years after menarche in women with early menarche (Apter, *et al.*, 1989, Henderson, *et al.*, 1985). Similarly, later age at menopause increases the number of ovulatory cycles, and may therefore lead to increased risk. For every one-year increase in the age at menopause, the risk of breast cancer increases by approximately three percent (Collaborative Group on Hormonal Factors in Breast Cancer, 1996).

Higher parity and early age at first birth have both been associated with decreased lifetime incidence of breast cancer (Chie, *et al.*, 2000, Kelsey, 1993, Pathak, *et al.*, 2000). Women having their first child before the age of 20 have about half of the risk compared to those having their first child after the age of 30. The mechanism of the protective effect of parity is not completely understood but it probably due to early full differentiation of mammary gland cells, making them less susceptible to carcinogenic transformations (Russo, *et al.*, 2000). Also prolonged lactation has been associated with decreased risk (Lipworth, *et al.*, 2000). Many of the above mentioned hormone related factors have recently been shown to be more strongly associated with oestrogen receptor (ER) and progesterone receptor (PR) positive than negative breast cancers (Enger, *et al.*, 2000, Huang, *et al.*, 2000).

1.4.7.3 Body size

Obesity has been related to both higher endogenous oestrogen levels and increased risk of breast cancer in postmenopausal women who have most of their circulating oestrogen derived from conversion of androgen to oestrogen in adipose tissue (Hunter and Willett, 1993). Another theory suggests a more important role for the localisation of body fat measured as waist-to-hip ratio (Hall, *et al.*, 2000, Mannisto, *et al.*, 1996).

1.4.7.4 Exogenous oestrogen

Since sex hormones have become widely used by women, concern has been raised about their safety. It has not usually been possible to study the effects of single hormones; many are used either in combination or consecutively in the same patient. Therefore, risks are generally assessed in relation to the therapeutic goal of the treatment, *i.e.*, oral contraception or hormone replacement therapy (HRT).

The results from studies on the role of oral contraceptives in breast cancer have been controversial. However, a recent meta-analysis using data from 54 studies concluded that current use of oral contraceptives poses a slight (24%) increase in the risk, which disappears 10 years after the cessation of use (Collaborative Group on Hormonal Factors in Breast Cancer, 1996).The data from 51 studies indicated that risk of having breast cancer is slightly increased in women using HRT (Collaborative Group on Hormonal Factors in Breast Cancer, 1997). A 35% increase in the risk was seen for women who have used HRT for 5 years or longer, which is consistent with the effect of delay in the menopause. The increase in risk largely disappears 5 years after terminating the use of hormones. The combined oestrogen-progestin regimen is associated with greater increases in the risk than oestrogen alone (Schairer, *et al.*, 2000). In some studies, breast cancer in women who have ever used HRT tend to be less advanced at diagnosis and biologically less aggressive than those in never users (Holli, *et al.*, 1998). The overall mortality among HRT users has been shown to be lower but the benefit diminishes with longer duration of use (Grodstein, *et al.*, 1997).

1.4.7.5 Smoking

The studies on smoking and breast cancer susceptibility have given rise to controversial results, and the risk has at its best been only weakly associated with cigarette smoking (Palmer and Rosenberg, 1993). However, statistically significant effects have been seen for early age at starting, and for heavy, current, and passive smoking (Wartenberg, *et al.*, 2000).

1.4.7.6 Alcohol intake

Alcohol has been described as being one of the most constant enhancers of breast cancer risk. In a recent analysis pooling six cohort studies, alcohol was shown to increase breast cancer risk linearly with alcohol consumption from 1 to 5 drinks/ day (Smith-Warner, *et al.*, 1998). However only a 15% increase in the risk was found in a study of alcoholic women (Kuper, *et al.*, 2000).

1.4.7.7 Diet

The human diet contains a great variety of natural carcinogens and anticarcinogens (Sugimura, 2000). Many of these may act through the generation of oxygen radicals, which in turn may lead to DNA damage. Accordingly, a high intake of fat, especially polyunsaturated fatty acids, has been shown to increase breast cancer risk (Bartsch, *et al.*, 1999, Nair, *et al.*, 1999).

1.4.7.8 Ionising radiation

Ionising radiation has been shown to markedly increase breast cancer risk among young women who already received mantle radiation for Hodgkin's lymphoma (Bhatia, *et al.*, 1996). Also high incidence of breast cancer is seen in atomic bomb survivors in Japan (Tokunaga, *et al.*, 1994). It has also been shown that radiotherapy for an existing breast cancer increases the incidence of cancer in the contralateral breast (Boice, *et al.*, 1992).

1.4.7.9 Family history

The most prominent and therefore one of the best studied risk factors is a family history of breast cancer. Over 130 years ago, French physician Paul Broca (1866) reported multiple cases of breast cancer in a family. Since then multiple epidemiological studies have shown positive association between breast cancer and family history. The risk conferred by having a relative with breast cancer varies according to the closeness of kinship, number of affected relatives and age at which the relatives affected (Claus, *et al.*, 1990, Slattery and

Kerber, 1993). Having a first degree relative (mother, sister, and daughter) with breast cancer can increase a woman's risk of breast cancer by two fold. This risk is greater if the relative's age of breast cancer development is lower. Having more than one close relative with breast cancer is associated with even higher risk (Pharoah, *et al.*, 1997 and references therein). Ovarian cancer is also frequent in relatives of familial breast cancer patients (Go, *et al.*, 1983). Breast cancer families also show an early age of onset, increased incidence of bilateral disease, and, occasionally, the occurrence of male breast cancer. This familial clustering and also twin studies are suggestive of inherited susceptibility for familial cases (Peto and Mack, 2000). Hereditary breast cancer accounts for 5-10% of all cases of breast cancer (Broeders and Verbeek, 1997, Lynch, *et al.*, 1994). First evidence for an autosomal dominant breast cancer susceptibility gene with age-related penetrance was provided in 1984 (Williams and Anderson, 1984). This hereditary trait has been shown in several different studies ever since (Go, *et al.*, 1983, Iselius, *et al.*, 1992, Lindblom, *et al.*, 1993, Newman, *et al.*, 1988).

Studies to find genetic basis of breast cancer resulted in isolation of the first breast cancer susceptibility gene *BRCA1* in 1994 (Miki, *et al.*, 1994) followed by identification of *BRCA2* in 1995 (Wooster, *et al.*, 1995). Germline mutations in both *BRCA1* and *BRCA2* genes confer an increased risk of breast and ovarian cancer; the risk is particularly high for *BRCA1* mutation carriers. In families with multiple affected members, female *BRCA1* or *BRCA2* mutation carriers have an approximately 80% chance of developing breast or ovarian cancer by the age of 70 (Ford, *et al.*, 1998).

1.4.8 Predisposing genes

1.4.8.1 BRCA1

BRCA1 is a large gene located on chromosome 17q12-q21 (Hall, *et al.*, 1990). With 22 coding exons, it encodes a 220-kilodalton nuclear protein (Miki, *et al.*, 1994). Exon 11 constitutes more than 60% of the coding region (Miki, *et al.*, 1994). Depending on the population under study, germline mutations in *BRCA1* account for 15-45% of hereditary breast cancer cases, and 80% of families with both breast and/or ovarian cancers (Couch, *et al.*, 1997, Easton, *et al.*, 1993, Narod, *et al.*, 1995). Female mutation carriers of *BRCA1* also have 60%-80% lifetime risk of developing breast cancer (Easton, *et al.*, 1993,

Struewing, *et al.*, 1996) and 20%-40% lifetime risk of developing ovarian cancer (Easton, *et al.*, 1995, Struewing, *et al.*, 1996). Some studies have reported variation in BRCA1 breast and ovarian cancer risks with mutation position, suggesting that mutations toward the 3' end of the gene are associated with lower ovarian cancer risks (Gayther, *et al.*, 1995). The median age of diagnosis in *BRCA1* mutation carriers is 42 years which is significantly lower than the median age for unselected western European and US women (Easton, *et al.*, 1994).

1.4.8.2 BRCA2

BRCA2 is located on chromosome 13q12-13 (Wooster, *et al.*, 1994). It has 27 coding exons with exons 10 and 11 being the largest, and encodes a 384-kilodalton nuclear protein. Germline mutations of *BRCA2* account for 37% of familial breast cancer. However, only 15% of breast and/or ovarian cancers are due to mutations in *BRCA2* (Ford, *et al.*, 1998). There is evidence for an increased risk of several other cancers, including prostate cancer, pancreatic cancer, gall-bladder and bile-duct cancer, stomach cancer, and malignant melanoma in carriers of BRCA2 mutation (Breast Cancer Linkage Consortium, 1999). A recent study has reported that 2.3% of early-onset prostate cancers are due to *BRCA2* mutations (Edwards, *et al.*, 2003). The lifetime breast cancer risk for carriers of BRCA2 mutation is 60%-85% (Easton, *et al.*, 1997) and lifetime ovarian cancer risk is 10%-20% (Easton, *et al.*, 1997, Ford, *et al.*, 1998). It has been reported that families with a high proportion of ovarian cancers, relative to the frequency of breast cancer, tended to have mutations located within a 3.3-kb region in exon 11 (Gayther, *et al.*, 1997, Thompson and Easton, 2001). Men with germline mutations in *BRCA2* have 6% lifetime risk of developing breast cancer which is 100 fold greater than the baseline risk of the male population.

1.4.8.3 Other Breast cancer susceptibility genes

Although estimation from early studies was that familial early-onset female breast cancers could be due to mutations in *BRCA1* and *BRCA2* in 50% and 35% 0f cases respectively, later studies confirmed that the breast cancer predisposition in a majority (67%) of families with less than six cases of female breast cancer and no ovarian cancer is not due to *BRCA1* or *BRCA2* mutations (Ford, *et al.*, 1998, Huusko, *et al.*, 1998, Rebbeck, *et al.*,

1996, Schubert, *et al.*, 1997, Serova, *et al.*, 1997, Vehmanen, *et al.*, 1997a, Vehmanen, *et al.*, 1997b). In Britain, only 16% of the early-onset breast cancer patients are carriers of these mutations (Peto, *et al.*, 1999). Additionally *BRCA1* and *BRCA2* are not mutated in sporadic breast cancer cases.

Germline mutations in the *TP53*, *ATM*, *PTEN*, *STK11/LKB1*, and *CHEK2* genes have been reported to predispose individuals to breast cancer (Boardman, *et al.*, 1998, Lobaccaro, *et al.*, 1993, Malkin, *et al.*, 1990, Meijers-Heijboer, *et al.*, 2002, Srivastava, *et al.*, 1990, Swift, *et al.*, 1987, Tsou, *et al.*, 1997, Wooster, *et al.*, 1992). However these cases are very rare and are usually part of a multi-cancer and/or developmental abnormality syndrome (Iau, *et al.*, 2001). Overall, mutation in these known susceptibility genes account for 15-25% of familial risk and less than 5% of all cases of breast cancer (Easton, 1999, Nathanson, *et al.*, 2001).

1.4.8.3.1 Ataxia telangiectasia mutant (ATM)

Ataxia telangiectasia is an autosomal recessive disorder resulting from germline mutations in the *ATM* gene. It has a complex phenotype; cerebellar ataxia, neuromuscular degeneration, dilated ocular and facial small blood vessels (telangiectasia), immunodeficiency, chromosomal instability, and a substantially increased incidence of some cancers (Meyn, 1997, Morrell, *et al.*, 1986, Savitsky, *et al.*, 1995). Estimated frequency of the disorder is about 1 per 40,000-100,000 live births (Pippard, *et al.*, 1988, Swift, *et al.*, 1986). Lymphocytic leukaemia and non-Hodgkin's lymphoma (100% life time risk) appear to be the commonest forms of cancer, but solid tumours in various organs including breast and ovary are also associated with ataxia-telangiectasia (Hecht and Hecht, 1990, Meyn, 1997). Heterozygous carriers of *ATM* have 3-4 fold higher risk of developing breast cancer. Unaffected member of AT families have increased risk of breast cancer with increased risk varying from 1.5-9 (Nathanson and Weber, 2001). In one mutation analysis of 82 breast cancer patients with early onset disease (<45 years) who had been exposed to low dose radiation, increased rate of mutation was observed (Broeks, *et al.*, 2000). However, several other mutation analysis studies show that there is no difference between frequency of mutation in young breast cancer patients (<40 years) and control group (Chen, *et al.*, 1998, FitzGerald, *et al.*, 1997, Izatt, *et al.*, 1999, Shayeghi, *et al.*, 1998). These data suggest that mutations of *ATM* gene have a small role in breast

cancer development with population attributable risk of only 1-2% (Nathanson and Weber, 2001, Olsen, *et al.*, 2001). It has recently been suggested that missense mutations of *ATM* may be implicated in breast cancer susceptibility. In agreement with this, an intronic *ATM* mutation has shown to have a dominant negative effect in the presence of a wild type copy of ATM (Chenevix-Trench, *et al.*, 2002).

1.4.8.3.2 TP53

The most frequent point mutations in breast tumours are *TP53* mutations, however *TP53* mutation occurs in only 20-40% of breast tumours (Greenblatt, *et al.*, 1996, Ingvarsson, 2001). Despite the high frequency of somatic mutation of *TP53*, germline mutations of this gene are rare in familial breast cancer and are only seen in Li-Fraumeni syndrome, a rare syndrome of familial clustering of brain tumour, sarcoma, childhood leukaemia, adrenal carcinoma and breast cancer. Penetrance of developing breast cancer is 50% by age of 30 and 90% by age of 70 (Li, 1990, Malkin, *et al.*, 1990). Approximately 50% of Li-Fraumeni families show mutations in *TP53* gene (Brugieres, *et al.*, 1993, Malkin, *et al.*, 1992). A single nucleotide polymorphism in *TP53* has also reported to slightly increase the risk of breast cancer (Dunning, *et al.*, 1999).

1.4.8.3.3 CHEK2

Cell-cycle checkpoint kinase 2 (CHEK2) mediates the cellular response to DNA damage, leading to cell-cycle arrest at G_1 (Chehab, *et al.*, 2000). CHEK2 is phosphorylated by ATM in response to DNA damage, and phosphorylate p53 and BRCA1, which are involved in cell-cycle control, apoptosis, and DNA repair (Also see section 1.7.1 and Fig. 5). Germline mutations of *CHEK2* genes have reported in Li- Fraumeni syndrome and variant LFS, a combination of multicancer phenotypes that fails to meet the stringent criteria for classic LFS (Bell, *et al.*, 1999, Lee, *et al.*, 2001, Vahteristo, *et al.*, 2001).

It has recently been found that the 1100delC variant in *CHEK2* which is carried by ~1% of the population confers a 1.7-fold increased risk and thus acts as low penetrance breast cancer susceptibility gene (Meijers-Heijboer, *et al.*, 2002, Vahteristo, *et al.*, 2002). Two independent studies have reported a higher frequency of this mutation in non-BRCA1/BRCA2 familial breast cancer cases relative to healthy controls (Meijers-Heijboer,

et al., 2002, Vahteristo, *et al.*, 2002). A recent study also reported presence of the 1100delC in 18% of families with hereditary breast and colorectal cancer which is significantly higher than the frequency of this mutation in families with only breast cancer cases (Meijers-Heijboer, *et al.*, 2003).

1.4.8.3.4 PTEN

Mutations in phosphatase and tensin homolog (*PTEN*) are associated with a rare autosomal dominant disorder, Cowden syndrome which is responsible for hamartomas, adenomas and follicular carcinoma of thyroid, adenocarcinoma of gastrointestinal tract and 20-50% increased in lifetime risk of breast cancer (reviewed in Arver, *et al.*, 2000). *PTEN* mutations are absent or quite rare in sporadic or familial breast cancer without other components of Cowden syndrome and it is suggested that the role of this gene in development of breast cancer is quite small (Arver, *et al.*, 2000).

1.4.8.3.5 STK11/LKB1

The *STK11/LKB1*, a tumour suppressor gene which is associated with most cases of Peutz-Jegher syndrome was identified after it was observed that chromosome 19p was missing in intestinal hamartoma (Hemminki, *et al.*, 1997). Apart from gastrointestinal hamartomatous polyps, melanocytic spots of the lips, buccal mucosa and digits, this autosomal dominant syndrome is characterised by a 5-fold increased risk of early-onset breast cancer (Tomlinson and Houlston, 1997). No somatic or germline mutations of *STK11/LKB1* have been found in breast cancer patients and the contribution of mutations of *STK11/LKB1* to breast cancer is very small (Arver, *et al.*, 2000).

Since 75-85% of familial cases and 95% of breast cancer overall can not be explained by the above mentioned susceptibility genes, and family history still remains a strong predictive factor in patients with no detectable mutation in these genes (Claus, *et al.*, 1998), other unknown loci with different penetrances should be involved. Multiple approaches are now being used to identify these additional breast cancer susceptibility genes (Nathanson and Weber, 2001). In one model based on high-penetrance susceptibility loci like *BRCA1* and *BRCA2*, genetic linkage studies are being performed on

families with multiple cases of breast cancer. Based on this model, it is proposed that at least one other major breast cancer susceptibility gene exist (Phelan, *et al.*, 1996, Serova, *et al.*, 1997). Positive linkage was observed at the chromosome region 8p12-p22 in two German breast cancer families (Seitz, *et al.*, 1997), but was not confirmed in a larger study (Rahman, *et al.*, 2000). In a study of Finnish, Swedish and Icelandic breast cancer families, another plausible breast cancer susceptibility locus at chromosome 13q21 was identified (Kainu, *et al.*, 2000), however the finding was not confirmed by other studies (Du, *et al.*, 2002, Thompson, *et al.*, 2002). As genetic linkage studies have had limited success in identifying new breast cancer susceptibility genes, another model has been proposed in which multiple common, low penetrance alleles confer the risk in the majority of the cases.

1.4.8.4 Low penetrance candidate genes

Low-penetrance genes are defined as sequence variants or polymorphisms that might be associated with a slightly elevated risk for breast cancer. However, due to their frequent occurrence, these alterations might confer a much higher attributable cancer risk in the general population than rare mutations in high-penetrance genes (Nathanson and Weber, 2001). In table 2 some characteristics of high penetrance mutations are compared with low penetrance polymorphisms.

Characteristic	High penetrance mutations	Low penetrance polymorphisms
Gene frequency	Uncommon	Common (>1%)
Penetrance	High	Low
Relative risk	High	Low
Population attributable risk	Low	High
Role of environment	Modest	Essential
Study setting	Family	Population
Study type	Linkage analysis	Case-control study

Table 2: Comparison of characteristics of high penetrance mutations and single nucleotide polymorphisms.

Adapted from (Caporaso and Goldstein, 1995).

1.4.9 Single Nucleotide Polymorphisms (SNPs)

The most common polymorphisms in the human genome are single base-pair variants, also called single nucleotide polymorphisms (SNPs) which constitute about 90% of human genetic variations (Collins, *et al.*, 1998). A SNP is a stable substitution of a single base with a frequency of more than 1% in at least one population (Taylor, *et al.*, 2001). More than $2X10^6$ SNPs have been identified so far (Marth, *et al.*, 2001). The overall frequency of SNPs throughout the genome is estimated to be one in every 1-2 kilobase pairs (Sachidanandam, *et al.*, 2001, Venter, *et al.*, 2001). These variations in DNA sequence influence individual characteristics such as physical appearance, susceptibility to diseases and response to treatments. Based on the position of SNPs in the human genome they have been classified as those that occur within coding region of genes and those that fall in non-coding regions. Depending on whether a coding SNP leads to an amino-acid substitution or not, they are classified as non-synonymous (change in amino-acid) and synonymous (no change in amino-acid). The former group can also be sub-classified into non-conservative and conservative groups (Risch, 2000). Non-synonymous SNPs are potentially important because they lead to amino-acid changes which may have functional effects and phenotypic outcomes (Risch, 2000). It is estimated that 60000-250000 SNPs fall in the category of non-synonymous coding SNPs (Risch, 2000, 2001). Non-coding SNPs are also separated into those occur in the 5' untranslated region (UTR), 3' untranslated region and other non-coding SNPs. A SNP in the 5' promoter region could alter the rate of transcription or it may increase or decrease gene expression either by affecting mRNA stability when occurring in a gene's 3' untranslated region.

There are many less common single nucleotide variants that do not have sufficiently high frequency to meet the definition of a SNP; however they may have significant biological or clinical importance (Taylor, *et al.*, 2001).

Based on the biological plausibility, candidate low-penetrance genes are usually chosen from among the genes in which even a subtle change could have an effect on the biochemical pathways that influence carcinogenesis. For example, the genes involved in carcinogen and steroid hormone metabolism, cell cycle control, DNA damage recognition and repair, apoptosis and immune surveillance have been hypothesized as candidates for low-penetrance cancer susceptibility genes (Nathanson and Weber, 2001). Due to the rare appearance of low-penetrance alleles in cancer families, linkage analysis has low power to identify these genes (Houlston and Tomlinson, 2000). Either population-based association

studies or the evaluation of low-penetrance genes as modifiers of high-penetrance genes are currently the two most suitable approaches.

1.4.10 The role of environmental factors

Since 1775 when the first link was established between environmental exposures to coal tar and the development of scrotal cancer (Waldron, 1983), epidemiological studies have uncovered the essential role of environmental determinants in the development of cancer. However in many settings, tumours develop in only a fraction of the population exposed to an established human carcinogen. This could be due to inter-individual variations in genes whose products metabolise carcinogens or involve in repair of DNA damage. One subject may be10-200 times more sensitive than another (Venitt, 1994) and may therefore develop cancer, while others at the same level of exposure will not. Therefore carcinogenic risk of many exogenous or endogenous exposures may be modified by common genetic polymorphisms in different genetic pathways. On the other hand a genetic susceptibility gene may only be activated in the presence of a certain level of environmental exposure. Understanding how these low-penetrance genetic factors interact with the environment is of great importance in understanding tumour biology, determination of the population at risk and possibly in cancer prevention.

1.4.11 Association Study

Although a linkage analysis approach has been used successfully to map simple Mendelian diseases, it has not yielded consistent evidence for mapping complex disease genes. It has been suggested that linkage analysis may have less power for weak effect genes compared to case control studies (Risch and Merikangas, 1996).

An association study or case-control study is the study of the genetic influences on a complex trait by generating hypotheses about candidate genes that might have a role in the aetiology of the disease. In this type of study the correlation between genetic variants and the trait is assessed on a population scale. Allele frequencies in cases (*i.e.,* breast cancer patients) compared to of control, unaffected patients. However, a potential problem with case-control studies is population stratification, in which both disease risk and frequency of polymorphism/mutation vary among ethnic groups. Matching cases to controls

on ethnic background is one way of avoiding the problem of population stratification bias. However, determination of ethnicity is not always easy, particularly in a large-scale epidemiologic study. Another way to overcome the problem of population stratification is by using family based case-control studies. Two main forms of this study have been introduced; the case-parent study and case-sib study. The case-parent study compares the number of heterozygous parents who transmit one allele with the number of heterozygous parents who transmit the other allele to the affected offspring. In practice the use of this approach is limited to diseases that occur at young enough ages that parents of the cases are alive. In a Sib-case study the frequency of transmitted allele is compared between affected and non-affected siblings. The Sib-case study is used for diseases with variable age of onset for which parents of affected offspring are more likely not to be alive. Family-based case-control studies are valid on the assumption that parental alleles are transmitted with equal and independent probability in the population (Ewens and Spielman, 2001).

1.5 DNA damage and carcinogenesis

DNA is exposed to a variety of different damaging agents, which can be of either exogenous or endogenous origin. Wide range of damage to the DNA is the result of such exposures (Hoeijmakers, 2001a) which can subsequently disrupt the integrity of the genome. Monitoring these damages and maintaining integrity of the genes without significant alteration is an important function of a cell. In addition to efficient DNA repair, correct activation of cell cycle checkpoints is also of great importance for maintaining genome integrity. Normal cells can arrest the cell cycle following a damage occurs to DNA, whereas failure of cancerous cell to arrest their cell cycle because of defective checkpoints may allow replication of damaged DNA (Vogelstein and Kinzler, 1998).

Deficiencies in DNA damage signalling and repair pathways are fundamental to the aetiology of most human cancers. Genomic instability, caused by a wide range of molecular and/or chromosomal alterations in neoplastic cells, is observed in most of human cancer (Cooper DN, in Genetic basis of human cancer, 1998) and is suggestive of sustained genetic damage in all cancer cells (Coleman and Tsongalis, 1999).

1.6 DNA repair in mammalian cells

Several DNA repair pathways have been identified in mammalian cells (Fig. 4). These include Base excision repair (BER), Nucleotide excision repair (NER), Mismatch repair (MMR), Homologous recombination repair (HRR) and Non-homologous end-joining repair (NHEJ). On the whole, each of these repair pathways recognises and repairs one particular form of DNA damage, however they are overlapping in repairing DNA damages (Hoeijmakers, 2001b). The severity of DNA damage, or the context in which damage occurs (e.g. during DNA replication), will often dictate a repair strategy that places survival first and incurs genetic change (Friedberg, 1995).

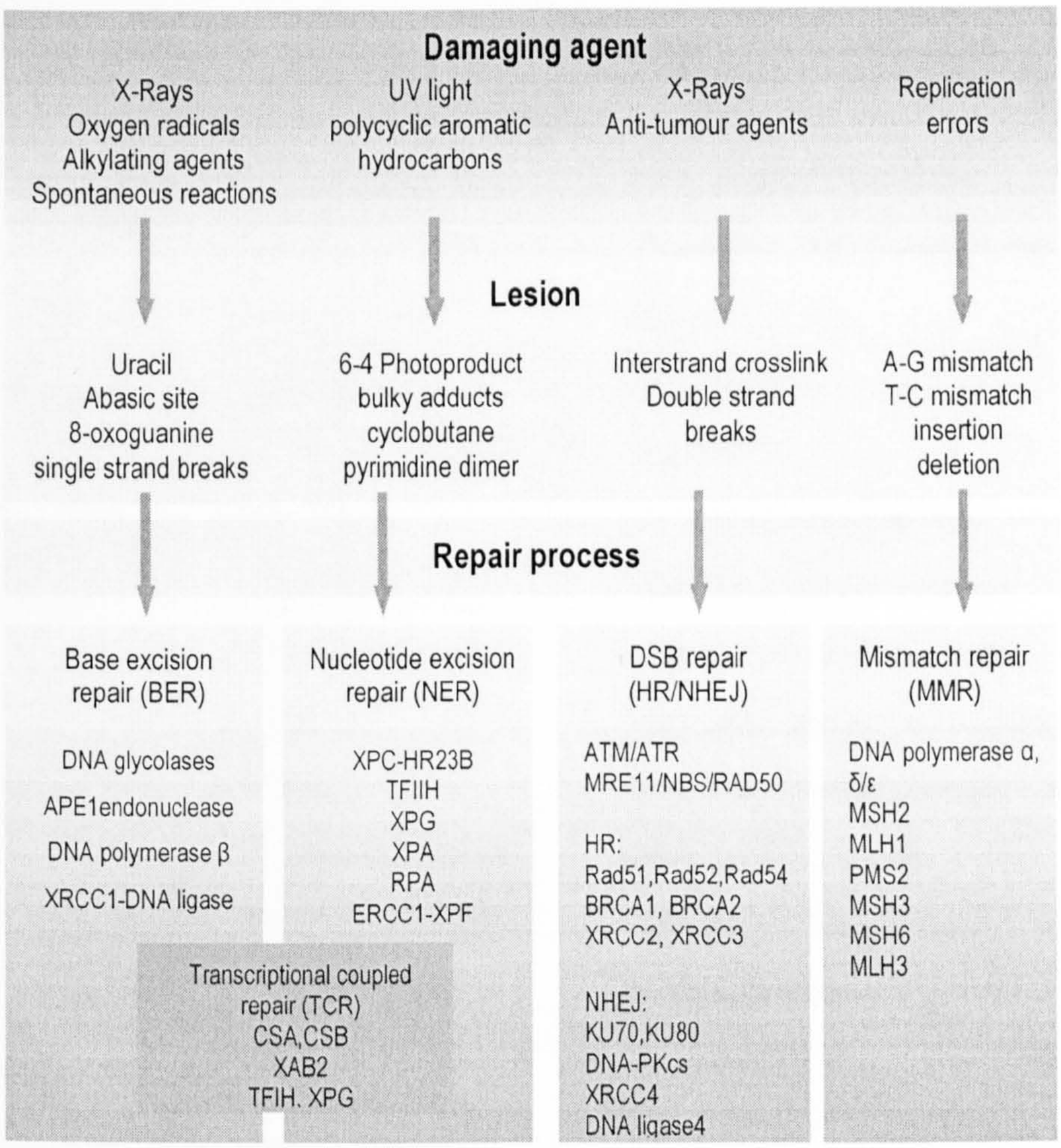


Figure 4: DNA damage and repair pathways.

DNA-damaging agents (top), examples of DNA lesions (middle), and the relevant repair mechanisms (bottom). The essential genes involved in each DNA repair pathway have been shown below the corresponding titles. HR, homologous recombination; NHEJ nonhomologous end-joining; TCR, transcription-coupled repair; BER, base excision repair; MMR, mismatch repair. Adapted from Hoeijmakers (2001), (Khanna and Jackson, 2001), (Svejstrup, 2002).

1.7 Chromosomal instability disorders that predispose to cancer

The consequences of loss of repair capacity are seen in a number of human syndromes and mutant cell lines (Table 3). These disorders show hypersensitivity to ionising radiation and some other environmental agents and often have multiple symptoms, including cancer proneness, neurological disorders, and immune dysfunction.

Gene	Defect	Hereditary syndrome	Cancer
ATM	DBS	Ataxia telangiectasia	Lymphoma, Leukaemia, Breast
MRE11A	DBS	AT-like disorder	Lymphoma
NBS1	DBS	Nijmegene breakage syndrome	Lymphoma
BRCA1	HR	Familial breast cancer 1	Breast, Ovarian, Prostate, Colon
BRCA2	HR	Familial breast cancer 2	Breast (female, male), Ovarian, Prostate, Pancreas
RECQL2	HR	Werner syndrome	Sarcomas
RECQL3	HR	Bloom syndrome	Common cancers
RECQL4	HR	Rothmund–Thomson syndrome	Osteosarcoma
CHEK1	CCC	Not reported	Colorectal and endometrial cancer
CHEK2	CCC	Li-Fraumeni syndrome	Breast, Sarcoma, Brain, Leukaemia
TP53	CCC	Li-Fraumeni syndrome	Breast, Sarcoma, Brain, Leukaemia
MSH2	MMR	HNPCC	Colon, rectum, Endometrium, Ovarian, Urinary organs
MLH1	MMR	HNPCC	Colon, rectum, Endometrium, Ovarian, Urinary organs
XPA-XPG	NER	Xeroderma pigmentosum	Skin
RB	CCC	Familial retinoblastoma	Retinoblastoma, Osteosarcoma
P16	CCC	Familial melanoma	Melanoma, Pancreas

Table 3: Genes involved in human chromosomal instability disorder with cancer predisposition.

Abbreviations: CCC, cell cycle control; DSB, double-strand break repair; HR, homologous recombination repair; MMR, mismatch repair; NER, nucleotide excision break repair; NR, not reported; TCR, transcription coupled repair. Adapted from (Bartek and Lukas, 2001), Hoeijmakers (2001), Svejstrup JQ (2002).

1.7.1 Ataxia telangictasia (AT)

Ataxia-telangiectasia is the best described of the radiosensitivity disorders. Cells derived from AT patients are hypersensitive to DSB-inducing agents (Gatti, 2001). Compared to normal cells, an elevated frequency of chromosomal aberrations is found both spontaneously and after irradiation of ataxia-telangiecasia cells that might be the result of deficiency in repair of DSBs by HR or NHEJ pathway (Jeggo, *et al.*, 1998, Levitt and Hickson, 2002, Thompson and Schild, 2002). Chen, *et al* (1999) also demonstrated that assembly of RAD51 foci in response to DSBs is defective in AT cells. A similar observation has been made by Morrison, *et al* (2000) in $ATM^{-/-}$ chicken DT40 cells. Another striking feature of ataxia-telangiectasia cells is that they fail to suppress DNA synthesis in response to ionising radiation (Painter, 1986). Several studies have also shown that ATM directly phosphorylates BRCA1 on several sites (Cortez, *et al.*, 1999, Gatei, *et al.*, 2000). BRCA1 is also phosphorylated by CHEK2, which acts downstream to the ATM (Lee, *et al.*, 2000). The activation of CHEK2 following induction of DSB is dependent on ATM (Matsuoka, *et al.*, 2000). The NBS1 protein, which is a part of NBS1/MRE11/RAD50 complex in HRR pathway, is also phosphorylated by ATM on several sites in response to DSBs (Wu, *et al.*, 2000, also see section 1.7.1 and Fig. 5). Activated ATM phosphorylates p53 on Ser15. Together these phosphorylations will eventually lead to cell cycle arrest and activation of the cellular pathway for repair of DNA damage (Figure 5).

1.7.2 Nijmegen breakage syndrome (NBS) and ataxia-telangiectasia like disorder (ATLD)

Apart from AT, there are two other phenotypically very similar chromosome breakage syndromes. Germline mutations in *MRE11A* give rise to an AT-like disorder (ATLD) (Stewart, *et al.*, 1999), whereas defects in *NBS1* result in Nijmegen breakage syndrome (NBS) (Carney, *et al.*, 1998, Varon, *et al.*, 1998). The clinical features of these two syndromes overlap, sharing the traits of cancer predisposition, immunodeficiency, hypersensitivity to radiation as well as chromosomal instability (Hoeijmakers, 2001b). Additionally, cells derived from affected individuals show very similar phenotypes which are also similar to that of ataxia-telangiectasia (Antoccia, *et al.*, 1997, Nove, *et al.*, 1986, Petrini, 2000, Taalman, *et al.*, 1983). This can be explained by the interacting roles of

these three proteins in the DSB pathway and the S-phase checkpoint control. MRE11, RAD50 and NBS1 form a nuclear complex, which is found in post irradiation cells at sites of DSBs (Carney, *et al.*, 1998, Maser, *et al.*, 1997) and is essential for normal cellular response to DSBs. ATM phosphorylates NBS1 on Ser343 in response to DNA damage, which is an event required for activation of NBS1/MRE11/RAD50 complex (Zhao, *et al.*, 2000).

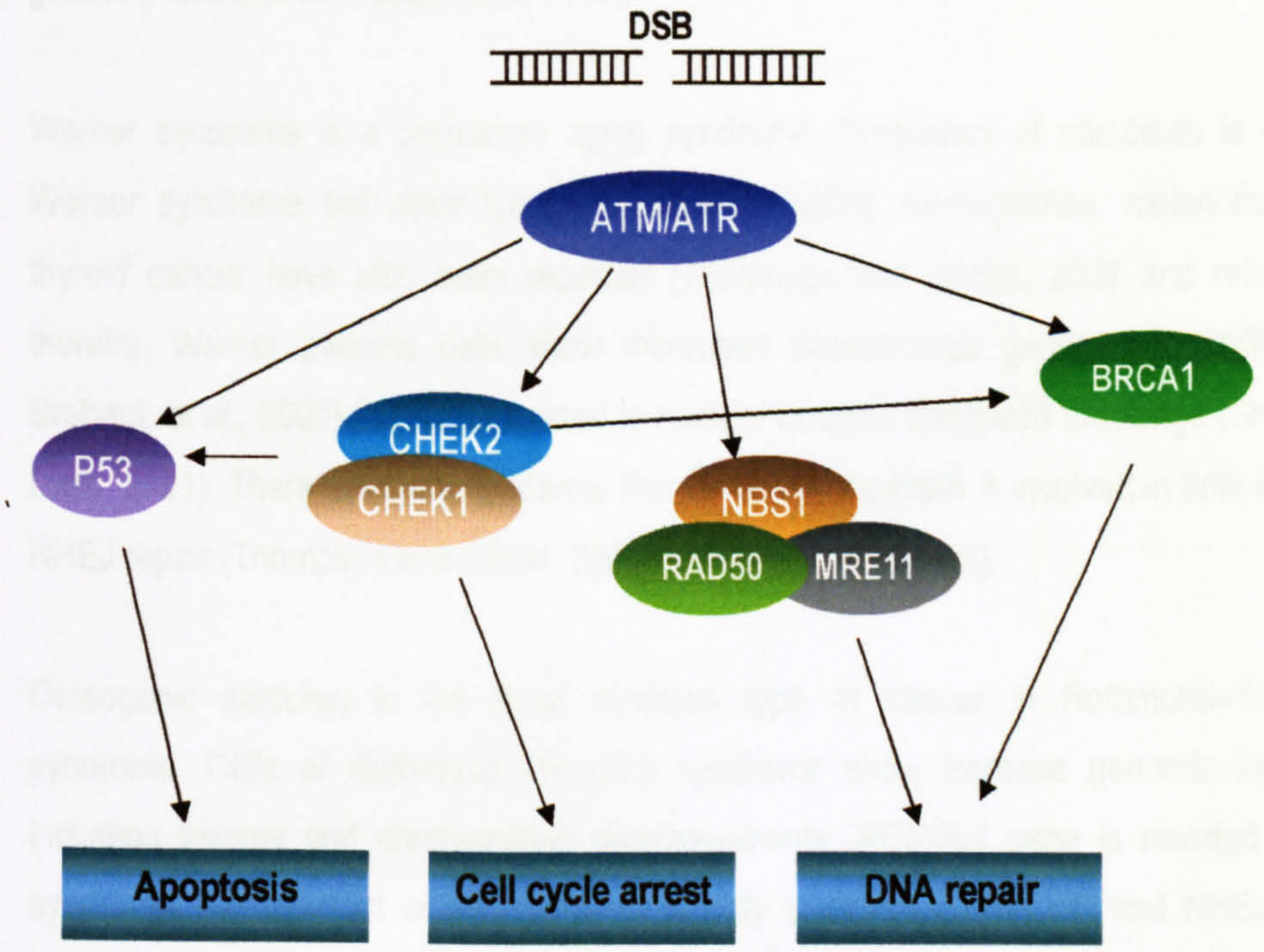


Figure 5: A schematic representation of the DNA DSB signaling pathway.

Modified from Khanna and Jackson (2001).

1.7.3 Bloom's syndrome, Werner syndrome and Rothmund–Thomson syndrome

Other chromosomal instability disorders with cancer proneness are Bloom syndrome, Werner syndrome and Rothmund–Thomson syndrome. These syndromes result from mutations in *RECQ* helicase family of genes that unwind DNA (Mohaghegh, *et al.*, 2001). Bloom syndrome results from a defective *BLM* gene and predisposes to a range of early onset cancers which are common in the population. Genetic instability is seen as high levels of spontaneously-occurring chromosomal aberrations and sister chromatid exchanges in cells of Bloom syndrome patients (Thompson and Schild, 2002); chromosomal sensitivity to X-rays has been found especially in cells in the G2 phase of growth (Aurias, *et al.*, 1985, Kuhn, 1980).

Werner syndrome is a premature aging syndrome. Frequency of sarcomas is high in Werner syndrome but other types of cancer including meningiomas, melanomas and thyroid cancer have also been reported (Thompson and Schild, 2002 and references therein). Werner patients cells show increased spontaneous genomic instability (van Brabant, *et al.*, 2000) but not increased in number of sister chromatid exchange (Shen and Loeb, 2001). There are some evidence that show WRN protein is involved in both HR and NHEJ repair (Thompson and Schild, 2002and references therein).

Osteogenic sarcoma is the most common type of cancer in Rothmund–Thomson syndrome. Cells of Rothmund–Thomson syndrome show increase genomic instability including trisomy and chromosomal rearrangements. *RECQL4* gene is mutated in this syndrome. The product of this gene is possibly involved in both HR and NHEJ repair (Thompson and Schild, 2002 and references therein).

1.7.4 Fanconi's anaemia

Fanconi's anaemia is a cancer-prone disorder, most commonly presenting with acute myeloid leukaemia (15,000-fold increased risk), although solid tumours are also found. Fanconi's anaemia cells show high levels of chromosomal aberrations and are hypersensitive to DNA cross-linking agents. It is an autosomal recessive disorder resulting

from mutations in one of eight genes called FANC genes (Joenje and Patel, 2001). It has been recently shown one of these genes, *FANCD1*, is infact the *BRCA2* (Howlett, *et al.*, 2002). Sequence analysis of *BRCA2* in FANCD1 patients revealed two truncating *BRCA2* mutations. Carrying two mutations in BRCA2 has already been shown to cause embryonic lethality in mice (Connor, *et al.*, 1997), however mice with mild (hypomorphic) mutations can survive to adulthood and show phenotypes such as small gonads, skeletal defects, and sensitivity to DNA cross linking-agents that can be seen in Fanconi's anaemia as well (Yu, *et al.*, 2000). Therefore it has been proposed that hypomorphic *BRCA2* mutations can cause Fanconi's anaemia. Studies have also shown that FANCD2 colocalise with BRCA1 to focal sites after DNA damage (Garcia-Higuera, *et al.*, 2001, reviewed in Thompson and Schild, 2002). Both FANCD2 and BRCA1 proteins are phosphorylated by ATM (Taniguchi, *et al.*, 2002).

Although these chromosomal instability disorders are rarely found in the population, studying the genetic basis of these disorders has established a link between DNA damage, genetic instability and cancer susceptibility. Most of mutant cells of the above-mentioned genes also show some degrees of cellular hypersensitivity to ionising radiation that can cause DNA double strand breaks and is a finding in cells from breast cancer patients as well. The link between cellular hypersensitivity to ionising radiation, DSBs and breast cancer is discussed in section 1.9 in more detail.

1.8 Repairing Double strand breaks

For the cell, double-strand breaks (DSB) are probably the most deleterious form of DNA damage because the genetic information in both strands of DNA molecules may be lost. DSB may arise from ionizing radiation (IR) such as X-rays, free radicals, chemicals, or during replication of single-strand breaks (SSB) (Khanna and Jackson, 2001). Because of the chromosomal fragmentation, translocations and deletions that can result from DSBs, repair of these lesions is important to prevent genome instability and the possibility of carcinogenesis (Kanaar, *et al.*, 1998). The presence of unrepaired DSB is sensed by a DNA damage response system and results in pausing of the cell cycle. Depending on the severity of the damage, cells may either arrest the cell cycle until the damage is repaired,

or if the damage is irreparable, proceed to apoptosis. A single DSB is sufficient to kill a cell if it inactivates an essential gene or triggers apoptosis (Rich, *et al.*, 2000).

There are two distinct and complementary mechanisms for DSB repair: homologous recombination (HR) and non-homologous end-joining (NHEJ) (Haber, 2000, Karran, 2000). When an intact DNA copy is available, HR can be carried out by using the homologous sequence from sister chromatid as a template to repair the damage. Otherwise, cells utilize the more error-prone NHEJ in which the two broken ends of DNA will be joined (See figure 6). It was presumed until recently that HR was the main repair pathway in yeast and NHEJ repair was the most predominant DSBs repair pathway in mammals, but emerging evidence indicates that HR, has a major role in repair of DNA DSBs in mammalian cells as well (Haber, 2000, Liang, *et al.*, 1998).

It has been proposed that Ku70 protein acts as a switch between the two DSB pathways (Goedecke, *et al.*, 1999). When present, Ku70 targets DSB for NHEJ by binding to DNA ends and attracting other factors, including MRE11. However, the absence of Ku70 allows participation of DNA ends together with MRE11 in the meiotic HR pathway. In HR, the DNA ends are first resected in the 5' to 3' direction by the exonuclease activity of the RAD50/MRE11/NBS1 complex (Paull and Gellert, 1998). Several proteins (Rad51, Rad52, Rad54, Rpa, BRCA1, BRCA2, XRCC2, and XRCC3) facilitate the identification and correct positioning of the DNA end with the homologous sister chromatid sequence. The 3' single-stranded tails invade the DNA double helix of the intact molecule, which is used as a template for DNA polymerase to restore information lost at the site of break (See figure 6). Following branch migration and ligation, Holliday junctions are resolved by resolvases to yield two intact DNA molecules (Khanna and Jackson, 2001). This mechanism provides a high fidelity and accurate repair of DSB. In contrast, NHEJ does not require an undamaged template: here, the two DNA ends are simply attached together using the end-binding Ku70/80 complex and DNA-PK_{cs}, followed by ligation by a DNA ligase IV/XRCC4 heterodimer (Haber, 2000, Hoeijmakers, 2001b, Karran, 2000, Khanna and Jackson, 2001). There are studies that show mutations in some of the genes involved in DSB detection and repair give rise to cancer or cancer-predisposing syndromes (Meijers-Heijboer, *et al.*, 2002, Meyn, 1997, Venkitaraman, 2002).

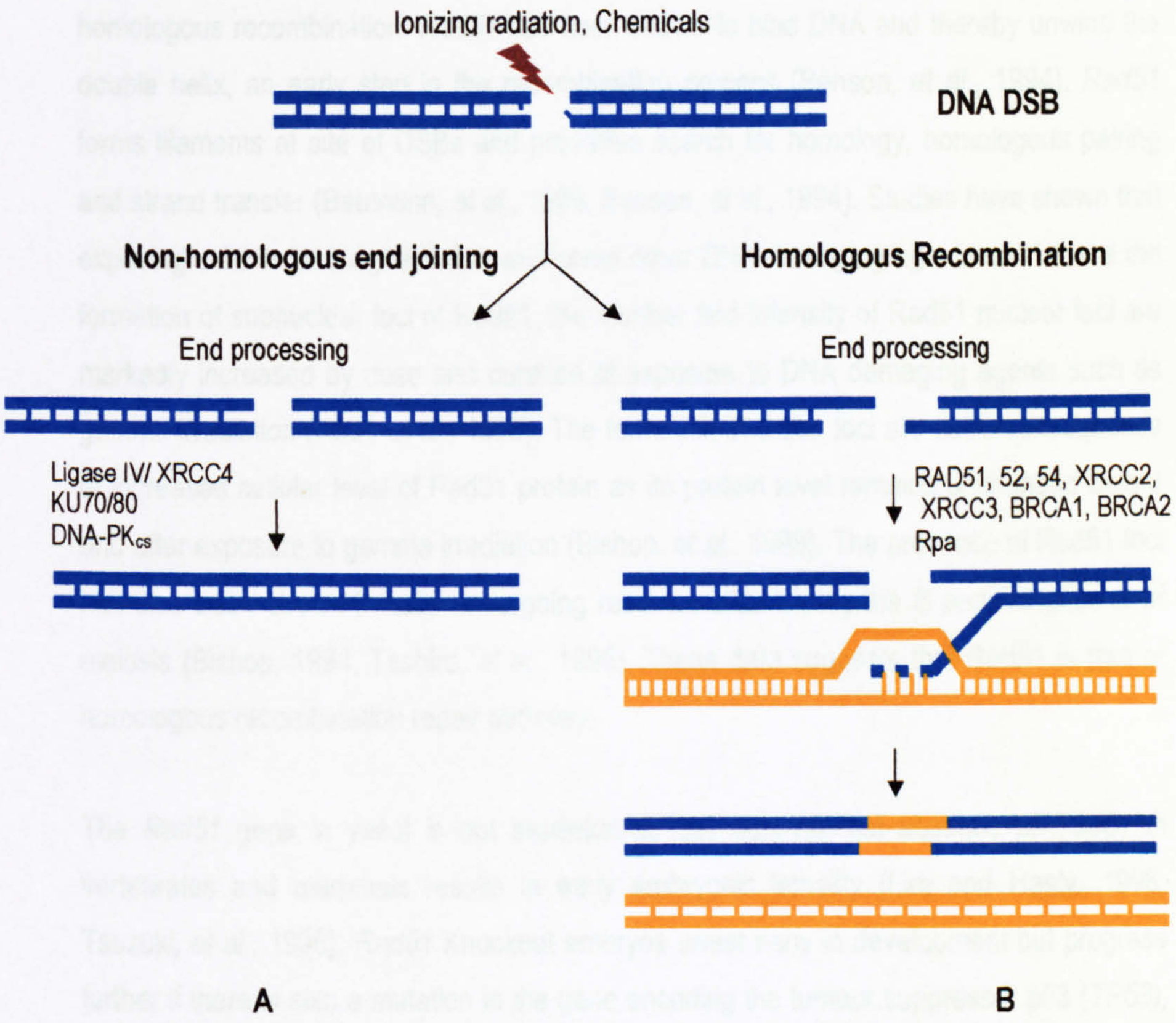


Figure 6: Repair of double strand breaks.

(A) double-strand break is rejoined end-to-end. (B) A double-strand break is repaired with the help of a homologous undamaged DNA (shown in orange). Strand invasion allows re-synthesis on complementary sequence, followed by a resolution of the strands and rejoining.

1.8.1 Genes involved in Homologous Recombination Repair

1.8.1.1 Rad51

Our understanding of the genes involved in HR comes mainly from studies of bacteria and yeast. In bacteria, RecA is the key recombination protein which catalyses DNA strand exchange (Eggleston and West, 1996). In yeast and humans the homologues of RecA have been identified as Rad51 and HsRad51 which show more than 50% amino acid similarity with RecA. The human *Rad51* gene product plays a central role in the process of homologous recombination. *Rad51* has been shown to bind DNA and thereby unwind the double helix, an early step in the recombination process (Benson, *et al.*, 1994). *Rad51* forms filaments at site of DSBs and promotes search for homology, homologous pairing and strand transfer (Baumann, *et al.*, 1996, Benson, *et al.*, 1994). Studies have shown that exposing cells to ionizing radiation and some other DNA damaging agents can induce the formation of subnuclear foci of Rad51, the number and intensity of Rad51 nuclear foci are markedly increased by dose and duration of exposure to DNA damaging agents such as gamma irradiation (Haaf, *et al.*, 1995). The formation of these foci are not a consequence of increased cellular level of Rad51 protein as its protein level remains unchanged before and after exposure to gamma irradiation (Bishop, *et al.*, 1998). The presence of Rad51 foci has also been shown in cells undergoing recombination during the S and G2 phases of meiosis (Bishop, 1994, Tashiro, *et al.*, 1996). These data suggests that Rad51 is part of homologous recombination repair pathway.

The *Rad51* gene in yeast is not essential for cell survival, but absence of *Rad51* in vertebrates and mammals results in early embryonic lethality (Lim and Hasty, 1996, Tsuzuki, *et al.*, 1996). *Rad51* Knockout embryos arrest early in development but progress further if there is also a mutation in the gene encoding the tumour suppressor p53 (*TP53*), possibly because of a reduction in programmed cell death. This may suggest that *Rad51* in mammals has functions in addition to recombination and repair, indicating a role in maintaining genome stability (Lim and Hasty, 1996). In another study deletion of *Rad51* in a chicken cell line resulted in accumulation of cells in G_2 and M stages of the cell cycle, and also accumulation of chromosomal breaks and early cell death, suggesting an important role for *Rad51* during replication (Sonoda, *et al.*, 1998).

1.8.1.2 Other genes involved in HRR

In addition to Rad51, other proteins, including Rpa (Replication Protein A), Rad52, Rad54 and five paralogues* of Rad51 are required for homologous pairing and strand exchange between homologous chromosomes. Rad51 paralogues are *XRCC2, XRCC3, Rad51B (Rad51L1), Rad51C (Rad51L2) and Rad51D (Rad51L3).*

Rpa is a single stranded binding protein and is required for efficient DNA strand exchange. It has been suggested that Rpa acts by removing secondary structure from single stranded DNA and providing a platform for Rad51 to act at the damaged site. It promotes efficient formation of Rad51 nuclear filaments (Sugiyama, *et al.*, 1997).

Rad52 also interacts with Rad51, facilitates loading of Rad51 onto Rpa-bound single stranded DNA, and is required for the strand exchange activity of Rad51 (Benson, *et al.*, 1998, Park, *et al.*, 1996). Deletion of Rad52 in mouse and chicken does not cause embryonic lethality and mutant cell lines do not show increased sensitivity to DNA damaging agents (Rijkers, *et al.*, 1998, Yamaguchi-Iwai, *et al.*, 1998).

Rad54 belongs to a family of DNA-dependent ATPases. It interacts with Rad51and facilitates the strand exchange activity of Rad51 in vitro. Homozygous deletion of Rad54 in mouse and chicken is tolerated, and results in increased cellular radiosensitivity and decreased homologous recombination (Bezzubova, *et al.*, 1997, Essers, *et al.*, 1997).

1.8.1.3 Rad51 paralogues

Rad51 paralogues (*XRCC2, XRCC3, Rad51B, Rad51C, and Rad51D*) share 20 to 30% sequence identity with *Rad51* and to each other. The homology is mainly clustered around two ATP biding domains, Walker Boxes A and B (Liu, *et al.*, 1998, Thacker, 1999, Thompson and Schild, 2001, Walker, *et al.*, 1982). The *XRCC2* and *XRCC3* (X-Ray Repair Cross Complementing) genes were identified on the basis of their ability to complement the sensitivity of mutant Chinese hamster ovary cell lines, irs1 and irs1SF (Fuller and Painter,

* Paralogues are genes that arose through duplication of an ancestral gene and acquired new functions (Thompson LH, 2001).

1988, Jones, *et al.*, 1987), to ionizing radiation and some DNA damaging agents. Other members of Rad51 family, Rad51B, *Rad51C*, *Rad51D* were identified by searching sequence database for Rad51-like sequences (Albala, *et al.*, 1997, Dosanjh, *et al.*, 1998, Pittman, *et al.*, 1998).

Cells deficient in *XRCC2* and *XRCC3* (irs1 and irs1SF respectively) show 2-3 fold increase in sensitivity to ionising radiation and ultraviolet light and are extremely (60-100 fold) sensitive to the DNA cross linking agent Mitomycin-C (Jones, *et al.*, 1987, Tebbs, *et al.*, 1995, Thacker, *et al.*, 1995). These cell lines also show an increase in the number of spontaneous and radiation-induced chromosomal breaks compared to the wild type cell lines V79 (XRCC2 proficient) and AA8 (XRCC3 proficient) (Cui, *et al.*, 1999, Fuller and Painter, 1988, Tebbs, *et al.*, 1995). The chromosomal instability of the deficient cell lines is complemented by transferring part of human chromosome 7 or 14 (containing *XRCC2* and *XRCC3* respectively) (Cui, *et al.*, 1999). Transfection of deficient cell lines with *XRCC2* and *XRCC3* cDNA showed similar results (Bishop, *et al.*, 1998, Liu, *et al.*, 1998, O'Regan, *et al.*, 2001). Further studies showed that Rad51 focus formation after exposure to ionising radiation and DNA damaging agents is defective in irs1 and irs1SF cell lines and is rescued by expression of XRCC2 and XRCC3, suggestive of requirement of XRCC2 and XRCC3 for Rad51 function (Bishop, *et al.*, 1998, O'Regan, *et al.*, 2001). More importantly, irs1 and irs1SF cell lines showed respectively 100 fold and 25 fold reductions in the level of homologous recombination (Johnson, *et al.*, 1999, Pierce, *et al.*, 1999). These findings suggest that the *XRCC2* and *XRCC3* genes are responsible for maintaining chromosome stability and are required for the repair of DNA breaks by homologous recombination (Cui, *et al.*, 1999, Liu, *et al.*, 1998).

Earlier studies suggested that XRCC3 interacts with Rad51 (Bishop, *et al.*, 1998, Liu, *et al.*, 1998) and also that Rad51 paralogues interact with each other in a single large complex (Schild, *et al.*, 2000). However, recent findings indicate that at least two separate complexes exist, in which XRCC2 interacts with Rad51B, Rad51C and Rad51D in one complex and XRCC3 interacts with Rad51C in a separate complex. The interaction between XRCC3 and Rad51 could not be confirmed (Liu, *et al.*, 2002, Masson, *et al.*, 2001, Wiese, *et al.*, 2002).

Targeted disruption of *XRCC2* in mouse results in embryonic lethality from mid gestation onward. Knockout mice also exhibit developmental abnormalities and neurological defects if they survive to later stages of gestation (Deans, *et al.*, 2000). Embryonic lethality has also been reported after disruption of Rad51B and Rad51D (Pittman and Schimenti, 2000, Shu, *et al.*, 1999). These findings indicate that genes involved in HRR are crucial for early embryonic survival and development possibly because of their role in repairing DNA damage in proliferating cells of the embryo (Deans, *et al.*, 2000). However, despite the importance of Rad51 paralogues, their precise function is not yet known, and it is thought that they act as accessory proteins for Rad51 filament assembly and recognition of DSB (Masson, *et al.*, 2001).

1.8.1.4 BRCA genes and HRR

One of the most exciting recent findings has been the involvement of the products of breast cancer susceptibility genes in DNA repair. Cells deficient in *BRCA1* or *BRCA2* spontaneously show a variety of chromosome aberrations (Moynahan, *et al.*, 2001a, Moynahan, *et al.*, 2001b, Patel, *et al.*, 1998, Yu, *et al.*, 2000) indicating that *BRCA* genes are essential for maintaining chromosome structure (Venkitaraman, 2002).

Both BRCA1 and BRCA2 interact with Rad51 (Chen, *et al.*, 1999, Mizuta, *et al.*, 1997, Scully, *et al.*, 1997, Sharan, *et al.*, 1997) and colocalise with Rad51 in Rad51 foci (Scully and Livingston, 2000) even in the S phase of untreated cells (Chen, *et al.*, 1999). Cells deficient in BRCA1 and BRCA2 are also defective in Rad51 focus formation (Bhattacharyya, *et al.*, 2000, Huber, *et al.*, 2001, Yu, *et al.*, 2000, Yuan, *et al.*, 1999). A recent model suggest that BRCA2 works directly to regulate the availability and activity of RAD51 in binding to ssDNA which is important in homologous pairing and strand exchange (Venkitaraman, 2002).

BRCA1 is phosphorylated by ATM which is primary sensor of radiation-induced DSB (Cortez, *et al.*, 1999). This phosphorylation suggests that BRCA1 could act as an intermediate factor between DNA damage recognition and repair (Thompson and Schild, 2002). Additionally it is known that BRCA1 interacts with the MRE11/RAD50/NBS1 protein complex after exposure to ionising radiation (Zhong, *et al.*, 1999). There is also some evidence that show homologous recombination is less efficient in BRCA1 deficient cell

lines (Moynahan, *et al.*, 1999). BRCA1 and BRCA2 deficiency result in early embryonic lethality (Connor, *et al.*, 1997, Gowen, *et al.*, 1996, Hakem, *et al.*, 1996, Sharan, *et al.*, 1997) suggesting that they are required for normal cell proliferation during embryogenesis (Welcsh, *et al.*, 2000). BRCA1 and BRCA2 deficient cell lines exhibit a mild sensitivity to ionising radiation and severe sensitivity to cross-liking agents (Bhattacharyya, *et al.*, 2000, Moynahan, *et al.*, 2001a, Moynahan, *et al.*, 2001b, Yu, *et al.*, 2000). In human BRCA1 deficient cells, increased sensitivity to cell killing by gamma rays and reduced repair of DNA double strand breaks can be partially corrected by the introduction of the normal gene, while mutant BRCA1 genes fail to restore these defects (Scully, *et al.*, 1999).

1.9 Breast cancer and DNA repair

Although hereditary breast cancer only constitutes a small fraction of the total number of cases, study of genes mutated in hereditary cases may provide clues for genetic basis of sporadic breast cancer. As mentioned above, there are many reports that show genes which are involved in maintaining genome integrity are mutated in some cases of breast cancer and account for a proportion of hereditary cases. Below some other evidence that links breast cancer to defects in DNA repair capacity are discussed.

1.9.1 Breast cancer is linked to ionizing radiation exposure

Clearly, ionising radiation (a major cause of double strand breaks) is an important environmental risk factor for breast cancer. The evidence of the breast carcinogenic effect of radiation is supported by studies on Japanese atomic bomb survivors (Tokunaga, *et al.*, 1994). Other studies on women treated with radiotherapy for non-malignant conditions such as thymus enlargement and tuberculosis also showed increased rate of breast cancer (Hildreth, *et al.*, 1989, Hrubec, *et al.*, 1989). Using mantle irradiation for treatment of Hodgkin's lymphoma in girls has also been shown to increase the risk of developing breast cancer (Bhatia, *et al.*, 1996). Furthermore there is some evidence that using radiation for treatment of existing breast cancer can increase the risk of cancer in contralateral breast by 3% (Boice, *et al.*, 1992).

The idea that radiation-induced chromosomal breaks correlates with increased risk of breast cancer is also supported by animal studies. In one study, a significantly higher rate of radiation-induced mammary carcinoma was observed in BALB/c mice compared to C57BL/6 mice. The difference was correlated with radiation-induced genomic instability in mammary tissue. After 16 population doublings, irradiated mammary cells from BALB/c mice showed significantly more chromatid breaks than C57BL/6 mice (Ponnaiya, *et al.*, 1997).

Studies have shown that spontaneous chromosome aberrations and sister chromatid exchanges, as markers of chromosomal instability, are statistically higher in cells of breast cancer patients and their healthy relatives compared to control individuals (Dhillon, *et al.*, 1995, Roy, *et al.*, 2001). The correlation between chromosomal radiosensitivity and breast cancer has also been extensively studied. In a large study, 135 women with single breast cancer were compared to 105 controls with no history of breast cancer. The number of radiation-induced chromatid breaks in lymphocytes was significantly higher in the breast cancer group than the control group (Scott, *et al.*, 1999). Three similar studies with relatively small numbers of breast cancer also reported an increase in median number of radiation-induced lymphocyte chromatid breaks in cases vs. controls (Helzlsouer, *et al.*, 1996, Parshad, *et al.*, 1996, Patel, *et al.*, 1997). Another study on bilateral breast cancer patients also showed that radiation induces a greater number of lymphocyte chromatid breaks in bilateral breast cancers compared to controls (Buchholz and Wu, 2001).

1.9.2 Chromosomal radio-sensitivity in breast cancer patients is a genetic trait

Further evidence that chromosomal radio-sensitivity is genetically controlled was provided by two studies. The first study reported that first-degree healthy relatives of breast cancer patients are 7 times more likely to have suboptimal DNA repair than the control group and their risk of developing breast cancer is 2.7 times higher (Patel, *et al.*, 1997). Another study also showed that 62% of first-degree relatives of radiosensitive breast cancer patients were radiosensitive, compared to 7% in first-degree relatives of less sensitive breast cancer patients (Roberts, *et al.*, 1999). The authors suggested that chromosomal radio-sensitivity is a marker for low penetrance predisposing genes in majority of breast cancer cases. They also suggested genes involved in processing of DNA damage (polymorphisms in

XRCC DNA repair genes) as candidate low penetrance genes (Roberts, *et al.*, 1999, Scott, *et al.*, 1999). Reduced repair capacity of DNA repair among relatives of breast cancer patients (in comparison to the normal population) leads us to the conclusion that DNA repair capacity is a genetic trait (Helzlsouer, *et al.*, 1995, Helzlsouer, *et al.*, 1996) and deficient DNA repair capacity may play an important role in the development of breast cancer.

1.9.3 Mutations in some genes involved in genome stability predispose to breast cancer

Mutations in *TP53*, a tumour suppressor gene whose role has been described as 'guardian of the genome' are associated with breast cancer in Li-Fraumeni syndrome (Faille, *et al.*, 1994, Malkin, *et al.*, 1990). Also germline mutations in *CHEK2*, cell cycle checkpoint have found in Li-Fraumeni syndrome families with multiple breast cancer cases (Bell, *et al.*, 1999). Mutant alleles of *ATM*, a gene that is involved in signalling DNA damage also linked to breast cancer (Athma, *et al.*, 1996, Easton, 1994). Two major breast susceptibility genes *BRCA1* and *BRCA2* have recently been shown to be involved in DNA repair. Specifically, both BRCA1 and BRCA2 co-localise with Rad51 following radiation-induced double strand breaks (Scully, *et al.*, 1997, Sharan, *et al.*, 1997). In addition BRCA1 has been shown to associate with Rad50-Mre11-p95 in order to direct a cellular response to ionising radiation (Zhong, *et al.*, 1999). Homozygous mutations of BRCA1, BRCA2 and ATM result in radiosensitive phenotype (Morimatsu, *et al.*, 1998, Sharan, *et al.*, 1997).

1.10 Outline of present study

Despite active efforts, the existence of additional major high-penetrance cancer susceptibility genes with similar harmful effects, as has been observed for *BRCA1* and *BRCA2*, remains questionable. As defects in DNA repair and cell cycle control are known to promote carcinogenesis, a proportion of inherited breast cancers might be attributable to mutations in the genes involved in these mechanisms. Cellular sensitivity of breast cancer patients to ionizing radiation (a known DNA damaging agent), susceptibility to breast cancer in chromosomal instability disorders such as Ataxia telangiectasia and participation of BRCA1and BRCA2 in repair of DNA damage by homologous recombination are some clues that suggest genes involved in processing and repair of DNA damage could play a role in genetic susceptibility to breast cancer. In particular single nucleotide polymorphisms of these genes that may have small effects on the function of the repair machinery are of interest. Therefore, we wanted to assess the role of single nucleotide polymorphisms of some of the central genes involved in the homologous recombination repair of double strand DNA damage in breast cancer. Furthermore, the tracking of genes important for tumorigenesis in sporadic disease might also open up new perspectives into familial breast cancer.

Another interesting area in the study of single nucleotide polymorphism is whether sequence variance as small as a SNP could really decrease the DNA repair capacity? In this study we tried to answer this question as well.

CHAPTER TWO

CHAPTER TWO-MATERIALS AND METHODS

List of tables and figures

Tables

Figures

2. Materials and Methods

2.1. Subjects and controls

Cancer patients and control individuals were recruited as follows:

2.1.1. Patients

Nine hundred and fifteen unrelated breast cancer patients were recruited from surgical outpatient clinics at the Royal Hallamshire Hospital, Sheffield, UK between November 1998 and June 2001.

Sixty seven paraffin-embedded breast tumour tissues from the above-mentioned unrelated breast cancer patients were also used for mutation detection.

A total of 187 individuals from breast cancer families were recruited from Sweden. High risk breast cancer families denote families with history of breast cancer in at least three 1st degree relatives whereas low risk breast cancer families were defined as families with 2 or less 1st degree relative or history of breast cancer in their 2nd degree relatives.

A total of 156 unrelated prostate cancer cases were recruited from urology clinics at the Royal Hallamshire Hospital, Sheffield, between 2001 and 2002.

One hundred and thirty one unrelated colorectal cancer patients were recruited from those receiving surgery in Sheffield or neighbouring hospitals.

Ninety eight unrelated bladder cancer cases were recruited from urology clinics at the Royal Hallamshire Hospital, Sheffield, between 1991 and 1996.

Thirty seven individuals from multi-case cancer families were recruited from regional oncology clinics at Weston Park Hospital, Sheffield. Multi-case cancer families address to families with history of a cancer in at least three 1st or 2nd degree relatives.

The diagnosis of cancer was confirmed for all above-mentioned patients by histopathologic examination of their cancer tissues by a histopathologist.

2.1.2. Controls

Individuals for control groups were recruited from the healthy population as follows:

Eight hundred and forty healthy women attending mammography screening in Sheffield Breast Screening Service were recruited between 2000 and 2002. Inclusion criteria for this group was limited those women whose mammogram showed no evidence of a breast lesion.

Four hundred anonymous healthy women were recruited from amongst blood donors in Sheffield during 1996.

Three hundred and thirty eight anonymous healthy men were also recruited from amongst blood donors in Sheffield.

2.1.3. Ethical approval

Ethical committee approval was obtained from South Sheffield Research Ethics Committee and informed written consent was obtained from all subjects (See appendix 1).

2.2. Materials

2.2.1. General Laboratory equipment and reagents

2.2.1.1. General Items

All chemicals used in the preparation of buffers and solutions purchased from Sigma-Aldrich or BDH Merck unless otherwise stated. Solvents were purchased from Fisher Scientific Ltd.

Deionised water for rinsing glassware was obtained by passing tap water through a reverse osmosis system. Water used for preparation of standard solutions and buffers was purified using Purite water system (Purite Ltd.).

Where required, solutions and equipment were sterilized by autoclaving at 20lb/inch^2 for 20 minutes before use (Boxer Laboratory Equipment).

All glassware was washed in RBS 25™ general purpose surfactant (Medline Scientific Ltd.), rinsed several times in tap water and finally in de-ionised water. It was then dried in a hot air oven at 80°C. Items requiring sterilisation were then autoclaved.

2.2.1.2. Plastic and disposable equipment

Plastic and Disposable Items were purchased from the following suppliers:

Item	Supplier
5ml plastic pipettes	Corning Incoporated
10 ml plastic pipettes	Corning Incoporated
10µl,20µl,200µl and 1ml pipette tips	Sarstedt Ltd.
Filtered 10µl,20µl,200µl and 1ml pipette tips	Starlab GMBH
0.5ml,1.5 ml and 2ml eppendorf tubes	Sarstedt Ltd.
15 ml centrifuge tubes	Iwakai Ltd.
6 well tissue culture plates	Greiner Labortechnik
25 Cm^2 tissue culture flasks	Nalge Nund Ltd
75 Cm^2 tissue culture flasks	Nalge Nund Ltd
100 mm tissue culture dishes	Sarstedt Ltd.
2ml, 5ml, 10ml Syringes	Becton Dickinson & Company
Sterile disposable Scalpels	Swann - Morton
96 well PCR plates	Advance Biotechnologies Ltd.
Thin walled PCR tubes	Intermountain Scientific Corporation
1.2ml Cryovials	Nalgene Ltd.
PCR flat cap strips	Advance Biotechnologies Ltd.
Syringe driven filter unit 0.22µm	Millipore
15ml Falcon® 2059 polypropylene tubes	Becton Dickinson
Genepulser® electroporation cuvette 0.4cm	BioRad
Centrifugal filter tube, 5000 NMWL	Millipore
Rehydration tray	BioRad Laboratories Ltd.

Table 1: Plastic and disposable equipment

2.2.1.3. General Laboratory Equipment

General laboratory instruments and machinery was supplied by the following suppliers.

Item	*Supplier*
Ice Machine	Scotsman Ice System Ltd.
P2, P10, P200 and P1000 Pipettes	Finpipette
P10 and P50 Multi channel Pipettes	Status
Electric Pipette	Jencons Scientific Ltd.
Co_2 Incubator	Sanyo, Gallenkamp plc.
Sterile containment II safety cabinet	Walker Safety Cabinet Ltd.
Hereaus Centrifuge	Sanyo
UV spectrophotometer	Eppendorf
PCR machines	MWG
TaqMan Analyser	Applied Biosystems
Water bath	Grant Instruments Ltd.
Vortex	Scientific Industries Inc.
Heating block	Grant Instruments Ltd.
Light Microscope	Olympus
Power Pac	Bio-Rad Laboratories Ltd.
Wet blotter	Bio-Rad Laboratories Ltd.
SSCP tank	Bio-Rad Laboratories Ltd.
Mini horizontal electrophoresis tank	Bio-Rad Laboratories Ltd.
Vertical electrophoresis tank-Mini Protean II	Bio-Rad Laboratories Ltd.
Midi horizontal electrophoresis tank	Hybrid Ltd.
UV Transilluminator	UVP Inc.
800W Microwave	Panasonic
Hotplate Magnetic Stirrer	Chemlab
PH meter	Denever Instruments
Electroporator	Bio-Rad Laboratories Ltd.
Orbital incubator	Gallenkamp
Orbital shaker	Stuart Scientific
ABI 7200 sequence detector	Applied Biosystems
ABI 377 sequencer	Applied Biosystems
Balance	Toledo
Protean II XL vertical electrophoresis tank	BioRad Laboratories Ltd.
Protean IEF system *For 2-D with immobilized pH gradients*	BioRad Laboratories Ltd.

Table 2: General laboratory equipment

2.2.1.4. Miscellaneous disposable laboratory equipment

Item	*Supplier*
Para film "M" laboratory film	American National Can Ltd.
Aluminium foil	Terinex Ltd.
Paper tissue	Kimberly-Clark Corporation
Autoclave tape	Rexam Medical Packaging
Latex examination gloves	Ansell Medical

Table 3: Miscellaneous disposable laboratory equipment

2.2.1.5. Cell Lines

Cell Line	*Origin*	*Growth Medium*
Irs1SF	Chinese Hamster Ovary	DMEM
AA8	Chinese Hamster Ovary	DMEM
SW480	Human Colorectal Carcinoma	DMEM
LM217E	Human skin fibroblast	DMEM

Table 4: Cell line used for cell culture studies

AA8 and irs1SF cell lines were kind gifts of Dr. Larry Thomson and SW480 was purchased from ATCC (American Type Culture Collection). LM217E was kind gift of Dr, John Murnane.

2.3. Methods

2.3.1. DNA extraction

2.3.1.1. Extracting DNA from whole blood

- Solution A: 0.3M Sucrose, 7mM Tris base, 5mM $MgCl_2$ $7H_2O$, 1% Triton X-100, pH: 8.0 autoclaved and stored at room temperature.
- Solution B: 0.3 M Tris base, 76mM EDTA, 0.15M NaCl, 100 ml 10% SDS in 1 final volume of litre, pH: 8.0, autoclaved and stored at room temperature.

10-20mls of frozen blood samples (in EDTA blood tubes), were thawed on ice and transferred to 50 ml Falcon tubes. The volume was made up to 45 ml with 1X solution A, gently mixed by inversion. The tubes were centrifuged (Hereaus) at 2000X g for 20 minutes with the centrifuge set at 4°C. The supernatant was carefully removed, leaving behind approximately 10 ml of diluted blood containing nuclei, cell walls, etc. A further 40 ml of 1X solution A was added to the tube, the contents mixed gently, and again centrifuged at 2000X g. After centrifugation the supernatant was completely removed, care being taken not to disturb the cellular pellet. Two ml of blood solution B was added to the pellet, and the pellet was disrupted by aspiration with a pipette several times. The resuspended pellet was transferred to a 15ml falcon tube, 500 µl of 5M sodium perchlorate was added and the lysates were mixed on a rotary wheel for 10 minutes at room temperature. The samples were then incubated at 65°C for 25 minutes in a water bath with occasional inversion. Two ml of cold chloroform (-20°C) was added to each tube and the samples were then mixed for 10 minutes on a rotary wheel at room temperature. The samples were then centrifuged at 2000X g in a bench-top centrifuge for 5 minutes. The aqueous phase (containing DNA) was transferred to new 15ml Falcon tubes, care being taken not to disturb the interface containing denatured proteins. Twice volume of cold (-20°C) absolute ethanol was added and the tubes were inverted gently to precipitate the DNA. The DNA was recovered by spooling on a sterile pipette. The DNA pellets were air dried and the DNA then resuspended in 500 µl of sterile water and left at 4°C overnight to dissolve. All samples were quantified by UV spectrophotometery at 260nm.

2.3.1.2. Extracting DNA from Paraffin-embedded tissue

Paraffin embedded breast tumour tissues were cut into 5 micron sections on a Reichert Jung 2035 rotary microtome using a fresh sterile blades and transferred into 2 ml eppendorf tubes. To remove the paraffin from tissue samples, 1.2 ml of xylene was added to each tube and vortexed vigorously. Samples were centrifuged at 8000X g for 5 minutes. The supernatant was removed and 1.2 ml of 96% ethanol was added to the pellet and mixed to remove the residual xylene. Samples were centrifuged at 8000X g for 5 minutes again and the ethanol was removed carefully by pipetting. Another 1.2 ml of 96% ethanol was added, mixed and centrifuged for 5 minutes again to ensure removal of traces of xylene. The ethanol was removed and the tubes were incubated at 37°C for 10-15 minutes until the ethanol had evaporated. The breast tissues prepared by this procedure were used for DNA extraction by QIAamp® DNA Mini Kit (QIAGEN). In this procedure the tissue was resuspended in a lysis buffer containing Proteinase K and incubated at 56°C in a shaking incubator (Stuart Scientific) overnight until the tissue was completely lysed. The rest of DNA extraction procedure involved binding of DNA onto a silica-gel-membrane and several washing steps to wash away protein and other contaminants. The DNA was eluted with an elution buffer. All DNA samples were quantified and kept at -20°C until use.

2.3.2. DNA quantification

In order to quantify extracted DNA, 1µl of DNA sample was mixed with 99µl sterile deionised water and mixed thoroughly. The concentration of the double strands (ds) DNA was determined using a UV spectrophotometer (Eppendorf) by measuring the absorbance of UV light at 260nM. An optical density of 1.0 was assumed to correspond to 50µg/µl DNA.

2.3.3. Polymerase Chain Reaction (PCR)

20µl final reaction volume using 1.1X PCR mastermix (ABgene) contains:
1.25 units *Taq* DNA polymerase, 75mM Tris-HCl (pH: 8.8), 20mM Ammonium Sulphate, 1.5mM Magnesium Chloride, 0.01% (v/v) Tween 20, 0.2mM each of dATP, dCPT, dGTP and dTTP.

All PCR reactions were prepared in a sterile cabinet in a dedicated area kept solely for that

purpose. Sample cross-contamination was avoided by using filter tips and sterilising the set-up area between users by ultra-violet irradiation. For PCR, Aliquots of 18μl of 1.1X PCR master mix (Abgene) were added to aliquots of 1μl of genomic DNA (100 ng) and 6-10* pmol of each forward and reverse primer. A negative control was always included, using 1μl ddH_2O in place of the template DNA to detect any possible contamination of reaction components. Reactions were overlaid with 20μl of mineral oil. Charged PCR tubes were then cycled in a DNA thermocycler (MWG) for 40 cycles, following an initial denaturing step of 3 minutes at 95°C. Each cycle consisted of 35 seconds at 95°C, 25 seconds at 52-61°C* and 25 seconds at 72°C. A final extension step of 72°C for 10 minutes was included to allow complete extension of the PCR product.

2.3.4. Agarose gel electrophoresis

- Agarose: Low melting temperature agarose was purchased from BioWhittaker Molecular Applications and used for agarose gel electrophoresis.
- 1X TAE: 30mM Tris base, 20mM glacial acetic acid and 2 ml 0.5M EDTA in 1 litre of water (pH:8.0).
- Loading dye: 0.25% Bromophenol blue, 0.25% xylene cyanol and 30% glycerol in water.
- Ethidium Bromide: Tablets of Ethidium bromide were supplied by Sigma. One tablet (100mg) was dissolved in 40ml of ddH_2O in a dark bottle and kept at room temperature.
- 100bp Molecular Weight Marker was supplied by Life Technologies Ltd and stored at 4°C until use.

Low melting agarose gel was dissolved in 1X TAE at a concentration of 1.5% in a microwave oven (Panasonic) until the solution was clear. The solution was allowed to cool to about 55°C, and then ethidium bromide was added to 0.025 μg/ml. The solution was poured onto a pre-prepared electrophoresis tray (BioRad) containing desired combs and allowed to set at room temperature. The comb was removed and the gel was transferred into an electrophoresis tank (BioRad) containing 1X TAE running buffer and the same concentration of ethidium bromide as the gel. DNA samples were mixed with loading dye and then loaded into the wells. Electrophoresis was performed at 70-100 volts for 30-60 minutes depending on the gel size. The electrophoresis was carried out at room

* *Vary between different experiments, see relevant section.*

temperature until the tracking dye was approximately 3/4 the way across the gel. The gel was removed from electrophoresis tank and bands were visualized on UV- Transilluminator (UVP Inc.). The sizes of DNA fragments were estimated by comparison to 100bp DNA size marker (Gibco BRL) run on the same gel.

2.3.5. Genotyping techniques

2.3.5.1. PCR- Single Stranded Conformation Polymorphism for XRCC3

- SSCP loading dye: 95% deionised formamide, 0.05% Bromophenol blue, 0.05% xylene cyanol and 0.1% NaOH.
- Acrylamide was purchased as 30% acrylogel solution from BDH Merck and kept at 4°C until use.
- 1X TBE: 70mM Tris base, 80mM Boric acid and 5 ml of 0.5M EDTA in 1 litre water (pH:8.0).
- Ammonium per sulphate (APS) was prepared fresh at 0.25% concentration each time and kept at 4°C.
- Fixer solution: 10% absolute ethanol and 0.5% acetic acid in ddH_2O, used for fixing gels.
- Silver nitrate was purchased from BDH Merck. To make a working concentration, 1g silver nitrate was dissolved in 1 litre of ddH_2O in a dark bottle, kept in room temperature and used for silver staining of 6-8 gels.
- Developer solution: 1.5% Sodium hydroxide and 0.75 ml of 37% formaldehyde.
- Sodium bicarbonate prepared at 0.75% concentration in ddH_2O and used for final fixation of gels after silver staining.

PCR-SSCP was used to screen for *XRCC3* exon 7 polymorphism, essentially as described by Orieta *et al* (1989) and explained in section 2.4.2. PCR was carried out as described in section 2.3.3. For SSCP, 1μl of aliquot of the PCR product was withdrawn and diluted with 10μl of loading dye. The diluted PCR products were denatured by heating to 95°C for 5 minutes, then rapidly cooled on ice, and 8μl of the product was then loaded in each well of an SSCP gel (12% acrylamide, 0.5X TBE, 160μl 0.25% fresh Ammonium Per Sulphate, and 50μl TEMED). Samples were electrophoresed for 3000 volt/hours overnight at room temperature in 1X TBE running buffer. Constant temperature was maintained using circulation of tap water. After electrophoreses, gels were transferred to a plastic container and fixed by shaking in fixer solution for 15 minutes. Gels were then washed with water and placed in 0.1% silver nitrate solution on a shaker for 20 minutes, washed with water twice and then placed in developer solution on a shaker for 25-35 minutes until the bands were visible. Gels were then placed in 0.75% sodium bicarbonate for 5 minutes and then

transferred into plastic bags, sealed and photographed.

2.3.5.2. Restriction Fragment Length Polymorphism (RFLP) analysis of the D213N variant

Genomic DNA was amplified for XRCC3 exon 7 using primers and conditions which were described in section 2.3.3. The restriction digest was carried out by mixing 7µl of PCR product with 1µl of Bovine Serum Albumin, 1µl Buffer and 10 units Hin *f*I (Promega) overlaid under 20µl of mineral oil. The Mixture was then incubated at 37°C for 2 hours. Gel electrophoresis was carried out by loading 10µl of the digested PCR product onto a 1.5% agarose gel.

2.3.5.3. Fluorescent 5' exonuclease assay (TaqMan™)

The mechanism of 5' exonuclease assay has been explained in section 2.4.3. All probes and primers for TaqMan™ genotyping were designed using Primer Express® software v.1.5 (Applied Biosystems) according to the manufacturer's protocol. Using a constant concentration of FAM probe and different concentrations of TET probe, the emission of FAM and TET was measured by use of the Sequence detector 7200 (Applied Biosystems) and the concentration of FAM and TET probes at which the same amount of fluorescence is emitted by each probe was calculated. The required concentration of the primers was also optimised in a similar fashion by using a constant concentration of the forward primer and different concentrations of the reverse primer. To find the samples which are used as controls a total of 48 random samples were selected. Using the optimised concentration of probes, primers and PCR mastermix a PCR reaction was set. The samples were amplified and analysed by the Sequence detector 7200 under allelic discrimination mode. Once samples with different genotypes (wild type, heterozygous, homozygous) were identified, they used in the following TaqMan screening of the same SNP as controls. The first 24 wells of each 96 well plates were used as controls in which no template DNA and samples with wild type, heterozygous and homozygous genotypes were loaded (6 wells per each control). PCR amplification was performed using 12.5µl of 2X PCR master mix (Applied Biosystems or Eurogentec), 20ng genomic DNA template, 50-300nM forward and 300-

900nM reverse primers, 25-50nM FAM-labelled probe and 50-150nM TET-labelled probe in a final volume of 25µl. The cycling conditions were 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 61-63°C for 1 minute.

2.3.6. Sequencing

2.3.6.1. Preparation of PCR product for sequencing

All samples for sequencing were PCR amplified in a 50µl reaction. The PCR reaction was checked by running out a 5 µl aliquot of the reaction product on a 2% agarose gel. After confirming that the reaction had worked and verifying the product was at the correct size, the remaining 45µl of PCR product was loaded onto a 1.5% agarose gel and electrophoresed at 50 volts for one hour at room temperature. The correct bands were visualised on UV- transilluminator, excised using a sterile scalpel blade, weighed and put in 1.5 ml eppendorf tubes. The DNA was extracted from the agarose gel using a Qiagen gel extraction kit according to the manufacturer's instructions. This involves melting the agarose gel, binding the amplified DNA product onto a silica matrix, washing off unincorporated primers, partially amplified products and other reagents, and then finally eluting the purified product into sterile water. All samples were then stored at 4°C until use.

2.3.6.2. Sequencing reaction

Samples of amplified genomic DNA which were extracted and purified as above were used for preparing the sequencing reaction with the SequiTherm EXCEL™ II DNA Sequencing Kit (Epicentre Technologies) as described originally by Steffens *et al* (1995). Reagents in the kit were gently thawed on ice and vortexed prior to use. All steps were performed on ice. First 7.2µl of 3.5x SequiTherm EXCEL™ II sequencing buffer was mixed with 1-2 pmoles of each labelled primers and 1µl of sample DNA (100ng/µl). The reaction volume was adjusted to 16µl with deionized H_2O and then 1µl of SequiTherm EXCEL™ II DNA polymerase was added. The reaction was mixed well by pipetting, and 4µl of the reaction mix was added to each well containing 2µl of termination mixes (A, C, G and T). The reactions were mixed well, overlaid with 30µl of mineral oil and amplified using a DNA

thermocycler (MWG) for 35 cycles at: 30 seconds of 95°C, 15 seconds at 50°C and 1 minute at 70°C. The samples were then mixed with 3µl of loading buffer included in the SequiTherm EXCEL™II DNA Sequencing Kit-LC (Epicentre Technologies) and denatured at 96 °C for 3 minutes prior to electrophoresis, which was performed with the Li-Cor IR2 4200-S DNA Analysis system (Li-Cor Inc.). Of each sample, an aliquot of 0.5-1µl was loaded on to a 6% Long Range gel by Dr Ian Brock (Institute for Cancer Studies) and run at 1500 V for 7-9 hours, depending of the length of the analyzed fragment. The data were analyzed using the AlignIR™ and BaseImagIR™ softwares (Li-Cor Inc.). Sequences were analysed using the Sequencher software 4.1 (Gene code corporation).

2.3.7. Extracting RNA from mammalian cells

XRCC3 RNA was extracted from LM217E cell line using the RNeasy kit (QIAGEN) according to the manufacturer instructions. The extraction is based on binding of total RNA to a silica-gel-based membrane and washing steps to wash away cell debris and other molecules followed by elution of total RNA with an elution buffer. DNase treatment of the extracted RNA was performed to ensure that any residual amounts of DNA were removed. Using the RNase-Free DNase (Promega), 8µl of extracted RNA was mixed with 1µl of 10X reaction buffer and 1µl of RNase-Free DNase and incubated at 37°C for 30 minutes. Then 1µl of DNase stop solution was added and the reaction incubated at 65°C for 10 minutes to inactivate the DNase and terminate the reaction.

2.3.8. RT-PCR

RT- PCR was performed using 12.5µl of 2X PCR master mix (ABgene), 10 pmol of each forward and reverse primers designed from human XRCC3 cDNA (See chapter 4, Table 1), 2µl of extracted RNA, 0.5µl of reverse transcriptase (AB gene) and 8µl water in total volume of 25µl. The amplification conditions consisted of 30 minutes of incubation at 47°C followed by 2 minutes at 94°C and 40 cycles of 35 sec at 95°C, 35 sec at 57°C and one minute at 72°C. A final step of 10 minute at 72°C was also applied as final extension. A negative control (excluding reverse transcriptase) was used for each sample to determine whether any PCR products originated from contaminating DNA. A 5µl of the amplified

product was loaded on a 1.5% agarose gel and the band was compared to the DNA size marker to ensure that the right size product has been amplified.

2.3.9. Cloning of the XRCC3 cDNA

- PCR2.1-TOPO kit and pcDNA3.1/V5-His© TOPO® TA Expression kit were purchased from Invitrogen and were used for cloning XRCC3 exon 7 and XRCC3 cDNA respectively. They contained SOC medium, competent cells and sequencing primers as well as plasmids and salt solution.
- Luria- Bertani (LB) medium was made by dissolving 25g of LB broth (Merck) in 1 litre of water. pH was adjusted to 7 with 5N NaOH. The medium was autoclaved at 15lb/inch^2 for 20 minutes and kept at room temperature.
- LB agar was made by dissolving 25g of LB broth in 1 litre of water. 15g agar was added and dissolved before autoclaving.

Cloning of human XRCC3 cDNA was performed using pcDNA3.1/V5-His© TOPO® TA Expression kit (Invitrogen, See section 2.4.5). Human XRCC3 cDNA was amplified by RT-PCR as described in sections 2.3.7 and 2.3.8. The cloning reaction was prepared using 3μl of fresh PCR product, 1μl salt solution (1.2M NaCl; 0.06 M $MgCl_2$) and 1μl of TOPO® vector in final volume of 5μl. The reaction was gently mixed and incubated at room temperature for 5 minutes and then incubated on ice.

2.3.9.1. Transformation of vector into competent cell

One vial of One Shot® TOPO10 chemically competent *E.coli* was gently thawed on ice. Two microliters of cloning reaction was added into the vial of competent cells and incubated on ice for 15 minutes. The reaction was then heat shocked at 42°C for 30 seconds and immediately transferred to ice. 250μl of room temperature SOC medium was added to the cells and incubated in the orbital incubator (200 rpm horizontally) at 37°C for 1 hour. The transformation reaction was spread on two pre-warmed culture plates (LB agar containing 50μg/ml Ampicillin) and incubated at 37°C overnight. Ten colonies were picked next day and transferred in 10 separate tubes containing 5 ml of LB medium and 50μg/ml

Ampicillin. Tubes were incubated in orbital incubator (200 rpm horizontally) at 37°C overnight.

2.3.9.2. Extraction of plasmid

Overnight *E.coli* Cultures were removed from incubator and centrifuged at 3000X g for 10 minutes at room temperature. The supernatant was discarded and plasmid was extracted from *E.coli* cells using QIAprep spin Miniprep kit (QIAGEN) according to manufacturer instructions. This method is based on alkaline lysis of the bacterial cells followed by adsorption of DNA onto silica in the presence of high salt, several washes and elution of DNA in water. All transformants were analysed for the correct orientation of the PCR product by sequencing (see sequencing section 2.3.6).

2.3.10. Site-directed mutagenesis

10X PCR reaction for site directed mutagenesis (Stratagene) contains:
100mM Kcl, 100mM $(NH_4)_2SO_4$, 200mM Tris-Hcl (pH 8.8), 20mM $MgSO_4$, 1% Triton X-100 and 1mg/ml nuclease-free bovine serum albumin

The QuickChange™ site directed mutagenesis kit (Stratagene) was used for in vitro re-creation of desired mutations in the *XRCC3* gene (See chapter 4). A pair of complementary mutagenic primers containing the desired base pair change at the site of the mutation was designed for each mutation according to the guidelines proposed by Stratagene. Then a mutagenic PCR reaction mix was prepared for each desired mutation in thin-walled tubes using 5μl of 10X reaction buffer, 2μl of ds plasmid containing *XRCC3* cDNA prepared as described above, 1.25μl (125ng) of each mutagenic oligonucleotide primer and 1μl of dNTP mix and adjusted to 50μl with ddH_2O. Then 1μl of P*fu* Turbo DNA polymerase (2.5U/μl) was added. The reaction mixes were overlaid with 30μl of mineral oil and mutagenic polymerase chain reaction was carried out in the DNA thermocycler (MWG) at 95°C for 30 sec as a denaturing step followed by 12 cycles of 95°C for 30 sec, 55°C for 1 minute and 68°C for 13 minutes. Following temperature cycling, the reactions were placed on ice for 2 minutes to cool them to <37°C. To digest the parental wild type plasmid and to

select for mutant plasmid, each post-PCR reaction was mixed with 1μl of Dpn I endonuclease. The reactions were mixed gently and thoroughly by pipetting, centrifuged for 1 minute and incubated at 37°C for 1 hour to digest.

To transform the mutant plasmids, XL1-Blue supercompetent cells (Stratagene) were gently thawed on ice. For each sample reaction, 50μl of the supercompetent cells was aliquoted into prechilled Falcon 2059 polypropylene tubes and mixed with 1μl of Dpn I-treated plasmid. As an additional control for verification of efficacy of transformation of supercompetent cells, 1μl of pUC18 control plasmid (0.1ng/μl, Stratagene) was added to 50μl of supecompetent cells in a separate falcon tube and mixed. All reactions were incubated on ice for 30 minutes, and then heat shocked at 42°C for 45 seconds followed by 2 minutes incubation on ice. 500μl of preheated (42°C) SOC medium was added to each reaction and the transformation reactions were incubated at 37°C in a shaking incubator (225 rpm) for 1 hour. 250μl of each transformation reaction was then plated on agar plates (two plates per each sample) containing 50μg/ml Ampicillin. Also 50μl pUC18 control (in 200μl SOC medium) was plated on an agar plate containing the same amount of Ampicillin. All plates were incubated at 37°C overnight. The next day the plates were removed from incubator. The numbers of colonies in pUC18 control plates were more than 250 indicating the transformation was successful. Five colonies were picked for each sample, cultured in 10ml of broth containing 50μg/ml Ampicillin in 20ml tubes and incubated at 37°C overnight in an orbital incubator (200rpm, Gallenkamp). Samples (5 per each mutant) were taken out of incubator the next morning and were centrifuged at 3000X g for 10 minutes at room temperature. Supernatants were discarded and plasmids were extracted from the bacterial pellets using Qiaprep spin Miniprep (QIAGEN) according to the manufacturer's instructions. All created mutations were confirmed by direct sequencing using the XRCC3 primers (See section 2.3.6 and also chapter 4 table 1).

2.3.11. Tissue culture

- Phosphate Buffered Saline (PBS) was prepared by dissolving one tablet (Oxoid Ltd.) in 100ml distilled water and was autoclaved before use.
- Dimethyl Sulphoxide (DMSO) was supplied by BDH Merck, prepared at 10% (v/v) in tissue culture media and used for freezing cells.
- Tissue culture media: Dulbecco's modified eagle's medium (DMEM) supplemented with 4.5g/L Glucose and L-Glutamine was purchased from BioWhittaker Ltd. and stored at 4°C until use. All tissue culture media was supplemented with 9% foetal calf serum and checked for sterility before use.
- Foetal Calf Serum (FCS) was purchased from Helena BioSciences, aliquoted in 50ml tubes and stored at -20°C until use.
- Non-essential Amino Acids was purchased from BioWhittaker Ltd. and stored at 4°C until use.
- Presept was supplied by Johnson and Johnson Medical. One tablet of Presept containing 50% Sodium Dichloroisocyanurate was dissolved in 1 litre of sterilised water and kept in room temperature.
- Trypsin was purchased as a 2.5% solution from Gibco BRL and prepared at 1:20 trypsin in sterile PBS and stored at - 20°C until use.
- Versene/EDTA (ethyelenediamine tetra-acetic acid) was supplied by BDH Merck. 0.2g EDTA was dissolved in 1litre of PBS, aliquoted in 10ml bottles, autoclaved at 10lb/inch^2 for 10 minutes and stored at -20°C until use.
- Geneticin (G418 sulphate) was purchased from Invitrogen, Dissolved in fully supplemented growth medium to make a stock concentration of 100mg/ml, filtered using a 0.22 micron filter and kept in 4°C prior to use. The stock made fresh each time and any surplus after use was discarded.
- Lipofectamine reagent was purchase from Invitrogen and kept at 4°C until use.

All tissue culture procedures were carried out under sterile conditions in a containment level 2 laboratory cabinet in order to prevent any microbial contamination. All cell lines were maintained in a 37°C, 5% CO_2, humidified atmosphere incubator. All references to cells incubated at 37°C within this thesis were cultured under these conditions. Cells were routinely passaged at about 80-90% confluence, every 3-4 days.

2.3.11.1. Freezing cells

For freezing, 1X10^7 cells were resuspended in 10ml of culture media and centrifuged at 500Xg. The culture media was removed and cells were slowly resuspended in freezing medium (10% DMSO, 20% FCS, 70% DMEM), care being taken to have a uniform distribution of cells in the medium. The cell suspension was then transferred into 1.2 ml cryovial tubes and stored at -80°C overnight before transfer to liquid nitrogen.

2.3.11.2. Thawing cells

Cryovials of frozen cells were transferred to a 37°C water bath, for rapid thawing to limit the toxicity of the DMSO. Once thawed, cells were centrifuged at 400X g to remove any traces of DMSO, then resuspended in culture medium and transferred to a 25 cm^2 culture flask.

2.3.12. DNA transfection into mammalian cells

2.3.12.1. Electroporation

Chinese Hamster Ovary (CHO), XRCC3 deficient cell lines (irs1SF) were cultured in 75 cm^2 tissue culture flasks until they became confluent. They were then washed, trypsinized and counted. Approximately 15X 10^6 cells were centrifuged and resuspended in 1ml of PBS. Approximately 15µg of plasmids (in 50µl of ddH_2O) carrying wild type or mutant DNA were mixed with 750µl of resuspended cells (in PBS) and transferred into pre-chilled 0.4cm electroporation cuvettes. Electroporation was carried out with the Bio-Rad Gene Pulser at 400 V and 125 µF. The cells were incubated on ice for 10 minutes before and after electroporation. 200µl of post-electroporation cells for each transfection were then plated onto 100mm culture dishes and incubated at 37°C for two days before addition of antibiotic selection media.

2.3.12.2. Lipofectamine™ treatment

Chinese Hamster Ovary (CHO), XRCC3 proficient cell lines (AA8) and human colorectal carcinoma cell line (SW480) were cultured in 100mm tissue culture dishes until became 90% confluent. One microlitre of extracted plasmid for human XRCC3 D213N and wild type human XRCC3 (see section 2.3.9.2) was added to 199µl of serum-free medium in separate tubes (marked A). In other tubes (marked B) 20µl of Lipofectamine was added to 200µl of serum-free medium. The contents of tubes A and B were mixed together and incubated at room temperature for 30 minutes before use. The same mixture without any plasmid was prepared as a control and incubated at room temperature along with the other tubes.

Culture medium was removed from the cells and they were washed with serum-free medium. The mixture containing plasmid and Lipofectamin was added to 1.6ml of serum-free medium, which was then added to the cells and incubated at 37°C for 5 hours. After 5 hours the transfection mixture was removed and 10ml of normal medium containing 9% foetal calf serum was added and incubated at 37°C overnight. Transfectants were used for drug selection as described in section 2.3.13.

2.3.13. Drug selection

A total of $5X10^5$ cells were seeded onto 100mm plates and incubated at 37°C overnight until the cells adhered to the plates. The plates were removed from the incubator the next day, washed with PBS, and fresh culture medium containing 100 µg/ml of G418 sulphate was added. The plates were incubated at 37°C for 10-12 days until drug-resistant colonies formed. Three to six separate clones for each construct (depending on constructs) were picked, expanded and tested in cellular toxicity assays.

2.3.14. Determination of expression of transfected XRCC3 constructs

2.3.14.1. Extraction of mRNA from mammalian cell lines.

Poly-A^+ mRNA was extracted from mammalian cell lines using Oligotex™ Direct mRNA Kit (QIAGEN) according to the manufacturer's instructions. The extraction is based on binding of polyadenylated RNA (poly-A tail mRNA) to a dT oligomer ($dC_{10}T_{30}$) linked to the surface of polystyrene-latex particles and washing away other non-polyadenylated molecules. Then the poly-A^+ mRNA is eluted from its binding by destabilizing the dT:A hybrid using an elution buffer provided in the kit. The extracted mRNA stored in -20°C.

2.3.14.2. RT-PCR

RT- PCR was performed using the same method and primers as described in section 2.3.8 and chapter 4, table 1. A negative control (excluding reverse transcriptase) was used

for each sample to eliminate the possibility that any PCR products originated from contaminating DNA.

2.3.15. Western blotting

2.3.15.1. Extracting Protein from Mammalian cell lines

- RIPA buffer: 1% Nonidet P-40, 0.5% Sodium deoxycholate, 0.1% SDS in PBS stored at 4°C.
- Phenyl Methyl Sulfonyl Fluride (PMSF) was purchased from Calbiochem- Novabiochem Corporation, dissolved in Isoprapanol (57mM) in concentration of 10 mg/ml and stored at -20°C.
- Protein electrolysis (sample) buffer: 1ml glycerol, 0.5ml β-mercaptoethanol, 3ml 10% SDS, 1.25ml 1M Tris-base pH 6.7, 1mg Bromophenol Blue and stored at -20°C.

Chinese Hamster Ovary cell lines irs1SF and AA8 were cultured in 100 mm tissue culture dishes until they reached 80-90% confluence. The culture medium was removed and the cells were washed with PBS twice to remove all traces of growth medium. 1 ml of trypsin was added to each dish and cells were incubated at 37°C for 3 minutes for detachment. 5ml fresh culture medium were added to cells and gently mixed. The contents of each dish were transferred to a fresh 15ml centrifuge tube and centrifuged at 1000X g for 4 minutes. Supernatants were discarded and cell pellets were resuspended in PBS and pelleted again by centrifuging under the same conditions. PBS was discarded and 200μl of ice cold (4°C) RIPA buffer and 2μl of PMSF were added to the cells and mixed gently but thoroughly by pipetting. The contents of the tube were transferred to a fresh 1.5 ml eppendorf tube, incubated on ice for 30 minutes and centrifuged at 14000Xg in a bench-top microfuge at 4°C for 10 minutes to pellet out cell debris. The supernatants (containing protein) were removed, care being taken not to disturb the pellets. One microliter of the protein extract was mixed with 199μl of PBS and used for protein quantification, and the rest of extracted protein was mixed with 100μl of electrophoresis buffer, placed as aliquots of 30μl into several 0.5ml tubes and stored at -70 °C until use.

2.3.15.2. Protein quantification

Proteins extracted from mammalian cell lines were quantified prior to use in western blot. Coomassie™ plus-200 protein assay kit (PIERCE) was used for protein quantification. First

BSA standard (25000μg/μl) was diluted in different concentrations of 1, 5,10,15,20 and 25μg/μl, and 90μl of each diluted BSA was loaded into miniplate wells. They were used as controls to compare the absorbance of sample proteins with the standards. Then 150μl of each extracted protein sample (diluted 1:200 in PBS) was mixed with 150μl Coomassie reagent. The mixture was aliquoted into 3 wells of a miniplate, 90μl in each well for each sample. The absorbance was read at 595nm using an Anthos Denley 2001 plate reader (ANTHOS). The absorbance values from the spectrophotometery report were then entered into excel file where the values for the samples were compared with the values of the standards and the protein concentration for each sample were calculated graphically.

2.3.15.3. SDS Poly Acrylamide Gel Electrophoresis

- Ammonium Persulphate was prepared fresh at a concentration of 10% in ddH_2O and stored at 4°C.
- TEMED: was purchased from Sigma and stored at room temperature.
- Acrylamide: 30% acrylogel solution was supplied by BDH Merck and kept at 4°C until use.
- Molecular weight marker: Pre-stained protein marker was purchased from Bio-Rad and kept at -20°C until use.
- 10X TGS (Running) buffer: 30g Tris-base, 144g Glycine, 10g SDS adjust up to 1000cc with ddH_2O, autoclaved and stored at room temperature.
- Sodium Dodecyl Sulphate (SDS) was purchased from Sigma. 10g of SDS dissolved in 100ml of ddH_2O on a heating stirrer and kept at room temperature.
- To make 5% upper stacking gel: 0.83 ml of 30% acrylamide, 0.63 ml of Tris base pH: 6.8, 50μl 10% of SDS, 50μl of 10% APS, 5μl of TEMED (to be added last) in final volume of 50 ml.
- To make 8% SDS-PAGE gel: 2.6 ml of 30% acrylamide, 3.75 ml of Tris base pH: 8.8, 100μl 10% of SDS, 100μl of 10% APS, 8μl of TEMED (to be added last) in final volume of 10 ml.

Equivalent amounts of each protein sample (varying between 20 to 30μl depending on the samples), diluted in protein electrophoresis buffer, were heated to 100°C for 5 minutes in a heating block. They were then loaded onto the 5% upper stacking gel which concentrates the proteins prior to entering the 8% lower resolving SDS-PAGE gel which separate the proteins according to their size. Gels were run at 150 volts at constant voltage for 1.5 hours along with 10μl of pre-stained high molecular weight protein marker (BioRad). Electrophoresis was stopped when the dye front was about 1 cm from the bottom of the gel.

2.3.15.4. Blotting to PVDF membrane

- Towbin (transfer) buffer: 20mM Tris-base, 0.2M Glycine, 200ml Methanol adjust up to 1000cc with H_2O, autoclaved and stored at 4°C.
- PBS-T: Phosphate Buffered Saline plus 0.1% Tween- 20, stored at room temperature.
- Blocking Solution: 5% dried low fat skimmed milk (Marvel) in PBS-T, stored at 4°C.
- Filter paper: 3mm filter papers were supplied by Bio-Rad.
- Immun-Blot™ PVDF (polyvinylidene difluoride) membrane (0.2μm thick) was purchased from Bio-Rad and used for transfer of protein from gel.

After electrophoresis the gel was removed from the gel cassette and placed in cold (4°C) transfer buffer for 10 minutes to equilibrate. The transfer membrane (Polyvinylidene Difluoride (PVDF) membrane, Bio-Rad) was soaked in 100% methanol for 2 minutes and then soaked in cold (4°C) transfer buffer for 10 minutes. Filter papers (BioRad) were also soaked in cold (4°C) transfer buffer for 2 minutes. The transfer cassette (Bio-Rad) was assembled according to the manufacturer's instructions and the gel was sandwiched between filter papers and PVDF membrane making sure the gel was correctly oriented, care was taken to avoid air bubbles between gel, transfer membrane and filter papers (See section 2.4.4). The protein transfer was performed at 10 volts for 30 minutes. After transfer was completed the PVDF membrane was incubated in blocking solution on a rocking platform in the cold room (4°C) overnight.

2.3.15.5. Immunoblotting

The PVDF membrane was removed from the blocking solution and washed with PBS-T 3 times for a minimum of 5 minutes each. Then it was placed in primary anti-XRCC3 antibody (Santa Cruz Biotechnology) diluted (1:200) in blocking solution (3% non-fat milk in PBS-T), and incubated at 4°C on rocking platform for one hour. The membrane was then washed with PBS-T 3 times for a minimum of 5 minutes each to remove unbound antibody. The membrane was then incubated with Anti-goat IgG/horseradish peroxidase conjugate secondary antibody (Santa Cruz Biotechnology) diluted (1:5000) in blocking solution (3% non-fat milk in PBS-T) at 4°C on rocking platform for one hour. The membrane was then washed 4 times with PBS-T for minimum of 10 minutes each.

2.3.15.6. Detection

The XRCC3 protein was detected using the western blotting detection reagent ECL (Amersham pharmacia biotech) according to the instruction stated by the manufacturer. The membrane was taken out of PBS-T and excess PBS-T was drained off with Kimwipe (Fort James Ltd.). The membrane was placed on cling film and the ECL reagent was added to the top of the membrane with a pipette, making sure that all parts of the membrane were covered by ECL. After one minute, excess ECL was drained and membrane was dried using Kimwipe. The membrane was then placed in thin plastic wrap, placed in a film cassette and was exposed to X-Ray film (Kodak) in the dark for 15 minutes.

2.3.15.7. Developing of X-Ray films

Exposed X-Ray films were developed in the dark, by immersing in X-Ray developer (Kodak; diluted according to the enclosed instruction) for 2 minutes. Films were washed with tap water, then "fixed" by placing in X-Ray fixer (Kodak; diluted according to the enclosed instruction) for 5 minutes. Films were rinsed in tap water for 5 minutes and allowed to air dry.

2.3.16. Two-Dimension gel electrophoresis

- Sample buffer: 8M Urea, 2% CHAPS, 50mM DTT, 0.2% Bio-Lyte, 0.001% Bromophenol Blue. The buffer was distributed into small aliquots and kept at -20°C until use.
- Equilibration buffer: 10ml Glycerol, 12.5 ml of 1.5 mM Tris (pH: 8), 18g Urea and 1g SDS made up to 50ml with ddH_2O.
- Equilibration buffer I: 400mg DTT was added to 20ml of Equilibration buffer.
- Equilibration buffer II: 400mg Iodoacetamide was added to 20ml Equilibration buffer.
- Immobilized pH gradient (IPG) gel strips were bought from BioRad and kept at -20°C until use.
- To make 100ml of 14% SDS Acrylamide gel: 46.5ml of 30% Acrylamide, 25ml of 1.5mM Tris pH: 8.8, 1ml 0f 10% SDS, 0.5ml of 10% fresh APS, 27ml of ddH_2O and 50µl of TEMED.

Proteins were extracted from irs1SF (XRCC3 deficient) and transfected XRCC3 D213N irs1SF cells as explained in section 2.3.15.1. Total protein were quantified as described in section 2.3.15.2 and equal amount of protein for each of the two cell lines was loaded into

5K Dalton Centrifugal filter tubes and centrifuged at 12000Xg for 20 minutes to filter larger proteins.

Sample buffer was added to 30µg of protein to reach the final volume of 300µl for each sample. The whole volume (300µl) of each sample was loaded as a line in channels of a rehydration tray. Next, using forceps, the coversheets of IPG strips (17cm,pH: 3-100) were peeled off and the stripes were placed down onto the samples, care being taken not to induce any air bubble between strip and sample which may interfere with the even distribution of sample in the strip. Each strip was overlaid with 2-3ml of mineral oil along the length of the strip. Strips were rehydrated under active conditions using Protean® IEF cell at 50 volts for 12-16 hours at room temperature and at 40000 volt-hours afterward according to the manufacturer's instructions (Also see section 2.4.7). When the Isoelectric Focusing was finished, the strips were taken off the tray and placed in equilibration buffer I and II for ten minutes each and then loaded onto a 14% pre-prepared SDS polyacrylamide gel and fixed in place with melted 1% agarose. The gel cast was mounted in the electrophoresis tank where electrophoresis was performed at 16 mA/gel for 30 minutes and 24mA/gel for 5 hours. After electrophoresis was completed the gels were removed from the tanks and silver staining was performed using Owl silver staining kit according to the manufacturer's instructions (Owl Separation Systems). When required, western blotting was performed on gels after electrophoresis was finished as described at sections 2.3.15.4-7.

2.3.17. Drug sensitivity tests

- Camptothecin was supplied by Sigma as powder. Stock concentration of 25mM in DMSO was prepared and kept at -20°C.
- Mitomycin-C was supplied by Sigma, Stock concentration of 3µM in DMEM was made and kept at-20°C.
- Thymidine was purchased from Sigma. Stock concentration of 100mM was made and kept at -20°C until use.

Cell survival assays were performed by exposing cells from individual variant or wild type clones to the DNA cross-linking agent Mitomycin-C (MMC), Topo isomerase I inhibitor Camptothecine (CPT) and Thymidine. A total of 500 cells for each clone were seeded into 100 mm dishes in 10ml medium containing 100 µg/ml of G418, and incubated at 37°C

overnight. Desired concentrations of MMC (0-100 nM), CPT (0-75 nM) and Thymidine (0-10 μM) were added and plates were incubated for 10-12 days at 37°C. Plating efficiency was assessed in triplicate for each clone. Colonies were stained with Methylene blue (0.4% methyline blue in 50% methanol) and counted. Cell survival curves were obtained by plotting the survival percentage of each clone against concentration of the drug. Survival percentage was corrected for plating efficiency in the absence of the drug.

2.3.18. Statistical analysis

The results of all genotyping were entered into Excel file 2000 (Microsoft). Statistical significance of difference between case and control groups was performed using calculation of Odds ratio and 95% Confidence Interval (See section 3.2.6 for detail of statistical tests).

The cell survival percentage resulting from drug sensitivity tests were entered into Excel 2000, means and standard deviations were calculated for each drug concentration. The result of cell survival and their standard deviations were then entered into Cricket Graph (Computer associates) and were used for generating drug sensitivity graphs (See chapter 4).

2.4. Mechanism of some techniques used in this study

2.4.1. Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a rapid procedure for the *in vitro* enzymatic amplification of a specific segment of DNA, using two oligonucleotide primers that hybridize to opposite strands, and flank the target DNA (Taylor, 1992; Saiki *et al.*, 1988). A repetitive series of cycles involving template denaturation, primer annealing and the extension of the annealed primers by a heat stable DNA polymerase results in the exponential accumulation of the specific fragment. Three stages are involved (a) Denaturation in which the double stranded DNA is separated into single strand DNA by heating. (b) Annealing in which the primers anneal to flanking regions of the target sequence and (c) Extension in which the annealed primers are extended by the *Taq* DNA polymerase enzyme. In the next cycle the DNA is denatured, and fresh primers bind to repeat the process.

PCR is central to a wide range of molecular biology techniques, including mutation detection, sequencing and *in vitro* site directed mutagensis.

2.4.2. Single Strand Conformation Polymorphism analysis

SSCP is a PCR base technique which is widely used for the detection of sequence variation in DNA such as mutations or normally occurring DNA polymorphisms (Orieta *et al.*, 1989a, 1989b).The technique is based on the principle that the mobility of double-stranded DNA of a given length in gel electrophoresis is relatively independent of nucleotide sequence. In contrast, the mobility of single strands can vary considerably as a result of only small changes in nucleotide sequence. Therefore molecules differing by as little as a single base change may form different conformations relative to the wild type sequence and migrate differently in a non-denaturing polyacrylamide gel, resulting in band shifts (Fig.1).

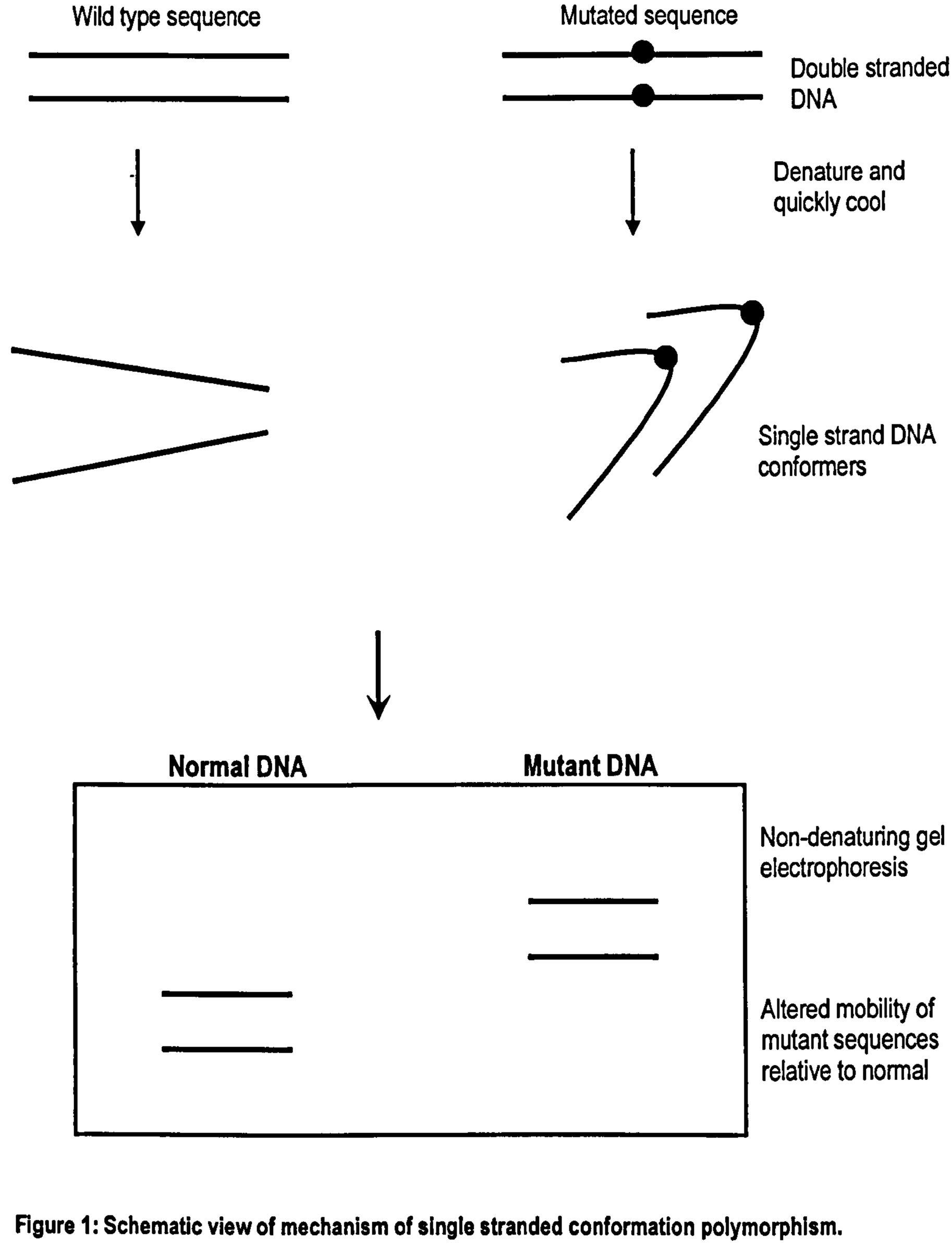


Figure 1: Schematic view of mechanism of single stranded conformation polymorphism.

2.4.3. 5' Nuclease assay (TaqMan®)

TaqMan® is a mutation detection method which is used for the detection of specific, known mutations or polymorphisms. It is based on hybridization of fluorogenic probes, specifically designed for each allele, to the template DNA during PCR. The probes are labeled with different fluorescent reporter dyes, generally FAM (6-carboxy-fluorescein) and TET (6-carboxy-4,7,2',7'-tetrachloro-fluoroscein) at the 5' end and with a quencher called TAMRA (6-carboxy-N,N,N',N'-tetramethyl-rhodamine) at its 3' end. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence. During PCR, forward and reverse primers hybridize to a specific sequence of the target DNA. The TaqMan™ probe hybridizes to a target sequence within the PCR product. The 5' nuclease activity of *Taq* polymerase cleaves the TaqMan™ probe if it is matched to the template DNA; the reporter dye and quencher dye are separated upon cleavage, resulting in increased fluorescence of the reporter which is detected by a detector after amplification is completed (Fig.2). In a case of mismatch between the probe and target sequence, caused by mutation or polymorphism in one or both alleles, the probe is displaced but is not cleaved and no fluorescence is detected. A substantial increase in just one fluorescent signal indicates homozygosity, while similar increase in both signals indicates heterozygosity for the specific alleles.

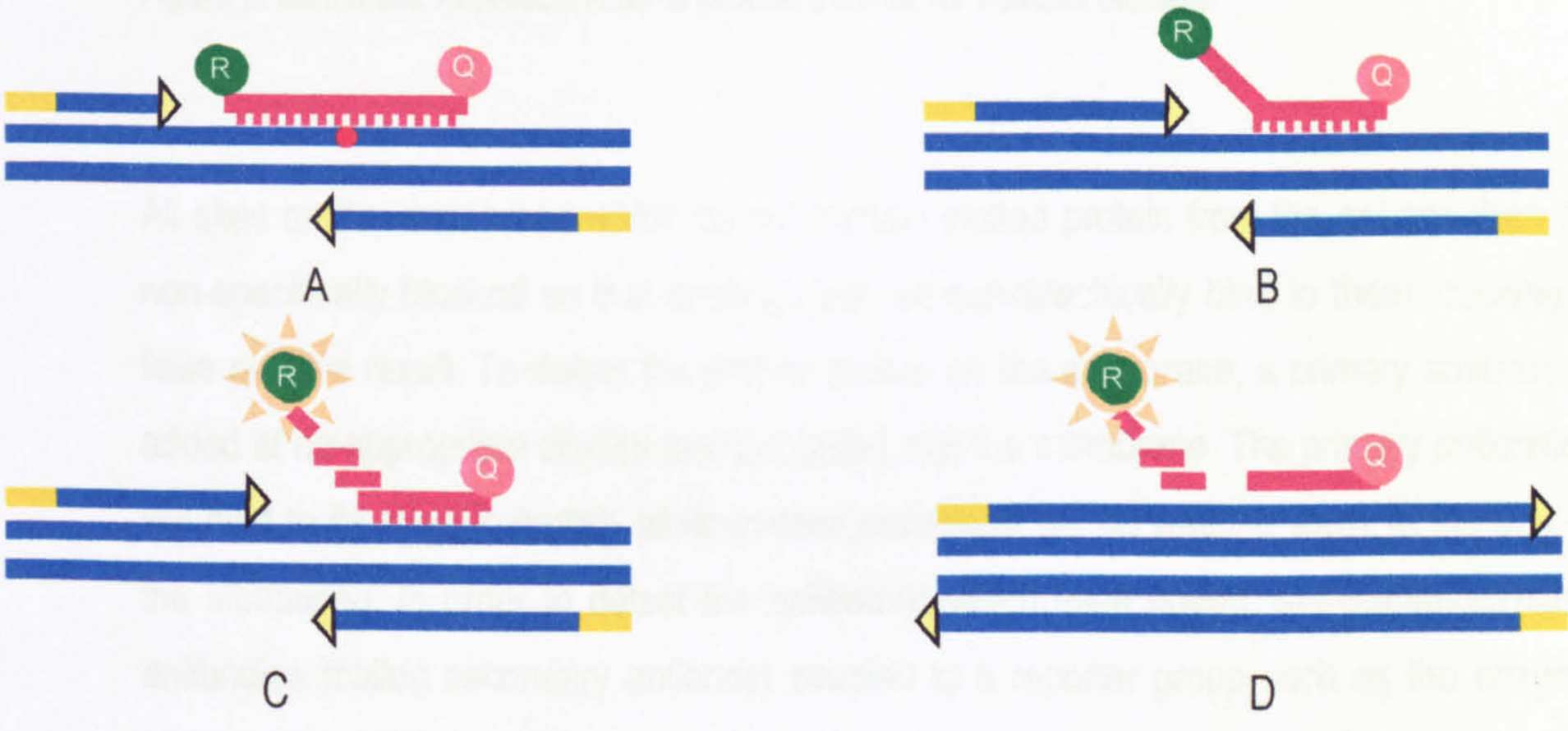


Figure 2: Schematic representation of 5' nuclease assay (TaqMan)

A; showing hybridization of probe to target sequence during PCR, B; Strand displacement by *Taq* Polymerase, C; cleavage of fluorescent bounded probe and D; completion of PCR. R, Reporter; Q, quencher; arrow, *Taq* polymerase; Red dot, polymorphic base.

2.4.4. Western blot

Sufficiently separated proteins in an SDS-PAGE can be transferred to a solid membrane for western blot analysis. For this procedure, an electric current is applied to the gel so that the separated proteins transfer through the gel and onto the membrane in the same pattern as they separate on the SDS-PAGE (Fig.3).

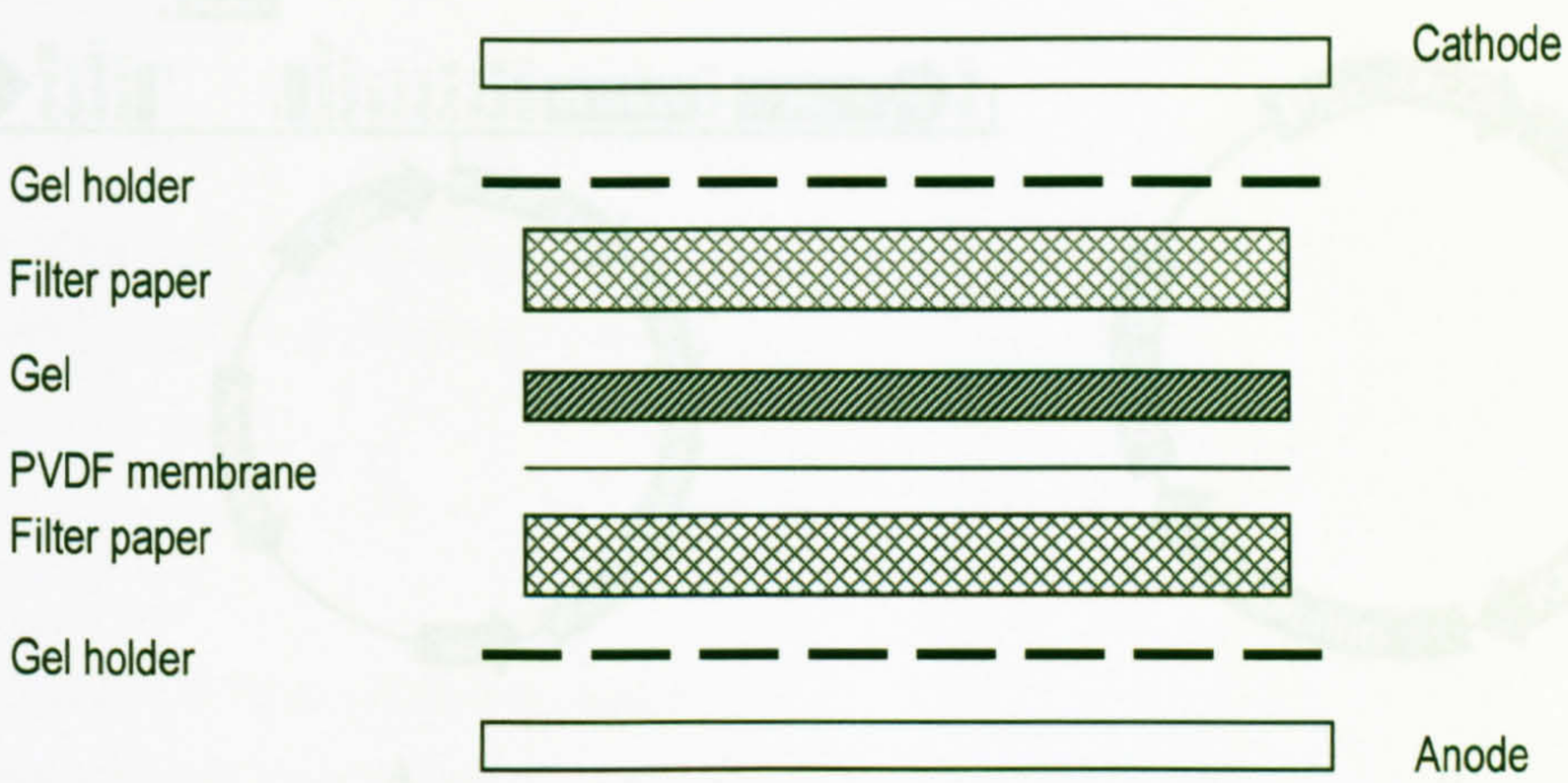
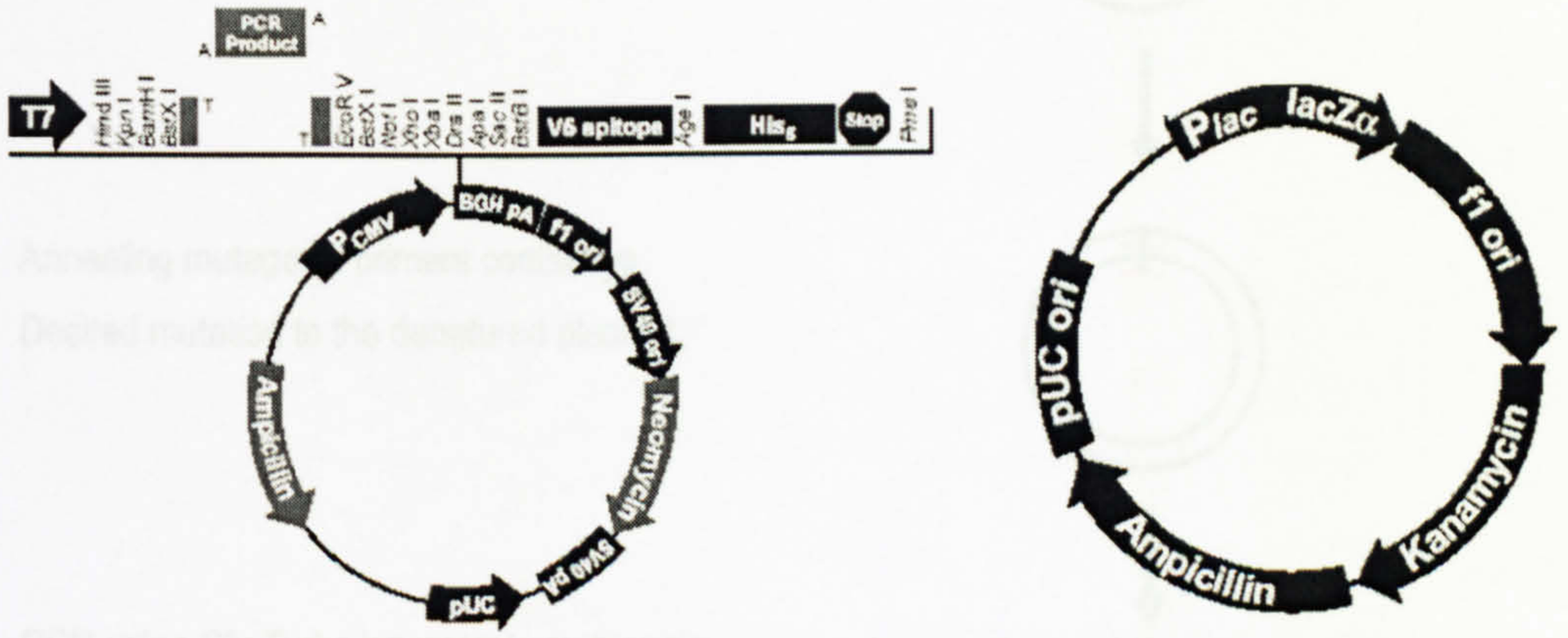


Figure 3: Schematic Representation of protein transfer for western blotting.

All sites on the membrane which do not contain blotted protein from the gel can then be non-specifically blocked so that antibody will not non-specifically bind to them, causing a false positive result. To detect the protein blotted on the membrane, a primary antibody is added at an appropriate dilution and incubated with the membrane. The primary antibodies will bind to its specific protein while excess antibodies will be washed away at the end of the incubation. In order to detect the antibodies which have bound, anti-immunoglobulin antibodies (called secondary antibody) coupled to a reporter group such as the enzyme alkaline phosphatase are added. Finally after excess second antibody is washed free of the blot, a substrate is added which will precipitate upon reaction with the conjugate resulting in a visible band where the primary antibody bound to the protein.

2.4.5. Cloning

The pcDNA3.1/V5-His© TOPO® plasmid vector is a linearized vector with single 3' thymidine (T) overhangs for TA cloning and Topoisomerase I which is covalently bound to the vector. The PCR product which has been amplified using *Taq* polymerase has a single deoxyadenosine (A) residue at its 3' end. Binding of the T overhangs from the vector and the A overhangs from the PCR product allows ligation of PCR insert with the vector.



A B

Figure 4: Vector map of pcDNA3.1 and pcDNA23.1

Vector map for pcDNA3.1/V5-His© TOPO® TA vector (A) and for pCR 2.1-TOPO® vector (B) showing multiple cloning site and their restriction digest sites.

2.4.6. Site-directed mutagenesis

In vitro site-directed mutagenesis is a PCR-based technique which is used for studying protein structure-function relationships and gene expression. There are several approaches to this technique. In QuickChange™ site directed mutagenesis kit, High-fidelity *Pfu*-turbo® polymerase which is a non-displacement DNA polymerase is used. It will extend synthesis directly from each of the mutant primers. Thus, mutant primers become part of replicated plasmid. Treatment of DNA after PCR with the restriction enzyme, DpnI, selectively destroys the parental (non-mutated) DNA while leaving the newly mutated DNA intact. This selectivity is ensured because the parental DNA has been methylated when cloned in bacteria (*E. coli* strain which is used for transformation is *Dam*$^+$, meaning it

contains methylating enzyme). DpnI will only digest methylated DNA. Since the PCR-amplified (mutated) DNA has not been used to transform bacteria, it remains unmethylated and is not digested.

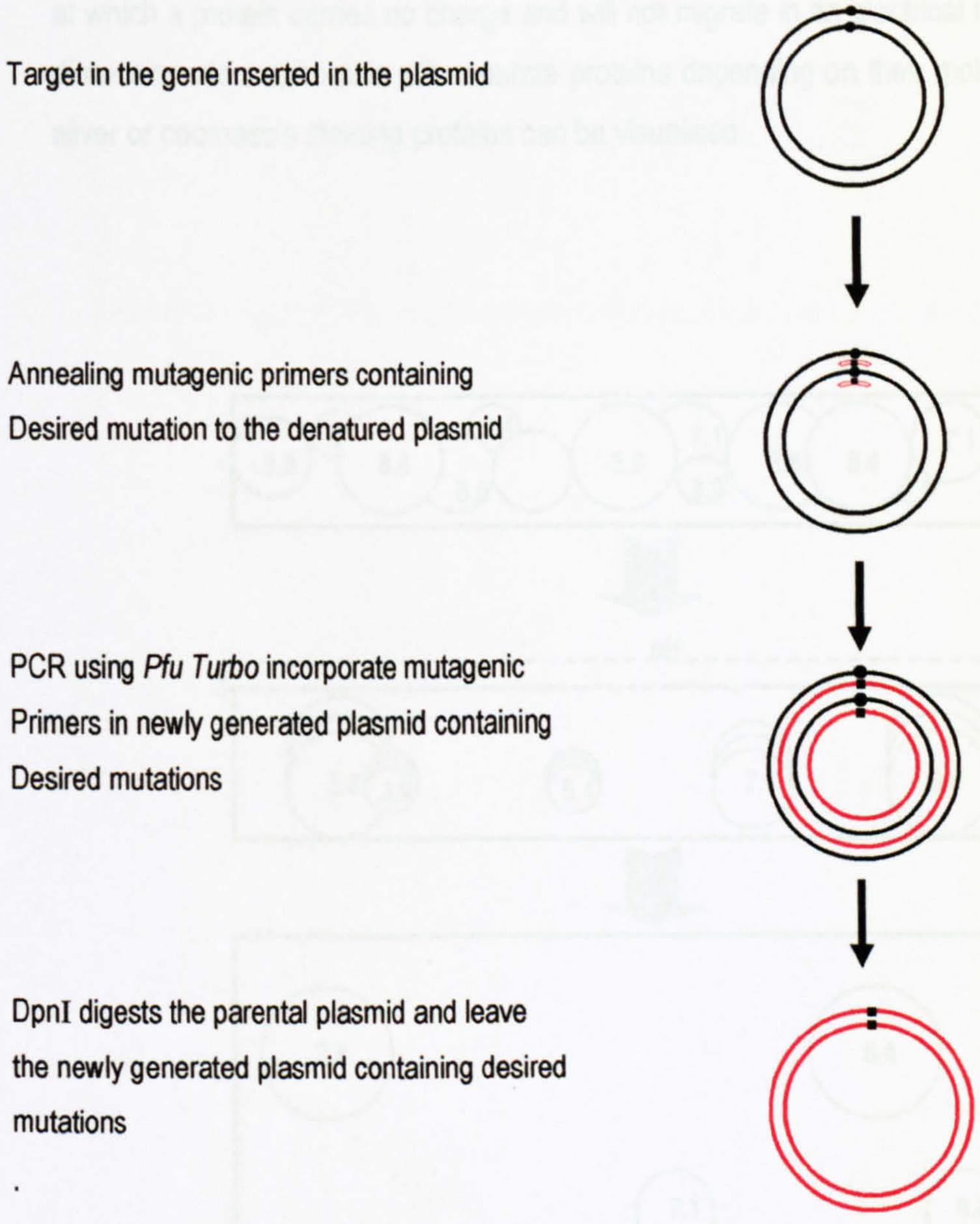


Figure 5: Schematic representation of different steps of "*in vitro* site-directed mutagenesis"

Adopted from protocol manual Stratagene.

2.4.7. Two-Dimensional Electrophoresis for Proteomics

Proteome analysis is a direct measurement of proteins in terms of their presence and relative abundance [Wilkins et al. 1996]. 2-D polyacrylamide gel electrophoresis (2-D PAGE) is a method by which it is possible to separate and analyse hundreds of proteins in a single gel. Proteins are first separated on the basis of their Isoelectric Points (pI), the pH at which a protein carries no charge and will not migrate in an electrical field. The second-dimension electrophoresis will separate proteins depending on their molecular weight. By silver or coomassie staining proteins can be visualised.

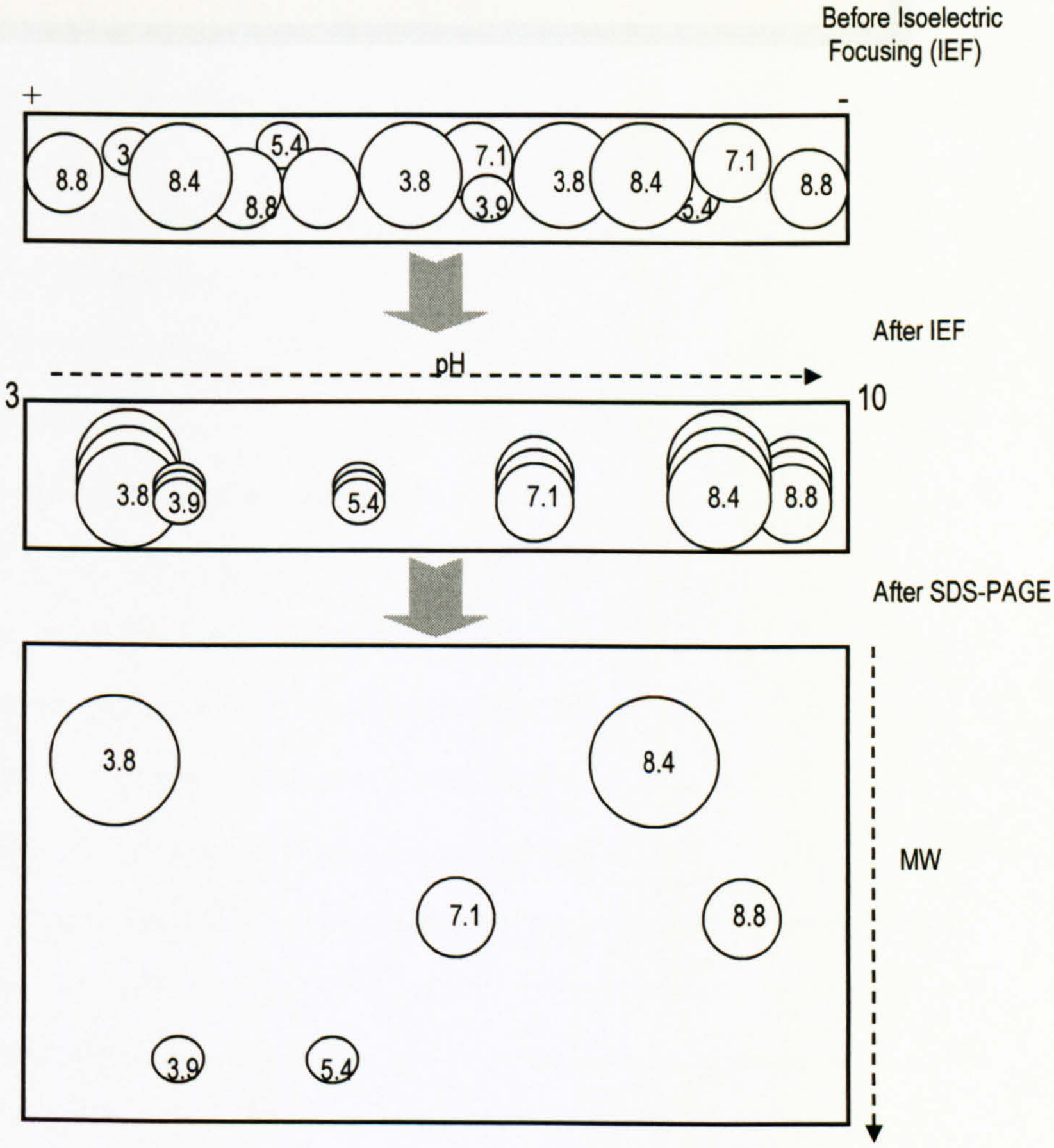


Figure 6: Schematic representation of mechanism of 2-D electrophoresis for proteomics

Adopted from protocol manual BioRad.

CHAPTER THREE

CHAPTER THREE- THE ASSOCIATION BETWEEN SNPs OF HRR GENES AND BREAST CANCER SUSCEPTIBILITY

List of tables and figures

Tables

Figures

3. The association between HRR gene polymorphisms and breast cancer susceptibility

3.1. Introduction

Despite extensive efforts, the search for highly-penetrant breast cancer susceptibility genes other than *BRCA1* and *BRCA2* has not been successful so far. Only 5% of breast cancer cases are related to mutations in *BRCA1, BRCA2* and few other high-penetrance genes. Although these mutations are very rare in the population, they confer a high risk of developing breast cancer among carriers. Whilst research is still being done to find other high-penetrance breast cancer susceptibility loci, common genetic variants or polymorphisms have been proposed as low-penetrant alleles for susceptibility to breast cancer. They are more frequent in the population therefore they could contribute to a larger proportion of breast cancer cases. Inter-individual's response to the environment or susceptibility to diseases may be linked to the presence of DNA sequence variations within critical genes. Therefore subtle change(s) in protein production or function resulting from SNPs in gene(s) whose products are important in cancer prevention could ultimately lead to carcinogenesis through the activation/ inactivation of a certain cellular pathways. SNPs in genes involved in oestrogen hormone metabolism, carcinogen metabolism, DNA damage recognition and repair, cell cycle controls, immune surveillance and apoptosis are among the candidates.

The mammalian genome is regularly exposed to a variety of toxins, from both the external and internal environment, and thus is subjected to DNA damage that can cause genetic instability. There is also some evidence that an acquired genetic instability is fundamental

to tumour development. An accumulation of DNA damage causes the disruption of normal cellular functions and enables the clonal expansion of abnormal cells to form a tumour. Defects in DNA repair mechanisms are known to be responsible for inherited cancer-prone syndromes such as Xeroderma Pigmentosum and hereditary non-polyposis colon cancer (HNPCC) (Fishel, *et al.*, 1993, Robbins, *et al.*, 1974). Therefore DNA repair genes have a critical role in protecting cells from carcinogenesis (Mohrenweiser and Jones, 1998).

Exposure to ionizing radiation has been identified as a risk factor for breast cancer. Ionizing radiation exposure damages DNA in many ways, including the induction of DNA double strand breaks. Improper or inefficient repair of DSBs can result in the accumulation of chromosomal translocations, deletions, insertions and other gene rearrangements which can subsequently lead to carcinogenesis (Elliott and Jasin, 2002, Jeggo, 1998, Pastink, *et al.*, 2001). Hypersensitivity to ionizing radiation and defective DNA repair has been reported in breast cancer patients and their healthy relatives compared to healthy individuals without any family history of breast cancer (Helzlsouer, *et al.*, 1996, Kovacs and Almendral, 1987, Parshad, *et al.*, 1996, Patel, *et al.*, 1997, Roberts, *et al.*, 1999, Scott, *et al.*, 1999). Also higher levels of radiation-induced cellular G_2 delay have been observed in breast cancer patients and healthy women with family history of breast cancer (Lavin, *et al.*, 1992) Hu JJ, 1999). These studies provide evidence that cellular and chromosomal radiosensitivity resulting from deficiencies in DNA repair is inherited in breast cancer patients and their families. In addition, the products of the breast cancer susceptibility genes *BRCA1* and *BRCA2* are involved in the homologous recombination DNA repair pathway, which repairs DNA double-strand breaks (Karran, 2000). BRCA1 and BRCA2 deficient cell lines and knockout mice are defective in homologous recombination repair and show radiation hypersensitivity (Moynahan, *et al.*, 2001, Ponnaiya, *et al.*, 1997). Since in the absence of *BRCA1* and *BRCA2* mutations, a family history of breast cancer constitutes a significant risk factor (Lynch, *et al.*, 1989, Spitz and Bondy, 1993), and considering that DNA repair deficiency in breast cancer families is inherited (Price, *et al.*, 1997, Roberts, *et al.*, 1999), it has been proposed that DNA repair genes (particularly those responsible for repairing DSBs) could be low-penetrance candidate genes for breast cancer susceptibility (Mohrenweiser and Jones, 1998, Price, *et al.*, 1997, Roberts, *et al.*, 1999).

As described in section 1.8.1.3, RAD51 and five other homologous genes (*RAD51B* (RAD51L1), *RAD51C* (RAD51L2), *RAD51D* (RAD51L3), *XRCC2* and *XRCC3*) have been identified in human and are involved in the HRR pathway of DSB repair. *XRCC2* and *XRCC3* mutant cells show moderate hypersensitivity to ionizing and UV radiation but extreme sensitivity to DNA cross-linking drugs such as Mitomycin C and cisplatin (Fuller and Painter, 1988, Jones, *et al.*, 1987, Liu, *et al.*, 1998). Both XRCC2 and XRCC3 are required for the assembly and stabilisation of Rad51 foci (Bishop, *et al.*, 1998, O'Regan, *et al.*, 2001) and cells lacking *XRCC2* and *XRCC3* show low levels of homologous recombination (Brenneman, *et al.*, 2000, Johnson, *et al.*, 1999, Liu, *et al.*, 1998, Pierce, *et al.*, 1999).

A number of SNPs have been identified in genes of HRR pathway, including the *XRCC2*, *XRCC3*, *BRCA2* and *RAD51* genes, and these can be used for generating association studies. As DNA repair genes contribute to the prevention of tumour development, the study of their genetic variants whose effect could lead to inefficient repair capacity, provide a means of identifying a population that may be at particular risk of developing tumour due to an environmental exposure (e.g. radiation).

Genetic association studies provide enough power for identifying low-penetrance alleles (See section 1.4.11). In association studies, genotype frequencies of three possible genotypes of a SNP are compared between cases and controls. The approximate relative risk of disease for each genotype can then be estimated relative to the group carrying common homozygote allele (Dunning, *et al.*, 1999). This approach allows estimation of the risk according to the number of copies of the risk allele.

We therefore hypothesized that single nucleotide polymorphisms of HRR genes which are responsible for repair of DSBs could be associated with breast cancer by alteration of DNA repair capacity or fidelity. In addition, as a consequence of the complex physical interaction of the proteins, combinations of variant forms of these recombination repair genes may have more dramatic effects on overall DNA repair capacity than expected from the effects of single genes in isolation. We therefore tested the association of polymorphisms of these genes in association with breast cancer. We chose coding SNPs in *XRCC2* and *XRCC3* genes that change an amino acid. The polymorphisms of these genes had already

suggested as low penetrance candidate genes for breast cancer susceptibility in the literature (Roberts, *et al.*, 1999). Various BRCA2 polymorphisms have already studied in association with breast cancer and only a weak effect have been observed for the BRCA2 N372H polymorphisms (Healey, *et al.*, 2000). To the best of our knowledge no coding RAD51 SNP in a high frequency has been identified in the population so far, and only a 5' UTR polymorphism has suggested to act as a modifier of risk in *BRCA2* mutation carriers (Levy-Lahad, *et al.*, 2001). We therefore tested whether this polymorphism could act as a breast cancer predisposing allele in sporadic breast cancer patients.

3.2. Materials and methods

3.2.1. Patients and controls

The patient cohort comprised 522 Caucasian breast cancer patients, from South Yorkshire, UK who were diagnosed and treated for breast cancer at the Royal Hallamshire and Rotherham District General Hospitals between November 1998 and June 2000. Less than 5% of patients at these clinics are secondary referrals; thus the cases are reasonably representative of the populations of South Sheffield and Rotherham. Women were included in the study if they had a diagnosis of breast cancer for which they had undergone surgery, so that pathology data were available. The histo-pathologic diagnosis was confirmed for all breast cancer cases (BCC) by a pathologist*. Demographic, family history, clinical and histo-pathologic data were collected for all patients. The control populations comprised 399 anonymous female healthy blood donors and 500 females from amongst those attending the Sheffield mammography screening programme from October 2000 to February 2001. Women in the latter group were included only if they had no mammographic evidence of breast lesion at the time of screening. All women on the community health index between the ages of 50 and 65 are invited for screening, and the uptake rate in Sheffield is on average 80%. Demographic data, environmental risk factors and family history data were collected from mammography screening controls (MSC) by a research nurse* using the same questionnaire as used for the cases (see appendix). Age and sex were recorded for the blood donor controls (BDC). This study was approved by the South Sheffield Research Ethics Committee and informed written consent was obtained from all subjects (See appendix).

3.2.2. DNA variants

The polymorphism in exon 3 of the *XRCC2* gene at nucleotide position 31479 (Genbank accession number AC003109) is a G to A substitution which changes arginine 188 to histidine. This variant was identified by sequencing polymerase chain reaction (PCR) products amplified from the genomic DNA of primary human fibroblast cultures from

*See appendix for list of collaborators

several different individuals by our collaborators Paul O'Regan and John Thacker*. The polymorphism in exon 7 of the *XRCC3* gene is a C/T substitution at nucleotide position 18067 (Genbank accession number AF037222) which changes a threonine 241 to methionine (Shen, *et al.*, 1998). The polymorphism in exon 10 of the *BRCA2* gene at nucleotide position 1342 (Genbank accession number NM_000059) is an A to C substitution which changes asparagine 372 to histidine (Healey, *et al.*, 2000). The polymorphism in 5' UTR of the *RAD51* gene at nucleotide position 135 (Genbank accession number D14134) is a G to C substitution (Wang, *et al.*, 2001).

3.2.3. DNA extraction

Genomic DNA was extracted from peripheral blood lymphocytes using 20ml of whole blood as described in section 2.3.1.1. The DNA concentration of all samples was then quantified (See section 2.3.2) and all samples were diluted to 100ng/µl for general PCR amplification and 5ng/µl for 5' nuclease PCR (TaqMan™) assays.

3.2.4. Genotyping techniques

3.2.4.1. PCR-SSCP

PCR-SSCP was used for genotyping all cases and controls for the XRCC3 T241M polymorphism. PCR amplification for *XRCC3* exon 7 was carried out as described in section 2.3.3 except that 6 pmol of forward and reverse primers (Table 1) was used. The amplification condition was 95°C for 3 minutes, followed by 35 cycles of 94°C for 35 sec, 61°C for 25 sec and 72°C for 25 sec. A final extension of 72°C for 5 minutes was also applied.

SSCP genotyping of the XRCC3 exon 7 was also performed as described in section 2.3.5.1. A 12% polyacrylamide gel and 5500 volt hours were used for electrophoresis. DNA bands were then visualised by silver staining (see section 2.3.5.1). PCR-SSCP was also used for re-genotyping 15% of the samples that had already genotyped for the XRCC2

* See appendix for list of collaborators

R188H polymorphism by TaqMan™ as described below. For PCR amplification, 100ng genomic DNA was mixed with 10pmol each forward and reverse primers (Table 1) and 18µl 1.1X PCR master mix. The amplification conditions consisted of 3 minutes at 94°C followed by 35 cycles of 30 sec at 95°C, 20 sec at 56°C and 20 sec at 72°C. Final extension was for 5 minutes at 72°C. SSCP genotyping was carried out as described above with electrophoresis for 3000 volt hours on 10% non-denaturing polyacrylamide gel.

3.2.4.2. Fluorescent 5' nuclease PCR assay (TaqMan™)

The TaqMan™ assay system was used for genotyping of 3 SNPs in the *XRCC2, BRCA2* and *Rad51* genes. It was also used for re-genotyping 15% of samples for XRCC3 T241M variants which had already been genotyped using PCR-SSCP.

Primers and probes for TaqMan™ assays were designed using Primer Express v1.5 software (ABI) and were made by MWG (Table 1). The required amount of primers and probes for each experiment was adjusted as described in section 2.3.5.3. All DNA samples were diluted to 5ng/µl.

PCR-SSCP primers		
	XRCC2	Forward: 5' ACT CTG AGG AAA TGT TCT CA 3'
	exon 3	Reverse: 5' AGT TGC TGC CAT GCC TTA CA 3'
	XRCC3	Forward: 5' CCA CAG GAC ACC TTG TTG GA 3'
	exon 7	Reverse: 5' AAA TAC GAG CTC AGG GGT GC 3'
XRCC2		
R188H		
	Primers	Forward: 5' CTG AGG AAA TGT TCT CAG TGC TTA GA 3'
		Reverse: 5' TGA TGA GCT CGA GGC TTT CTG 3'
	Probes	Detecting XRCC2 R188:
		5' FAM- TGT AAA TGA CTA TCG CCT GGT TCT TTT TGC -TAMRA 3'
		Detecting XRCC2 188H:
		5' TET- TTG TAA ATG ACT ATC ACC TGG TTC TTT TTG CAA C -TAMRA 3'
XRCC3		
T241M		
	Primers	Forward: 5' GGC CAG GCA TCT GCA GTC 3'
		Reverse: 5' CTT GGT GCT CAC CTG GTT GAT 3'
	Probes*	Detecting XRCC3 T241:
		5' FAM- ACG CAG CGT GGC CCC C -TAMRA 3'
		Detecting XRCC3 241M:
		5' TET- CAC GCA GCA TGG CCC CC -TAMRA 3'
BRCA2		
N372H		
	Primers	Forward: 5' CTG AAG TGG AAC CAA ATG ATA CTG A 3'
		Reverse: 5' AGA CGG TAC AAC TTC CTT GGA GAT 3'
	Probes	Detecting BRCA2 N372:
		5' FAM- ATT CAA ATG TAG CAA ATC AGA AGC CCT TTG A -TAMRA 3'
		Detecting BRCA2 372H:
		5' TET- TCA AAT GTA GCA CAT CAG AAG CCC TTT GA -TAMRA 3'
RAD51 135G/C		
	Primers	Forward: 5' AGC TGG GAA CTG CAA CTC ATC T 3'
		Reverse: 5' CCG CGC TCC GAC TTC A 3'
	Probes*	Detecting *RAD51 135C*:
		5' FAM- CAA CGC CCC TGG CTT ACG CTC -TAMRA 3'
		Detecting *RAD51 135G*:
		5' TET- CAA CGC CCG TGG CTT ACG CT -TAMRA 3'

Table 1: Primers and probes used for PCR-SSCP and TaqMan™ genotyping

Underlined base pairs indicate site of polymorphisms.

PCR amplification for TaqMan assays was carried out as described in section 2.3.5.3. The amount of forward and reverse primers and probes, were different in each assay, and are shown in table 2. The final reaction volume was adjusted to 25µl with ddH_2O. All reactions were prepared in 96 well PCR plates (AB gene). Six wells each of wild type, heterozygous and homozygous positive control and negative (no DNA) controls were included for each plate. PCR amplification conditions were 50°C for 2 minutes and 95°C for 10 minutes, followed by cycling conditions which generally consisted of 40 cycles of 95°C for 15 seconds and 61-63°C for 1 minute (see table 2 for exact annealing temperature for each assay). All amplified samples were analyzed using ABI sequence detection system 7200 (PE Biosystems) under allelic discrimination mode.

	Primers concentration (nM)		**Probes concentration (nM)**		**Annealing temperature**
	Forward	Reverse	FAM	TET	
XRCC2 R188H	300	300	25	50	63°C
XRCC3 T241M	300	300	50	100	62°C
BRCA2 N372H	300	300	25	100	62°C
RAD51 G135C	900	900	50	150	61°C

Table 2: TaqMan probe and primer concentrations.

Probes and primers concentration and annealing temperature for 5' nuclease PCR (TaqMan™) for *XRCC2, XRCC3, BRCA2* and *RAD51* genotyping.

3.2.5. DNA Sequencing

DNA sequencing was used to confirm sequence variants in the XRCC2 and XRCC3 genes following PCR-SSCP. PCR for sequencing reactions was carried out in a 50µl volume using the same conditions and primers as described in sections 3.2.4.1 and 2.3.6.

3.2.6. Statistical analysis

All data were entered into a Microsoft Access database and exported to Stata version 6.0 for the statistical analyses. DNA was available for 522 breast cancer cases, 496 mammography screening controls, and 399 blood donor controls. The power of study for each SNP was calculated by using Stata software version 6.0 using Sampsi command (Stata, 2000). Chi-squared tests on 2x2 contingency tables and the Wilcoxon rank sum test was used for comparison of variables between BCC and MSC (Kirkwood, 1988). The Odds ratios and 95% confidence intervals were determined for the genotype comparisons. Odds ratio was calculated by standard method (Bland and Altman, 2000). Confidence interval for the odds ratio was estimated by Woolf method (Woolf, 1955). Cases and mammography screening controls were matched for by age restricting analysis to those cases and controls diagnosed/recruited between ages 50-65 years. This is the age range of women routinely invited for mammography screening. Only 64 out of 496 controls in this study were outside this range, insufficient for analysis of other age strata. We also used extreme sampling, which has been shown to increase efficiency for genes involved in complex inheritance (Morton and Collins, 1998). The comparison groups for this analysis were defined as cases with age at diagnosis below the median age 57 years, with a positive family history (n=88), versus controls aged over 57 years with no family history of breast cancer (n= 181). Mantel-Haenszel test of homogeneity was used to calculate the significance of difference between the odds ratios for one SNP in the presence and absence of other HRR variant allele using Stata version 6.0 (Stata, 2000). Since data on exposure to X-Ray and ionising radiation was not available we were unable to analyse our data for the effect of gene-exposure interactions.

3.3. Results

3.3.1. Characteristics of patients and controls

Summary statistics for the risk factor variables for the BCC and MSC groups are shown in Table 3. Only age and sex data were available for the BDC group. Their age range was significantly lower than the BCC group (median 39, range 20-64; $p<10^{-12}$). There were no significant differences between the breast cancer cases and mammography screening controls for age at diagnosis/sampling (p=0.31), body mass index (p=0.14), or age at menarche (p=0.33). However, the breast cancer cases had fewer children (p=0.0009), a higher age at first pregnancy (p=0.0021), a higher age at menopause (p=0.0069) and a higher proportion of them had no children ($p=3.9x10^{-6}$). In addition, a higher proportion of breast cancer cases had at least one first or second degree relative with breast cancer (p=0.00074). Although there was no significant difference in median age between the breast cancer cases and mammography screening controls, the age range of the controls was narrower. This difference in age ranges probably accounts for the much higher proportion of controls who had received HRT and OCP ($p<10^{-12}$ and $p=2.3x10^{-11}$ respectively). HRT and OCP were not available to the older cases. For example, amongst those individuals born after 1940, for whom OCP would have been available during their reproductive years, there was no significant difference in OCP use between cases and controls (69.7% *vs* 71.9%; p=0.58).

3.3.2. The XRCC2, XRCC3, BRCA2 and RAD51 genotyping

To determine whether there is any association between carriage of variant alleles of DNA repair genes and susceptibility to breast cancer, we genotyped 522 independent breast cancer cases, 496 healthy women from mammography screening and 399 healthy female blood donors controls for four single nucleotide polymorphisms in *XRCC2, XRCC3, BRCA2* and *RAD51* genes using PCR-SSCP and TaqMan™ as described in section 3.2.4.2 (Figure 1). The different band patterns on the SSCP gel and genotype categories on the TaqMan™ analysis were clearly distinguishable between different genotypes and were confirmed by direct sequencing (Fig 1). To ensure the accuracy of the genotyping results from SSCP, 150 samples (15%) were also selected randomly and genotyped by TaqMan™. The result of genotyping with TaqMan™ was more than 99% in agreement with

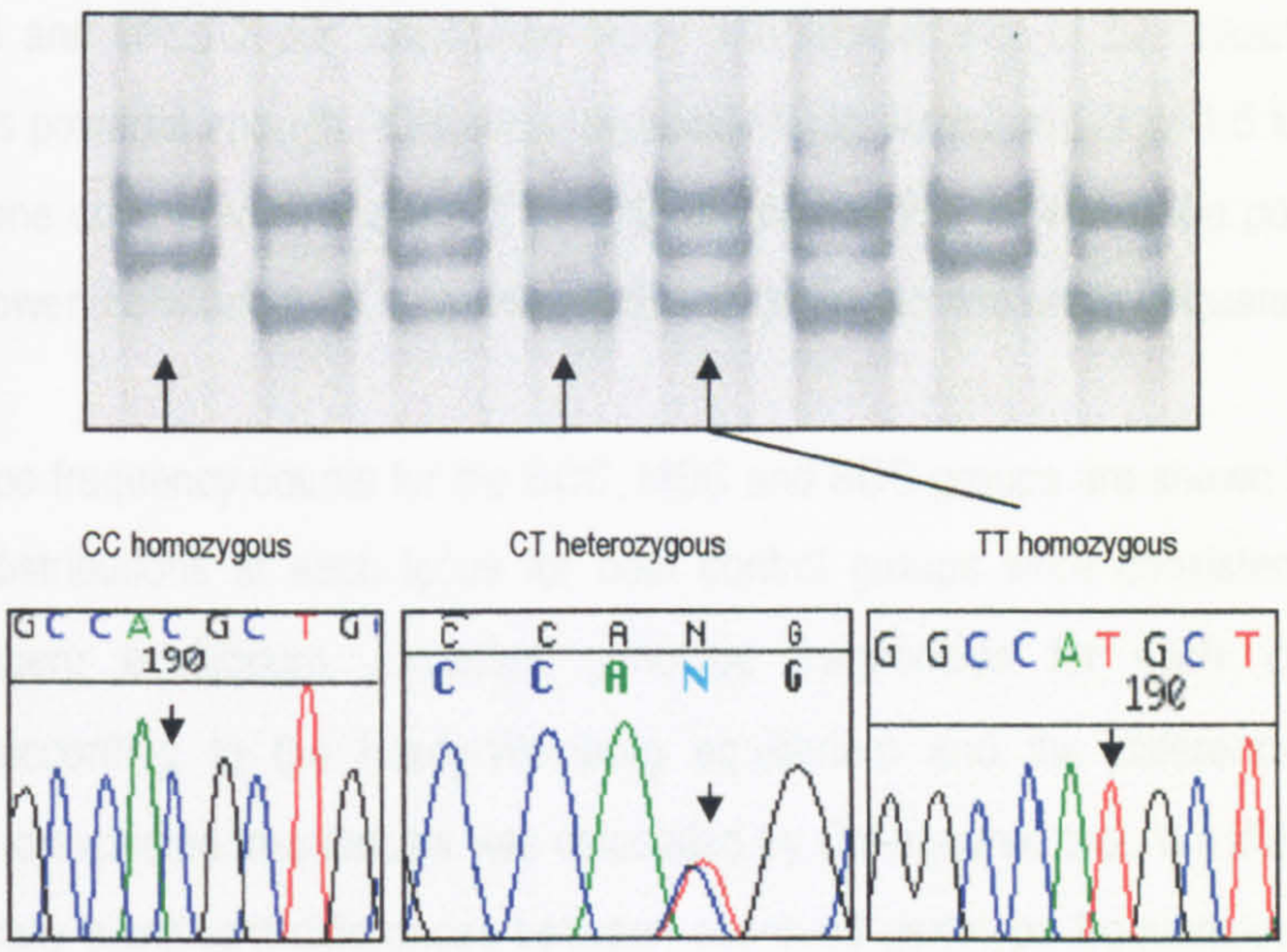
the result of genotyping obtained by SSCP. Also 25 samples from different band categories were randomly selected and subjected to direct sequencing that confirmed the result of both genotyping techniques. The result of TaqMan™ genotyping of the XRCC2, BRCA2 and RAD51 was also confirmed by SSCP and direct sequencing as described above.

	BCC			MSC			
continuous variables	**n**	**median**	**range**	**n**	**median**	**range**	**p[a]**
Age at diagnosis (yr)	522	57	28-89	494	57	45-77	0.31
BMI	498	25.9	9.2-44.2	493	26.2	18.7-52.9	0.14
Age at first pregnancy (yr)	419	24	16-41	447	23	16-40	0.0021
number of children	486	2	0-8	460	2	0-8	0.0009
Age at menarche (yr)	508	13	9-18	493	13	9-18	0.33
Age at menopause (yr)	420	50	29-61	416	49	24-59	0.0069
binary variables	**n**	**frequency**	**%**	**n**	**frequency**	**%**	**p[b]**
Children	486	434	89.3%	460	446	97.0%	3.85×10^{-6}
HRT	521	103	19.8%	496	220	44.4%	$<10^{-12}$
OCP	521	209	40.1%	496	303	61.1%	2.28×10^{-11}
BrCaFH	521	168	32.3%	496	113	22.8%	0.00074

Table 3: Characteristics of breast cancer patients and controls.

[a] Wilcoxon rank sum test. [b] Chi-squared test. Age at diagnosis indicates age at diagnosis for cases and age at blood sampling for controls. BCC: breast cancer cases, MSC: mammography screening controls. n indicates number of observations available for that variable. BMI: body mass index. HRT: hormone replacement therapy, OCP: oral contrceptive pill. BrCaFH: breast cancer family history (at least one first or second degree relative with breast cancer).

A



B

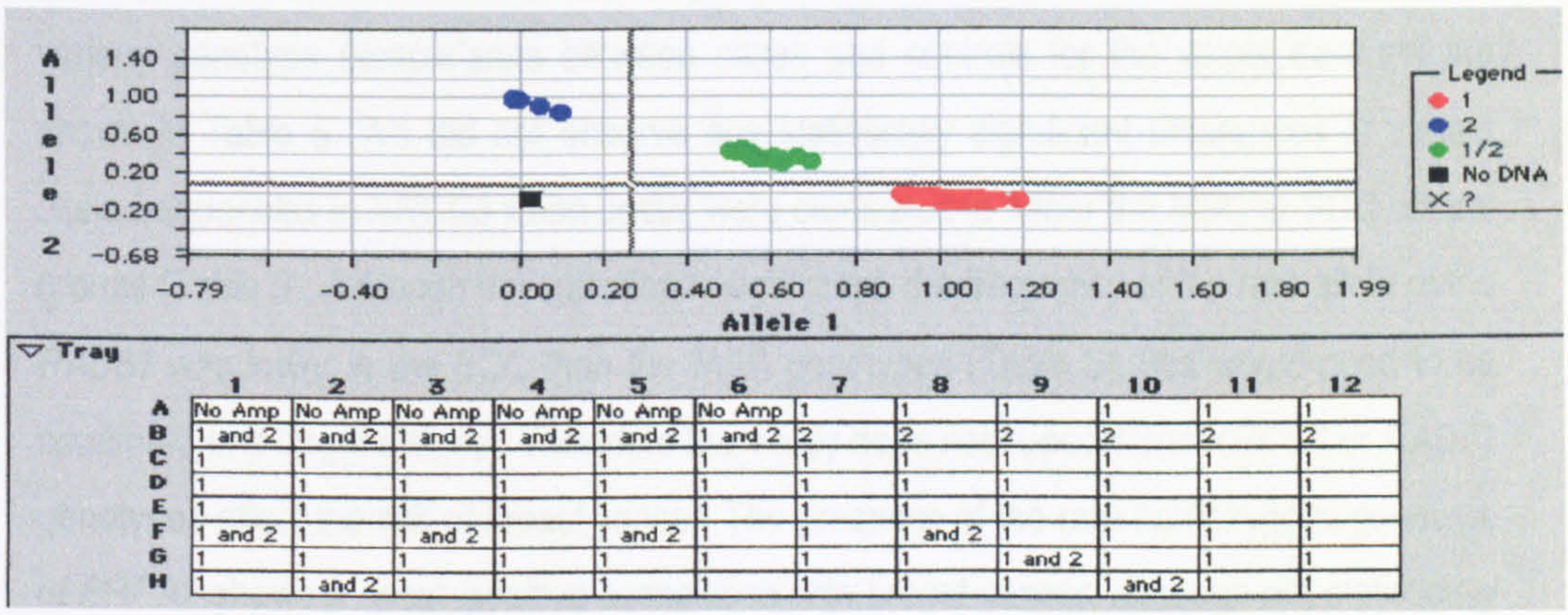


Figure 1: PCR-SSCP and TaqMan genotyping.

A; Image of SSCP assay for XRCC3 T241M genotyping carried out on 12% non-denaturing polyacrylamide gel. Chromatogram of DNA sequencing has also shown under each relevant genotype. Arrows indicate different band patterns related to each genotype (arrows indicates sites of polymorphisms). **B**; Image of TaqMan™ assay for XRCC2 R188H genotyping. X axis: detects allele1 (wild type) and labelled with FAM, Y axis: detects allele 2 (variant allele) and labelled with TET, Red dots: samples with wild type sequence, green dots: heterozygote samples and blue dots: rare homozygote samples. The result of sequencing for each genotype has also shown (arrows indicates sites of polymorphisms).

3.3.3. Analysis of genotyping data

For XRCC3 and BRCA2 our association study with sample size of 522 cases and 500 controls was powerful enough (83%, 88% respectively) to detect an OR of 1.5 for carriage of at least one copy of variant allele. For XRCC2 and RAD51, however, the power of the study was lower, 65% and 64% respectively, due to the lower rare allele frequency of these SNPs.

The genotype frequency counts for the BCC, MSC and BDC groups are shown in Table 4. Genotype distributions at each locus for both control groups were consistent with the Hardy-Weinberg equilibrium. Expected genotype frequencies for each locus were calculated according to the Hardy-Weinberg equilibrium and the difference between observed and expected frequencies was calculated by Chi-squared test. We did not detect any statistically significant differences between observed genotype frequencies and what we expected under the Hardy-Weinberg equilibrium (Table 4). The odds ratios for the various genotype comparisons between cases and controls for the whole data set are shown in Table 5. We did not observe any statistically significant differences in variant allele frequencies in *XRCC3* when cases were compared to either the MSC or BDC control groups (Table 5). Although not statistically significant, the frequency of the rare allele of the *RAD51* was lower in the BCC than the MSC genotypes (Table 5); this would need to be confirmed in a larger dataset. Therefore this study does not support that *XRCC3* or *RAD51* genotypes affect the risk of breast cancer. The presence of the rare homozygous genotype of *BRCA2* shows a weak positive association with breast cancer, although not statistically significant possibly due to small sample size. We also did not observe any significant difference in the frequency of BRCA2 heterozygote allele when cases were compared to either of MSC or BDC controls (Table 5). The presence of the A allele of XRCC2 was associated with the increased risk of breast cancer when cases were compared to the BDC controls (odds ratio 1.58 (1.07, 2.34)). There is some evidence that carriage of the XRCC2 HH genotype is more associated (2.43 (0.48, 12.14)) with an increased risk of breast cancer than carrying one variant allele 1.59 (1.06, 2.36)), although this does not reach statistical significance probably due to small size sample. This positive association was reduced in the comparison between BCC and MSC controls (odds ratio 1.15 (0.82, 1.62)), and fell just short of statistical significance when the two control groups were combined (odds ratio 1.32 (0.98, 1.79)) (Table 5).

		Observed genotype frequency			v allele frequency %	Total	H-W expected genotype frequency			Deviation form HWE
		w/w n (%)	w/v n (%)	v/v n (%)		n	w/w n	w/v n	v/v n	x^2/p
XRCC2	BCC	431 (83.0%)	82 (15.8%)	6 (1.2%)	9%	519				-
	MSC	419 (85.0%)	71 (14.4%)	3 (0.6%)	7.8%	493	419	71	3	$1.7X10^{-5}$/ 0.99
	BDC	350 (88.8%)	42 (10.7%)	2 (0.5%)	5.8%	394	349	43	1	0.36/ 0.54
XRCC3	BCC	201 (38.6%)	248 (47.6%)	72 (13.8%)	37.6%	521				-
	MSC	196 (39.7%)	228 (46.1%)	70 (14.2%)	37.2%	494	194	230	68	0.07/ 0.77
	BDC	145 (37.0%)	188 (48.0%)	59 (15.0%)	39%	392	145	186	59	0.02/ 0.87
BRCA2	BCC	260 (50.2%)	213 (41.1%)	44 (8.5%)	29.1%	517				-
	MSC	253 (52.4%)	192 (39.8%)	37 (7.6%)	27.5%	482	252	192	36	0.004/ 0.94
	BDC	192 (49.3%)	172 (44.2%)	25 (6.4%)	28.5%	389	198	158	31	2.7/ 0.09
RAD51	BCC	449 (87.1%)	65 (12.6%)	1 (0.19%)	6.5%	515				-
	MSC	411 (84.9%)	70 (14.4%)	3 (0.6%)	7.8%	484	410	70	3	0.0001/ 0.99
	BDC	325 (83.3%)	63 (16.1%)	2 (0.5%)	8.6%	390	325	60	3	0.32/ 0.53

Table 4: XRCC2, XRCC3, BRCA2 and RAD51 genotype frequencies.

XRCC2, XRCC3, BRCA2 and RAD51 genotype and rare allele frequencies in breast cancer cases (BCC), mammography screening controls (MSC) and blood donor controls (BDC). HWE: Hardy-Weinberg Equilibrium. w/w; wild type/ wild type, w/v: wild type/variant, v/v: variant/ variant. The actual w and v alleles for each SNP are as follows: XRCC2 R188H; w/w: His/His, w/v: His/Arg, v/v: Arg/Arg; XRCC3 T241M; w/w: Thr/Thr, w/v: Thr/Met, v/v: Met/Met; BRCA2 N372H; w/w: Asp/Asp, w/v: Asp/His, v/v: His/His; RAD51 G135C: w/w: g/g, w/v: g/c, v/v: c/c
X^2, Chi-squared test. HWE: Hhardy-Weinberg equilibrium

Case/ control	Odds ratio (95% CI) w/w *vs* w/v	Odds ratio (95% CI) w/w *vs* v/v	Odds ratio (95% CI) w/w *vs* w/v+v/v
XRCC2			
BCC	N/A	N/A	N/A
MSC	1.12 (0.79,1.58)	1.94 (0.48,7.84)	1.15 (0.82,1.62)
BDC	1.59 (1.06,2.36)	2.43 (0.48,12.14)	1.58 (1.07,2.34)
MSC+BDC [a]	1.29 (0.95,1.76)	2.14 (0.65,7.06)	1.32 (0.98,1.79)
XRCC3			
BCC	N/A	N/A	N/A
MSC	1.06 (0.81,1.38)	1.05 (0.71,1.54)	1.05 (0.82,1.36)
BDC	0.94 (0.7,1.25)	0.9 (0.6,1.35)	0.93 (0.71,1.22)
MSC+BDC	1.00 (0.79,1.27)	0.98 (0.7,1.37)	1.00 (0.80,1.25)
BRCA2			
BCC	N/A	N/A	N/A
MSC	1.07 (0.83,1.40)	1.15 (0.72,1.85)	1.09 (0.85,1.40)
BDC	0.91 (0.69,1.20)	1.29 (0.76,2.19)	0.96 (0.74,1.25)
MSC+BDC	1.00 (0.79,1.25)	1.21 (0.80,1.84)	1.03 (0.83,1.28)
RAD51			
BCC	N/A	N/A	N/A
MSC	0.85 (0.59,1.22)	0.30 (0.03,2.94)	0.82 (0.57,1.18)
BDC	0.74 (0.51,1.08)	0.36 (0.032,4.00)	0.73 (0.5,1.06)
MSC+BDC	0.80 (0.58,1.10)	0.32 (0.04,2.81)	0.82 (0.6,1.13)

Table 5: Odds ratios and 95% CI between cases and controls

Odds ratios and 95% confidence interval (CI) between cases and controls for XRCC2, XRCC3, BRCA2 and RAD51.
a Combined mammography screening controls and blood donor controls. Other abbreviations as table 4.

3.3.4. Relationship between genotype and age of diagnosis

Data on age at diagnosis, family history of breast cancer, tumour histology types, tumour grade, and vascular invasion were available for 500 cases. We examined the significance of each polymorphism related to some of these variables.

Age is one of the important risk factors for breast cancer. We therefore performed a number of analyses to study whether carriage of these variant alleles conferred increased risk of breast cancer in a specific age group. Since 87.4% of women in MSC were in age group of 50-65 years, the age range for which women routinely attend mammography screening, we compared breast cancer cases of 50-65 years old with the age matched group from MSC. No significant differences in variant allele frequency were observed when carriage of at least one variant allele of XRCC2, *XRCC3, BRCA2* and *RAD51* was compared between cases and controls, and the size of the odds ratios were consistent with the whole dataset analysis (Table 6). The median value of age for both BCC and MSC was 57 years, therefore we analysed the genotyping results from BCC and MSC below 57 years and also BCC and MDC above 57 years. However no difference between cases and controls was observed for carriage of rare allele frequency in both age groups (Table 6).

3.3.5. Relationship between genotype and family history

The effect of some variants is only seen in those with positive family history of breast cancer (eg. *CHEK2* 1100delC, (Meijers-Heijboer, *et al.*, 2002). To determine the effect of these sequence variants in combination with family history in association with breast cancer, we selected cases with a positive family history of breast cancer and compared them to MSC with no family history of breast cancer. As shown in table 7, we did not observe any difference for the effect of the variant alleles compared to the analysis of the whole dataset. Although not statistically significant, the odds of having breast cancer were higher in cases with positive family history for carriers of *BRCA2* variants relative to odds of having cancer from comparison of BCC and MSC in whole data set.

To increase power to detect weak genetic effects, it has been proposed that extreme sampling may be useful (Morton and Collins, 1998). Therefore we selected cases of

younger onset (below the median value of 57 years) with a positive family history of breast cancer and compared them with older controls (above 57 years) that had no family history of breast cancer. The odds ratios of having breast cancer in cases carrying at least one variant allele in *XRCC2*, *XRCC3*, *BRCA2* or *RAD51* genes are all slightly increased relative to the whole dataset comparison of BCC with MSC (Table 8). In particular, there was a suggestive effect for carriage of the *XRCC2* A allele when younger onset cases with a positive family history were compared to older controls with no family history, (odds ratio 1.82 (0.91,3.64); Table 8). Although not statistically significant these results are consistent with the idea that in familial cases a combination of factors may act together to increase the effect of a particular allele.

Case/ control	Genotype			Total	Odds ratio (95% CI) w/w *vs* w/v	Odds ratio (95% CI) w/w *vs* v/v	Odds ratio (95% CI) w/w *vs* w/v+v/v	Unadjusted OR w/w vs w/v+v/v
	w/w	w/v	v/v					
XRCC2								1.15 (0.82,1.62)
BCC 50-65 years	209 (81.3%)	47 (18.2%)	1 (0.3%)	257				
MSC 50-65 years	368 (85.1%)	63 (14.6%)	1 (0.2%)	432	1.31 (0.86, 1.98)	1.76(0.1, 28.29)	1.32 (0.87, 1.99)	
BCC ≤57	217	45	1					
MSC ≤57	220	40	1		1.14 (0.71, 1.81)	1.01 (0.06,16.3)	1.13 (0.71,1.80)	
BCC >57	214	37	5					
MSC >57	200	31	2		1.11 (0.66,1.86)	2.33 (0.44,12.17)	1.18 (0.72,1.95)	
XRCC3								1.05 (0.82,1.36)
BCC 50-65 years	104 (40.4%)	114 (44.3%)	39 (15.1%)	257				
MSC 50-65 years	175 (40.6%)	193 (44.7%)	63 (14.6%)	431	0.99 (0.71, 1.39)	1.04 (0.65, 1.66)	1.00 (0.73, 1.37)	
BCC ≤57	93	135	36					
MSC ≤57	103	119	36		1.25 (0.86,1.82)	1.10 (0.64,1.90)	1.22 (0.85,1.74)	
BCC >57	108	111	38					
MSC >57	94	108	33		0.89 (0.60,1.31)	1.00 (0.58,1.72)	0.91 (0.64,1.31)	
BRCA2								1.09 (0.85,1.40)
BCC 50-65 years	130 (50.5%)	106 (41.2%)	21 (8.1%)	257				
MSC 50-65 years	222 (52.8%)	165 (39.2%)	33 (7.8%)	420	1.09 (0.79, 1.52)	1.08 (0.60, 1.95)	1.09 (0.80, 1.49)	
BCC ≤57	130	110	22					
MSC ≤57	130	100	23		1.1 (0.75,1.51)	0.95 (0.50,1.80)	1.07 (0.75,1.51)	
BCC >57	130	103	22					
MSC >57	123	92	14		1.05 (0.72,1.53)	1.48 (0.72,3.03)	1.11 (0.78,1.59)	
RAD51								0.82 (0.57,1.18)
BCC 50-65 years	224 (87.8%)	30 (11.8%)	1 (0.4%)	255				
MSC 50-65 years	357 (84.7%)	61(14.5%)	3 (0.7%)	421	0.78 (0.49, 1.25)	0.53 (0.54, 5.13)	0.77 (0.48, 1.22)	
BCC ≤57	226	34	0					
MSC ≤57	215	39	1		0.82 (0.50,1.36)	-	0.82 (0.50,1.36)	
BCC >57	223	31	1					
MSC >57	196	31	2		0.87 (0.51,1.49)	0.43 (0.04,4.88)	0.85 (0.50,1.43)	

Table 6: Odds ratios between cases and controls adjusted for age.

Genotype frequencies, odds ratios and 95% confidence interval (CI) in subgroups of breast cancer cases (BCC) and mammography screening controls (MSC). Other abbreviations as table 4.

Case/ control	Genotype			Total	Odds ratio (95% CI) w/w *vs* w/v+v/v	Unadjusted OR w/w vs w/v+v/v
	w/w	w/v	v/v			
XRCC2						1.15 (0.82,1.62)
BCC +ve FH	124 (82.6%)	24 (16 %)	2 (1.3%)	150		
MSC –ve FH	337 (86%)	52 (13.2%)	3 (0.8%)	392	1.18 (0.29,1.99)	
XRCC3						1.05 (0.82,1.36)
BCC +ve FH	62 (39.4%)	67(42.6 %)	28(17.8%)	157		
MSC –ve FH	157 (40.2%)	179(45.8%)	54 (14%)	390	1.00 (0.68,1.45)	
BRCA2						1.09 (0.85,1.40)
BCC +ve FH	75 (50%)	60(40 %)	15(10%)	150		
MSC –ve FH	212 (55.7%)	138(36.3%)	30 (7.8%)	380	1.26 (0.86,1.84)	
RAD51						0.82 (0.57,1.18)
BCC +ve FH	133 (89.2%)	16 (10.7 %)	0	149		
MSC –ve FH	326 (85.3%)	54 (14.1%)	2 (0.6%)	382	0.70 (0.38,1.26)	

Table 7: Odds ratios between cases and controls adjusted for family history

Genotype frequencies, odds ratios and 95% confidence interval (CI) in subgroups of breast cancer cases (BCC) and mammography screening controls (MSC). +ve FH: positive family history of breast cancer, -ve FH: negative family history of breast cancer. Other abbreviations as table 4.

	Genotype			n	Odds ratio(95% CI) any v *vs* ww	Unadjusted OR (95% CI) BCC *vs* MSC, any v *vs* ww
	w/w	w/v	v/v			
XRCC2						1.15 (0.82,1.62)
BCC≤57+FH	67 (79.7%)	16(19.1%)	1 (1.2%)	84		
MSC>57-FH	158(87.7%)	20(11.1%)	2 (1.1%)	180	1.82 (0.91,3.64)	
XRCC3						1.05 (0.82,1.36)
BCC≤57+FH	30 (34.8%)	41 (48.8%)	15(17.8%)	86		
MSC>57-FH	71(39.2%)	81 (44.7%)	29 (16%)	181	1.20 (0.70,2.05)	
BRCA2						1.09 (0.85,1.40)
BCC≤57+FH	42 (50%)	35 (41.6%)	7 (8.4%)	84		
MSC>57-FH	102(58.2%)	59 (33.7%)	14(8%)	175	1.39 (0.82,2.35)	
RAD51						0.82 (0.57,1.18)
BCC≤57+FH	71(85.5%)	12 (14.4%)	0	83		
MSC>57-FH	149(85.1%)	25 (14.2%)	1 (0.5%)	175	0.96 (0.46, 2.03)	

Table 8: Odds ratios between cases and controls adjusted for combined age and family history

Genotype frequencies, odds ratios and 95% confidence interval (CI) for extreme comparison between breast cancer cases (BCC) and mammography screening controls (MSC). ≤57+FH: younger BCC with family history of breast cancer; >57 –FH older controls without family history of breast cancer.
Other abbreviations as table 4.

3.3.6. Relationship between genotype and histopathological markers

Histopathological data was available on 493 tumours from our data set. The majority (294; 59.6%) of tumours were of the ductal NOS type, 49 (9.9%) were lobular, 102 (20.6%) were ductal special types and the remaining 49 (9.9%) were other types. The rate of carriage of the XRCC2 H allele was 32.7% in the lobular type, compared to 16% in the ductal NOS type and 14% in the ductal special types (p=0.001). Although this preliminary result suggests that the *XRCC2* rare allele is significantly associated with the lobular carcinoma of breast, however due to small sample size (n=49), this should be repeated in a bigger dataset. We however could not find any association between rare variants of the other genes and breast tumour histology. No significant association was observed between the site of tumour and carrying variant alleles in any of the genes (Table 9). Carriage of the *RAD51* rare allele seems to have a preventive role against vascular invasion (OR: 0.36 (0.14, 0.95), p=0.03), although this also needs to be confirmed in a bigger data set (Table 9).

3.3.7. Preliminary analysis of interaction between variant alleles of HRR in association with breast cancer

Given our hypothesis that the effects of variation in several proteins involved in homologous recombination repair may combine to produce susceptibility to cancer, we also tested for interaction between the *XRCC2, XRCC3, BRCA2* and *RAD51* polymorphisms. All possible pairwise interactions were tested in which the odds of cancer for carriage of at least one rare allele were calculated at the presence and absence of the other three variants. As shown in table 10, *XRCC2* A allele is associated with a more increased risk of cancer in the presence of *XRCC3* T allele (1.50 (0.97, 2.31), p=0.063) and absence of *BRCA2* C allele (1.60 (0.98, 2.60), p=0.065). Our data also show that the effect of *XRCC3* T allele increases in the presence of *XRCC2* A allele (1.86 (0.97, 3.55), p=0.063) and *BRCA2* C allele (1.35 (0.93, 1.96), p=0.068). Also for *BRCA2* rare allele the association with breast cancer is more evident at presence of *XRCC3* T allele than in its absence (1.32 (0.96, 1.82), p= 0.068), however the association of *BRCA2* C allele with breast cancer increase in the absence of *XRCC2* A allele (1.21 (0.92, 1.59), p=0.065). The odds of having cancer for *RAD51* C allele

shows more of a protection effect in the presence of rare *XRCC2* and *XRCC3* alleles (Table 10). The results suggest that the interaction of *XRCC2* and *XRCC3* genes is associated with increased susceptibility to breast cancer. Also the present study suggests that *XRCC3* and *BRCA2* interaction is more associated with breast cancer risk. However the association of *XRCC2* polymorphism with breast cancer increased in the absence of *BRCA2* and the effect of *BRCA2* on breast cancer susceptibility was more evident in the absence of *XRCC2*. Although all of the above results of interaction analysis are not statistically significant, they suggest hypothesis that could be tested in an independent dataset.

		w/w	w/v + v/v	Total	p
XRCC2					
Tumour histology	Lobular	33 (67.3%)	16 (32.7%)	49	0.001[a]
	Ductal specified	84 (84%)	16 (16%)	100	
	Ductal NOS	252 (86%)	41 (14%)	293	
Tumour site	Right-sided	202 (81.7%)	45 (18.3%)	247	
	Left-sided	212 (84.4%)	39 (15.6%)	251	0.42
	Bilateral	16 (80%)	4 (20%)	20	0.71
Vascular invasion	Yes	75	11		0.11
	No	273	69		
XRCC3					
Tumour histology	Lobular	17 (34.6%)	32 (65.4%)	49	0.53[a]
	Ductal specified	42 (41.5%)	59 (58.4%)	101	
	Ductal NOS	113 (38.4%)	181 (61.6%)	294	
Tumour site	Right-sided	91 (36.7%)	157 (63.3%)	248	
	Left-sided	106 (42%)	146 (58%)	252	0.21
	Bilateral	4 (20%)	16 (80%)	20	0.08
Vascular invasion	Yes	34	52		0.88
	No	133	211		
BRCA2					
Tumour histology	Lobular	30 (61.3%)	19 (38.7%)	49	0.14[a]
	Ductal specified	53 (53%)	47 (47%)	100	
	Ductal NOS	144 (49.3%)	148 (50.7%)	292	
Tumour site	Right-sided	117 (47.5%)	129 (52.5%)	246	
	Left-sided	134 (53.6%)	116 (46.4%)	250	0.17
	Bilateral	9 (45%)	11 (55%)	20	0.62
Vascular invasion	Yes	44	42		0.86
	No	171	170		
RAD51					
Tumour histology	Lobular	42 (85.71%)	7 (14.29%)	49	0.89[a]
	Ductal specified	90 (91.83%)	8 (8.17%)	98	
	Ductal NOS	246 (84.53%)	45 (15.47%)	291	
Tumour site	Right-sided	209 (85%)	37 (15%)	246	
	Left-sided	223 (90%)	25 (10%)	248	0.09
	Bilateral	16 (80%)	4 (20%)	20	0.32
Vascular invasion	Yes	80 (94.11%)	5 (5.89%)		0.03
	No	290 (85.45%)	49 (14.45%)		

Table 9: The association between HRR gene SNPs and histopathologic characteristics.

Comparison of genotype frequencies and histopathologic characteristics of breast tumours. NOS: not otherwise specified, Ductal specified includes: tubular, mucinous, medullary, papillary carcinomas. w/w: wild type/wild type; w/v+ v/v: carriage of at least one variant allele. [a] p calculated by comparison of lobular carcinoma and ductal carcinoma (ductal NOS+ ductal specified together).

			MSC OR (95% CI)[a]	Test of homogeneity p
XRCC2 1.32 (0.98,1.79)	XRCC3 C/T	T present	1.50 (0.97,2.31)	0.063
		T absent	0.77 (0.44,1.33)	
	BRCA2 A/C	C present	0.84 (0.51,1.36)	0.065
		C absent	1.60 (0.98,2.60)	
	RAD51 G/C	C present	0.60 (0.22,1.63)	0.16
		C absent	1.28 (0.89,1.84)	
XRCC3 1.00 (0.80,1.25)	XRCC2 G/A	A present	1.86 (0.97,3.55)	0.063
		A absent	0.95 (0.72,1.26)	
	BRCA2 A/C	C present	1.35 (0.93,1.96)	0.068
		C absent	0.84 (0.59,1.19)	
	RAD51 G/C	C present	0.62 (0.31,1.25)	0.11
		C absent	1.14 (0.87,1.50)	
BRCA2 1.03 (0.83, 1.28)	XRCC2 G/A	A present	0.63 (0.33,1.19)	0.065
		A absent	1.21 (0.92,1.59)	
	XRCC3 C/T	T present	1.32 (0.96,1.82)	0.068
		T absent	0.82 (0.55,1.22)	
	RAD51 G/C	C present	1.26 (0.64,2.46)	0.62
		C absent	1.05 (0.80,1.37)	
RAD51 0.82 (0.60, 1.13)	XRCC2 G/A	A present	0.43 (0.16,1.16)	0.16
		A absent	0.91 (0.62,1.35)	
	XRCC3 C/T	T present	0.65 (0.41,1.03)	0.11
		T absent	1.20 (0.66, 2.17)	
	BRCA2 A/C	C present	0.911 (0.54,1.51)	0.62
		C absent	0.76 (0.45,1.26)	

Table 10: Gene-gene interaction between HRR gene SNPs in breast cancer.

Studying interaction between XRCC2, XRCC3, BRCA2 and RAD51genes.

[a] Odds ratios and 95% confidence interval (CI) were calculated for carriage of at least one variant (v) allele of each gene between breast cancer cases (BCC) and mammography screening controls (MSC) at the presence and absence of rare variants of other genes.

3.4. Discussion

Deficient DNA repair has been reported in breast cancer patients and healthy women with a family history of breast cancer (Parshad, *et al.*, 1996, Patel, *et al.*, 1997, Roberts, *et al.*, 1999, Scott, *et al.*, 1999). Thus hereditary genetic defects in DNA repair capacity arising from genetic polymorphisms may be associated with breast cancer. We have used a case control series of 1416 individuals to determine whether there is any association between genotype of polymorphisms at HRR genes *XRCC2, XRCC3, BRCA2* and *RAD51* and breast cancer.

We observed a suggestive association between carriage of *XRCC2* GA/AA genotype and breast cancer (odds ratio for combined control groups 1.32 (0.98, 1.79)). Although not statistically significant, the carriage of *XRCC2* AA genotype was more associated with increased risk of breast cancer (2.14 (0.65, 7.06)). These results need to be confirmed in a larger study. Whilst we found no significant effect for age and family history per se, our results show that the association is greater in the selected cases and controls when younger cases with positive family history compared to older controls without family history of breast cancer (odds ratio 1.82 (0.91, 3.64)). Most of malignant breast tumours are ductal carcinoma; our data, however, indicate that carriage of *XRCC2* rare allele is more associated with lobular breast carcinoma (p=0.001). There is also some evidence on combinatory effect of *XRCC2* and *XRCC3* polymorphisms, as we detected that the association of *XRCC2* GA/AA genotype with breast cancer is higher at the presence of *XRCC3* rare allele (p=0.06). Our case-control study had 65% and 35% statistical power (α= 0.05) to detect odds ratio of 1.5 and 1.3 for carriage of the rare *XRCC2* allele (using BCC and MSC). If we suppose that the real odds ratio for carriage of rare *XRCC2* allele is 1.32 as we found in our study, a bigger case-control study consisting of at least 1600 cases and 1600 controls is needed to have enough power (80%) to detect this OR. Coincidentally and consistent with the present study, a significant positive association was also reported between the *XRCC2* AA genotype and breast cancer (Kuschel, *et al.*, 2002), thus providing confirmation of *XRCC2* AA as a risk factor for breast cancer. One of a few studies on the functional aspect of SNPs of DNA repair genes has been carried out on the *XRCC2* R188H polymorphism. Although the functional effect for R188H substitution is weak, our

collaborators showed that non-conservative substitution or deletion of amino acid position 188 causes significant deleterious effect on cell survival following DNA damage (Rafii, *et al.*, 2002). The small association between carriage of rare allele and breast cancer can reflect the small functional effect of this variant on the protein function.

We however did not find any association between carriage of rare alleles of *XRCC3*, *BRCA2* and *RAD51* and breast cancer. Although our study had 83% statistical power to detect odds ratio of 1.5, we did not detect any association between carriage of the *XRCC3* rare allele and breast cancer. The association of XRCC3 T241M polymorphism with cancer has been studied in number of experiments and produced rather conflicting results. A positive association was reported between carriage of rare allele and malignant melanoma, bladder cancer and breast cancer (Kuschel, *et al.*, 2002, Matullo, *et al.*, 2001, Winsey, *et al.*, 2000). Malignant melanoma and bladder cancer studies were carried out using a relatively small sample size (Table 11). Furthermore in a relatively larger study, the malignant melanoma association was not replicated (Duan, *et al.*, 2002) and the association with bladder cancer did not produce the same result and fell short of statistical significance (Stern, *et al.*, 2002). In a small study no association was found between carriage of *XRCC3* T allele and breast cancer (Smith, *et al.*, 2003). No association was also found between carriage of *XRCC3* rare allele and lung cancer (Butkiewicz, *et al.*, 2001, David-Beabes, *et al.*, 2001, Misra, *et al.*, 2003, Smith, *et al.*, 2003) and a weak association with squamous cell carcinoma of head and neck (SCCHN) was statistically insignificant (Shen, *et al.*, 2002). Despite having over 99% statistical power to detect an odds ratio of 2.36 (as in the melanoma study) or 2.77 (as in the bladder cancer study), our study did not have good power (49%) to exclude an effect of *XRCC3* if the true odds ratio were close to 1.3 as it is seen in most of other studies (breast cancer, SCCHN and bladder cancer; Table 11). Thus the lack of association in our dataset may be partly due to lack of power.

We observed a weak association between carriage of BRCA2 372HH genotype and breast cancer that is statistically non-significant. We also identified that the association between carriage of H genotype and breast cancer is statistically higher at the presence of XRCC3 M allele than in its absence. The results from other independent studies show that there is weak effect for BRCA2 372HH variant in association with breast cancer and ovarian cancer.

In one relatively large study using five independent data sets, a weak association was reported with breast cancer (OR=1.31 (1.07, 1.61)), although this effect was not statistically significant in all data sets analysed (Healey, *et al.*, 2000). A suggestive weak association was also reported with ovarian cancer in UK population (OR=1.25 (0.89, 1.74)) (Auranen, *et al.*, 2003). Two other studies using Australian populations also reported OR= 1.47 and 1.51 for breast and ovarian cancer respectively (Auranen, *et al.*, 2003, Spurdle, *et al.*, 2002). Thus our data on BRCA2 N372H polymorphism is consistent with these published results.

Cancer	Cases	Controls	OR (95% CI)	Reference
Breast cancer	2205	1826	1.30 (1.1,1.6)	Kuschel B, 2002
Breast cancer	162	302	0.95 (0.64,1.40)	Smith TR, 2003
Bladder cancer	124	85	2.77 (1.55,4.93)	Matullo G, 2001
Bladder cancer	233	209	1.30 (0.9,1.9)	Stern MC, 2002
Lung cancer	313	306	0.96 (0.69,1.34)	Misra RR, 2003
Lung cancer	331	687	0.92 (0.67,1.28)	David-Beabes GL, 2001
Lung cancer	96	96	N/A [a]	Butkiewicz D, 2001
Malignant Melanoma	125	211	2.36 (1.44,3.86)	Winsey SL, 2000
Malignant Melanoma	305	319	0.89 (0.65,1.24)	Duan Z, 2002
SCCHN	367	354	1.36 (0.89,2.08)	Shen H, 2002

Table 11: Studies on association between the XRCC3 T241M SNP and cancer.

[a] No difference was observed in genotype frequencies between cases and controls

No association was identified between carriage of the rare *RAD51* allele and breast cancer. Although carriage of the rare allele showed more protection against breast cancer, comparison of BCC with either of the controls could not reach any statistically significant association. Association of this polymorphism with breast cancer has been considered in few studies. Consistent with our result, in a large study no association was found for carriage of the heterozygous allele and the overall OR for carriage of the *RAD51* C allele

was 0.86 (0.68, 1.08) (Kuschel, *et al.*, 2002). However the rare *RAD51* allele appeared to be associated with increased risk of breast cancer in *BRCA1* and *BRCA2* mutation carriers (Levy-Lahad, *et al.*, 2001, Wang, *et al.*, 2001). We did not observe any evidence indicating that presence or absence of rare *XRCC2*, *XRCC3* or *BRCA2* alleles affect the association of the rare *RAD51* allele with breast cancer.

In conclusion the present study suggests that common SNPs of DNA repair genes may contribute to breast cancer susceptibility. The observed weak associations of XRCC2 R188H and BRCA2 N372H are consistent with the published studies. Due to the small effect of these polymorphisms, no single SNP is sufficient on its own to predict occurrence of a clinical phenotype with acceptable accuracy. Furthermore as our data suggest the effect of a specific SNP might not be apparent unless in the presence or absence of others. Therefore to explain the genetic basis of breast cancer a polygenic approach in which the combinatory effect of several common low-penetrant genes with each other and also with the environment seems to be needed (Pharoah, *et al.*, 2002).

CHAPTER FOUR

Chapter 4- The effect of rare sequence variants on the DNA repair activity of XRCC3

List of tables and figures

Tables

Figures

4. The effect of rare sequence variants on the DNA repair activity of XRCC3

4.1. Introduction

It is important for cells to have the capacity to correct DNA damage and maintain genome stability. This ability is lost in most cancer cells. DNA Double Strand Breaks (DSBs), caused by ionizing radiation and other forms of DNA damages are lethal if unrepaired. Cells, however, can repair DSBs through Homologous Recombination (HR) or Non Homologous End Joining (NHEJ) repair. HRR is an accurate and high- fidelity repair mechanism, whereas NHEJ repair is more error-prone (Khanna and Jackson, 2001) Defects in the process of HRR can cause mutations, chromosomal breakage, translocations and cancer, in part by shifting the repair of DSBs toward NHEJ (Brenneman, *et al.*, 2002). Therefore HR is necessary for maintaining genome stability and cells deficient in this repair pathway show high levels of chromosomal aberrations (Cui, *et al.*, 1999, Thacker, 1999).

Rad51, a eukaryotic homologue of *E.coli* RecA, is believed to play a central role in HR. Following DNA damage Rad51 forms nuclear foci, where HR and DSBs repair are presumably taking place. Two other proteins, Rad55 and Rad57, have been identified in yeast and form a heterodimer which interacts with Rad51 and dramatically enhance homologous pairing catalyzed by Rad51 (Sung and Stratton, 1996). No direct human homologues for Rad55 and Rad57 have been identified. However five genes (Rad51L1 (Rad51B), RAD51L2 (Rad51C), RAD51L3 (Rad51D), XRCC2 and XRCC3) with 20-30% sequence identity to Rad51 have been identified. This homology is mostly confined to two

highly conserved ATP binding sites termed Walker Boxes (Thacker, 1999). Very little is known about the precise function of these genes, however, it has been shown that Rad51 family members interact with each other and with Rad51 and facilitate the assembly of Rad51 at site of DSBs (Masson, *et al.*, 2001). XRCC3 interacts with both Rad51 and Rad51C and is required for the formation of damage-induced Rad51 sub-nuclear foci (Bishop, *et al.*, 1998, Kurumizaka, *et al.*, 2001, Masson, *et al.*, 2001) . Cells lacking XRCC3, in particular, show an elevated number of spontaneous and radiation induced chromosomal breaks and rearrangements (Cui, *et al.*, 1999, Fuller and Painter, 1988). These defects can be fully corrected by XRCC3 transfection (Tebbs, *et al.*, 1995), suggesting the importance of XRCC3 in maintaining genomic instability. Cell lines lacking XRCC3 such as irs1SF are also significantly sensitive to ionizing radiation and the DNA cross-linking agents cisplatin and Mitomycin-C (Cui, *et al.*, 1999, Tebbs, *et al.*, 1995). Moreover, irs1SF cells exhibit a 25-fold decrease in levels of homologous recombination (Pierce, *et al.*, 1999). Although XRCC3 was believed to act as an accessory to Rad51 for initiating HR, recent findings indicate that deficiency in the function of XRCC3 can affect both initiation and progression of HRR, and could result in chromosomal rearrangement/ instability by shifting DSBs repair from HR to error prone NHEJ repair (Brenneman, *et al.*, 2002). Chromosomal rearrangements, long gene conversion tracts and unstable heteroduplex DNA structure have been reported in XRCC3 deficient cells (Brenneman, *et al.*, 2002), suggesting a more important role for XRCC3 in maintaining genome stability. XRCC3 is also important for precise chromosome segregation at mitosis and preventing centrosome fragmentation (Griffin, *et al.*, 2000).

Given the importance of *BRCA1* and *BRCA2* in the prevention of breast cancer and the importance of Rad51 paralogues in the maintenance of chromosome stability, there has been considerable interest in the effect of mutations of these genes on cancer predisposition. Several single nucleotide polymorphisms in HRR genes including *XRCC3* have been identified and a number of studies have focussed on the potential role of these common polymorphisms as low-penetrance cancer susceptibility alleles (Healey, *et al.*, 2000, Matullo, *et al.*, 2001, Stern, *et al.*, 2002, Stern, *et al.*, 2002, Winsey, *et al.*, 2000), but thus far only limited information is available on the functional effects of such alleles. Although the functional significance of most of these polymorphisms has not yet been

determined, *In vitro* studies on some of them are suggestive of weak effect on the DNA repair capacity (Rafii, *et al.*, 2002), which needs further investigation.

In this study we report four novel single nucleotide polymorphisms in *XRCC3* gene. Although in rare frequency, one of them can drastically affect the function of the protein. We also investigated whether carrying this allele was associated with increased susceptibility to cancer.

4.2. Materials and methods

4.2.1. Cancer patients and controls

Genomic DNA was extracted from venous blood samples from subjects recruited as follows. Unrelated breast cancer patients were recruited from surgical outpatient clinics at the Royal Hallamshire Hospital, Sheffield, UK between November 1998 and June 2001. Healthy women attending mammography screening in Sheffield were recruited concurrently. This is the same cohort of patients and controls described in chapter 3. DNA samples from Swedish women with breast cancer and a family history of breast cancer were obtained by Annika Lindblom*. Healthy males and females were recruited from amongst blood donors in Sheffield. Patients with bladder and prostate cancer were recruited from urology clinics at the Royal Hallamshire Hospital Sheffield, and colorectal cancer patients were recruited from those receiving surgery in Sheffield or neighbouring hospitals. Multi-case cancer families were recruited from regional oncology clinics at Weston Park Hospital, Sheffield. Ethical committee approval was obtained from South Sheffield Research Ethics Committee and informed consent was obtained from all subjects (See appendix 1).

4.2.2. PCR amplification for SSCP genotyping

DNA amplifications were carried out using 100ng genomic DNA, 6pmol of each primer (Table 1), and 18.2µl 1.1×PCR master mix (AB gene) in final volume of 20µl. Cycling conditions were 5 minutes at 95°C followed by 35 cycles of 95°C for 35 seconds, 61°C for 25 seconds and 72°C for 25 seconds. A final extension of 5 minutes at 72°C was also applied after cycles were finished.

4.2.3. SSCP screening

SSCP screening of sample was performed as described in section 2.3.5.1. A 12% Polyacrylamide gel and 5500 volt hours were used for electrophoresis.

* see appendix for list and address of collaborators

4.2.4. TaqMan™ genotyping

DNA amplifications for 5'nuclease PCR were carried out using 12.5 µl 2X qPCR mastermix (Eurogentec), 20ng genomic DNA template, 50nM of forward and 300nM of reverse primers, 50nM FAM-labelled probe and 125nM TET-labelled probe (Table 1) in a final volume of 25µl. The cycling conditions were 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 63°C for 1 minute. Samples were then analysed using the ABI 7200 Sequence Detector to determine levels of FAM and TET.

4.2.5. Mammalian Cell Culture

Wild-type Chinese Hamster Ovary (CHO) XRCC3 proficient (AA8) and XRCC3-deficient (irs1SF) cells, human colorectal carcinoma (SW480) and human skin fibroblast cells (LM217E) were cultured in monolayer in Dulbecco's modified eagle's medium (Life Technologies Inc.) supplemented with 9% foetal calf serum. Cells were grown at 37°C in 5% CO_2 humidified incubator.

4.2.6. Site-directed Mutagenesis

Human *XRCC3* mRNA was extracted from the LM217E cell line as described in section 2.3.7 and was reverse transcribed to cDNA, amplified using *Pfu* Turbo polymerase (Stratagene) as described in section 2.3.8. The XRCC3 cDNA was then cloned into mammalian expression vector pcDNA 3.1*N*s-His Topo TA (Invitrogen) as stated in section 2.3.9. The mutagenic polymerase chain reaction was carried out using QuikChange site-directed mutagenesis kit (Stratagene) with mutagenic primers (Table 1) designed to create changes at desired positions (Also see section 2.3.10). All created mutations were confirmed by direct sequencing.

4.2.7. Transfection of mutant constructs into mammalian cell line

Approximately 15µg of plasmids carrying wild type and each mutant DNA were transfected into irs1SF cells by electroporation (Bio-Rad Gene Pulser at 400 V and 125 µF) as described in section 2.3.12.1. A total of $5X10^5$ cells were seeded into 100mm plates and incubated for 14 days in 100 µg/ml of G418.

4.2.8. RNA extraction and RT-PCR

Messenger RNA was extracted from the same clones for which drug sensitivity had already been determined using Oligotex Direct mRNA micro kit (QIAGEN) according to the manufacturer instructions. Since the hamster *XRCC3* sequence was not available on the database, mouse and human *XRCC3* sequence were aligned and RT- PCR primer designed to only amplify the human sequence (Table 1). RT-PCR was performed according to the condition described in section 2.3.14.2. A negative control (excluding reverse transcriptase) was used for each sample to eliminate the possibility that any PCR products originated from contaminating DNA.

4.2.9. Drug toxicity assay

Cell survival assays were performed by exposing cells from individual variant or wild type clones to the DNA cross-linking agent Mitomycin-C (MMC), Topo isomerase inhibitor Camptothecine (CPT) and Thymidine as described in section 2.3.17. Plating efficiency was assessed in triplicate for each clone.

4.2.10. Western Blotting

Cells were grown to sub-confluent levels in tissue culture plates; total protein was extracted and quantified using the coomasie plus protein assay (Pierce) as described in sections 2.3.15.1 and 2.3.15.2. A 20µg protein for each sample was subjected to electrophoresis on a 10% SDS-polyacrylamide gel. Following electrophoresis, the proteins were transferred onto polyvinylidene difluoride membrane (Bio-Rad) as described in sections 2.3.15.4 and 2.4.4. XRCC3 protein was detected using the XRCC3 antibody at a dilution of 1:200 in PBST, 3% non fat dried milk. Anti-goat IgG/ horseradish peroxidase conjugate was used as

the secondary antibody (Santa Cruz Biotechnology) at a dilution of 1:5000, and bands were visualized using ECL reagents (Amersham pharmacia biotech) and Kodak film (Kodak) according to the manufacturer's protocols.

4.2.11. 2-D gel electrophoresis

Proteins were extracted from irs1SF (XRCC3 deficient) and transfectant D213N irs1SF cells, quantified (Pierce) and passed through 5K Dalton centrifugal filter tubes at 12000 rpm for 20 minutes to filter larger proteins. To spread proteins according to their isoelectric point on horizontal direction, IPG strips were rehydrated with equal amount of protein in sample buffer for 12-16 hours at 50 volts at room temperature under active condition following by another 40000 volt hours (Bio-Rad) as described in section 2.3.16. The IPG strips were then loaded onto 14% SDS acrylamide gel and vertical electrophoresis was performed at 16 mA/ gel for 30 minutes and 24mA/gel for 5 hours. After electrophoresis was completed gels were stained as described in section 2.3.16. Western blotting was performed on gels before they stained (see sections 2.3.15.4-7).

4.2.12. Restriction Fragment Length Polymorphism (RFLP) Analysis

RFLP was carried out as described in section 2.3.5.2 using primers and conditions which were described at "PCR amplification" section 4.2.2.

4.2.13. DNA Sequencing

Genomic or plasmid DNA was amplified in volume of 50µl and was subjected to 2% agarose gel electrophoresis. The correct band was excised and the DNA was gel purified using DNA gel purification kit (Qiagen). Sequencing reaction was performed using human or vector-specific primers. For sequencing reactions, the SequiTherm EXCEL™II DNA Sequencing Kit-LC (Epicentre Technologies) was utilized as described in section 2.3.6.2.

PCR primers for SSCP	Forward: 5' CCA CAG GACACC TTG TTG GA 3' Reverse: 5' AAA TAC GAG CTC AGG GGT GC 3'
RT-PCR Primers	Forward: 5' ATG GAT TTG GAT CTA CTG GA 3' Reverse: 5' CAC CGT GTC AGT GGG ACT 3'
Mutagenic Primers	**P199L** Forward: 5'GTG AAT AAG AAG GTC C**T**C GTA CTG CTG TCT CGG 3' Reverse: 5' CCG AGA CAG CAG TAC G**A**G GAC CTT CTT ATT CAC 3' **D213N** Forward: 5'CGC CTG GTG GTC ATC **A**AC TCG GTG GCA GC 3' Reverse: 5'GCT GCC ACC GAG T**T**G ATG ACC ACC AGG CG 3' **T241I** Forward: 5'GGG GGC CA**T** **T**CT GCG TGA GCT GAG CAG TGA 3' Reverse: 5'GCA CTG CTC AGC TCA CGC AG**A** **A**TG GCC CCC 3' **T241M** Forward: 5'GGG GGC CA**T** GCT GCG TGA GCT GAG CAG TGA 3' Reverse: 5'GCA CTG CTC AGC TCA CGC AGC **A**TG GCC CCC 3'
TaqMan primers and probes	**Primers** Forward: 5'AGG TCC CCG TAC TGC TGT CTC 3' Reverse: 5' CTG TCA AAT TCA CAG CGG AAT G 3' **Probes** Detecting *XRCC3* D213: 5' FAM- CCA CCG AGT **C**GA TGA CCA CCA G-TAMARA 3' Detecting *XRCC3* N213: 5' TET- CCA CCG AGT TGA **T**GA CCA CCA GG-TAMARA3'
Vector-specific primers	T7: 5' TAA TAC GAC TCA CTA TAG GG 3' BGH Reverse: 5' TAG AAG GCA CAG TCG AGG 3'

Table 1: Probes and primers for the XRCC3 functional study.

SSCP, RT-PCR, and mutagenic primers for XRCC3, primers and probes for the *D213N* Taqman™ assay, and vector-specific (pcDNA 3.1) sequencing primers. Bold underlined nucleotides indicate the site of induced mutations.

4.3. Results

4.3.1. Four novel polymorphisms were identified in XRCC3 exon 7

As part of routine genotyping of the *XRCC3 T241M* polymorphism, we carried out PCR-SSCP analysis of exon 7 of the *XRCC3* gene using genomic DNA from 1416 women (526 breast cancer cases, 491 healthy women attending mammography screening and 399 healthy female blood donors). In addition to the published T241M variant, we found four novel variants in this exon, as indicated by extra bands not presented in the wild type samples (Fig. 1A). DNA sequence analysis of these variants revealed three heterozygous non-synonymous alterations; C/T (P199L), G/A (D213N), G/T (T241I) and a synonymous alteration C/T (L201L) (at position 17941, 17982, 18068 and 17946 respectively in GenBank accession no. AF037222) (Fig. 1B). We were not able to differentiate the extra sequencing band from sequencing artefact for P199L alteration by direct sequencing of genomic DNA. Therefore to confirm the sequencing results obtained from the PCR products, the amplification product of exon 7 was cloned into the pCR2.1-TOPO vector, transformed and cultured in *E.coli*. Seven colonies were selected; the plasmids were extracted and sequenced using vector-specific primers (M13 and T7 primers). Two of the colonies carried the variant copy of XRCC3 (T) and five showed the wild type (C) sequence at position 17941(See appendix 3).

4.3.2. The D213N substitution occurs in a highly conserved region

Alignment of XRCC3 protein with some other members of Rad51 family included hsRad51, hsXRCC2 and also mouse XRCC3 revealed that the *XRCC3 D213N* variant occurs in a highly conserved region of the protein, the ATP binding domain, known as the Walker box B (Walker, *et al.*, 1982). The *XRCC3* P199L and T241M/I variants fall close to this region and are conserved between human and mouse (Fig.2). Further alignments of XRCC3 with 23 other proteins of Rad51 family from different species also showed that D213 is conserved in species such as Arabidopsis thaliana, fission yeast, Drosophila melanogaster and mouse, suggesting that this site may be important in the function of the protein.

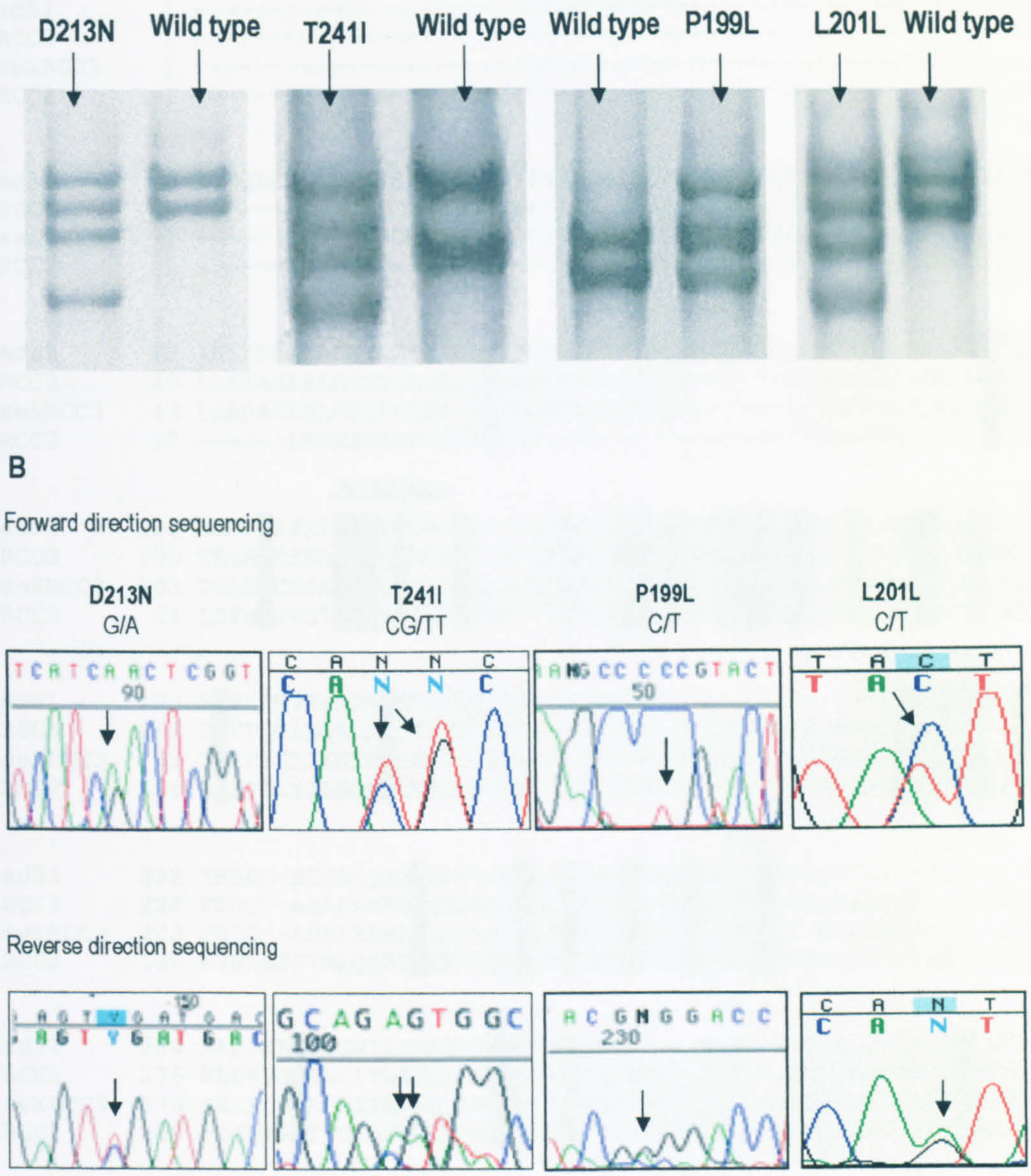


Figure 1: Novel variants in exon 7 of XRCC3.

Genomic DNA was amplified for XRCC3 exon 7. The resulting PCR products were denatured and subjected to electrophoresis on 12% non denaturing polyacrylamide gel (SSCP). A shows the results of SSCP analysis comparing band pattern identified in the novel variants and the wild type samples. B DNA sequences of novel variants were obtained by direct sequencing of amplified XRCC3 exon 7 in both forward and reverse directions using the primers listed in table 1. Arrows indicate site of novel variants.

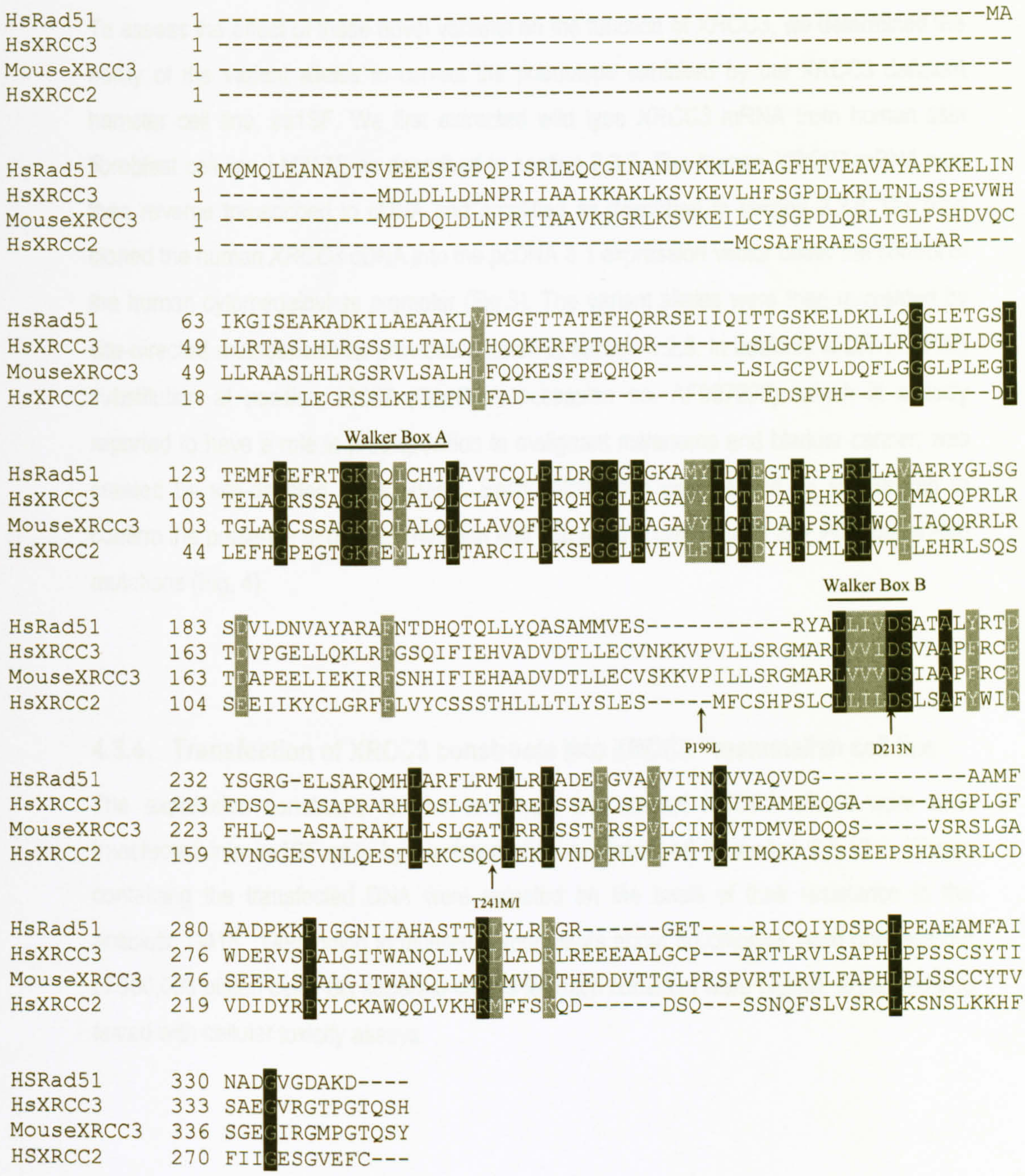


Figure 2: Amino acid sequence alignment

Amino acid sequences of the relevant regions of human RAD51, human XRCC3, mouse XRCC3 and human XRCC2 are shown. Black boxes indicate amino acid identity and grey boxes indicate conservation of charge. The positions of the Walker A and B boxes are shown by a horizontal line and the positions of the amino acid variants investigated are indicated by vertical arrows

4.3.3. Cloning and site-directed mutagenesis of the human XRCC3 gene

To assess the effect of these novel variants on the function of XRCC3, we determined the ability of the variant alleles to correct the phenotype exhibited by the XRCC3 deficient hamster cell line, irs1SF. We first extracted wild type *XRCC3* mRNA from human skin fibroblast cell line LM217E as described in section 2.3.7. The human *XRCC3* mRNA was then reverse transcribed to cDNA and amplified as described in section 2.3.8. We then cloned the human *XRCC3* cDNA into the pcDNA 3.1 expression vector under the control of the human cytomegalovirus promoter (Fig.3). The variant alleles were then re-created by site-directed mutagenesis as previously stated in section 4.2.6. In addition, a C/T (T241M) substitution at position 18087 (GenBank accession no. AF037222), which is already reported to have a role in predisposition to malignant melanoma and bladder cancer, was created by site-directed mutagenesis. Each substitution was verified by sequencing to confirm the presence of desired mutation and rule out the presence of any PCR-generated mutations (Fig. 4).

4.3.4. Transfection of XRCC3 constructs into XRCC3$^{-/-}$ mammalian cell line

The expression constructs for the wild type and variant *XRCC3* alleles were then transfected into irs1SF cells by electroporation as described in section 2.3.12.1. Clones containing the transfected DNA were selected on the basis of their resistance to the antibiotic G418. Depending in different experiments about 50 colonies were obtained out of 500,000 plated cells. Six separate clones for each construct were picked, expanded and tested with cellular toxicity assays.

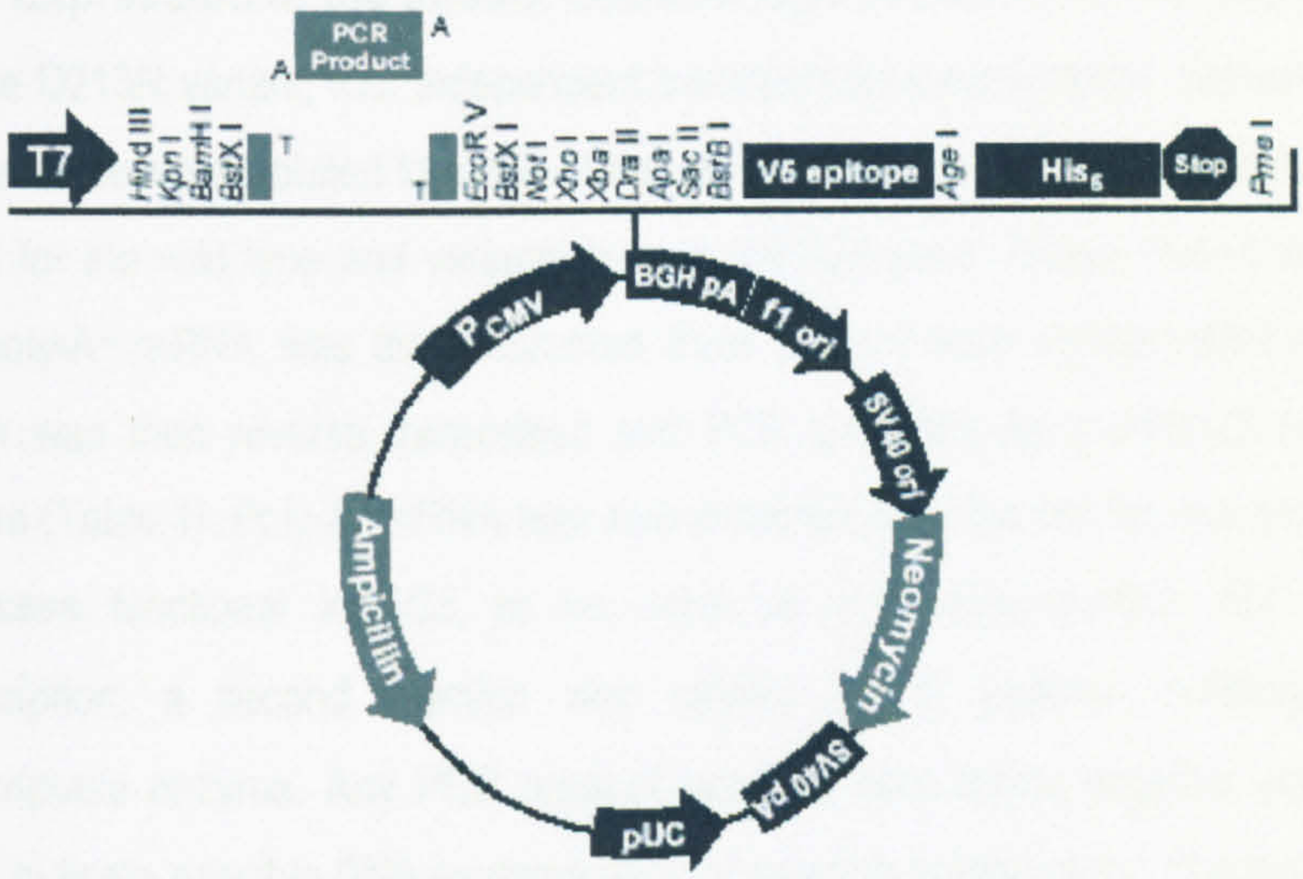


Figure 3: Schematic Map of pcDNA 3.1

Adopted from pcDNA3.1/V5-His© TOPO® TA Expression kit catalog supplied by Invitrogen.

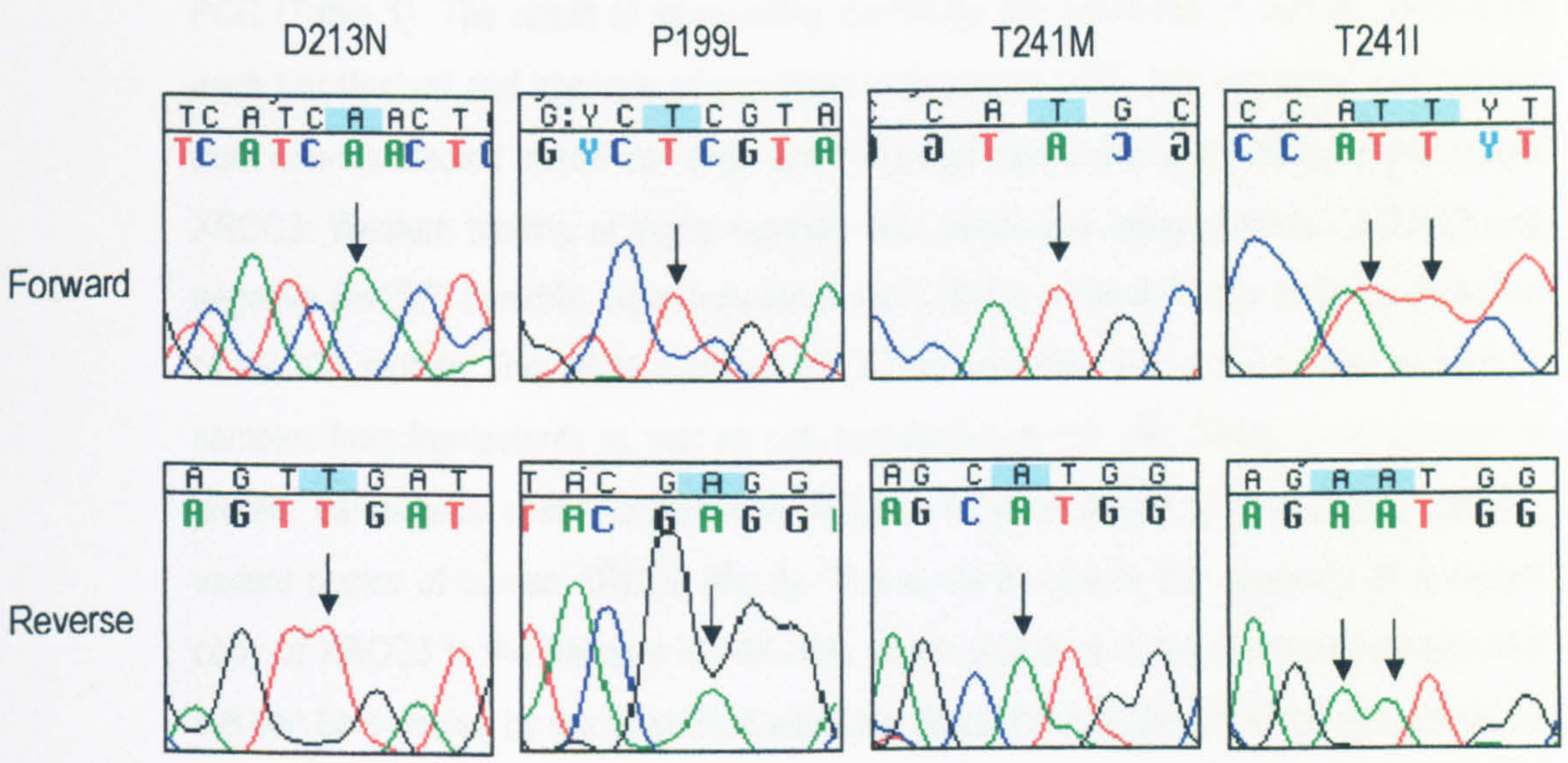


Figure 4: cDNA sequence of cloned XRCC3 after site directed mutagenesis

Human XRCC3 cDNA was cloned into pcDNA 3.1 expression vector. Novel mutations were created at desired positions using site directed mutagenesis. Created mutations were confirmed by sequencing using vector specific primers.

4.3.5. Expression of the mutant and wild type alleles in irs1SF cell line

For the D213N variant, four independent transfectants were isolated and two independent transfectants were isolated for each of the other constructs. Expression of the isolates was tested for the wild type and variants human *XRCC3* gene. These clones were expanded and poly-A$^+$ mRNA was then extracted from cells of each independent clone. Poly-A$^+$ mRNA was then reverse transcribed and PCR amplified using *XRCC3* human specific primers (Table 1). Poly-A$^+$ mRNA was also extracted from the human cell line LM217E that expresses functional *XRCC3*, to be used as a positive control. For each reverse transcription, a second reaction was carried out in parallel, omitting the reverse transcriptase enzyme. Any PCR product resulting from these negative control reactions would indicate possible DNA contamination of reaction components. The results of the RT-PCR reactions show that human primers have amplified the *XRCC3* mRNA from the positive control and the transfected cell lines but not from the non-transfected hamster cell line (Fig. 5A). Expression of the correct alleles was also confirmed by sequencing of whole length of *XRCC3* cDNA for each amplified product using the same primers used for RT-PCR (Table 1). The result of sequencing confirmed the presence of variant *XRCC3* for each transfectant and absence of any other mutation (Fig 5B). We extracted total protein from non-transfected irs1SF cell lines and individual clones carrying wild type and variant XRCC3. Western blotting of these samples was performed using positive (LM217E) and negative (irs1SF) controls. Goat polyclonal anti-XRCC3 antibody* was used for detection of XRCC3 protein. The XRCC3 protein (37.8 KD) was detected at the correct location in samples from tranfectants as well as non transfected irs1SF. No difference in strength of protein bands was observed between XRCC3 deficient irs1SF and cell lines carrying variant copies of human XRCC3 (Fig 6). This could be due to the presence of a mutant copy of XRCC3 in the hamster irs1SF cells which produces a non functional protein that still can be detected by the polyclonal antibody. Since the human XRCC3 protein would be likely to differ from the hamster protein on the basis of their isoelectric points and molecular weight, we tried to separate human XRCC3 from CHO cell XRCC3 using 2D gel electrophoresis followed by western blotting as described in sections 2.3.16 and 2.3.15.4-7. Although distribution of protein in samples from human was different from CHO on the gel (Fig.7), we did not detect any differences between two samples when XRCC3 was

* This antibody detects a peptide mapping near the amino terminus domain of XRCC3 of the human origin (Sanata Cruz Biotechnology, Inc.)

detected using polyclonal XRCC3 antibody which was the only commercially available antibody at the time of experiment. This also could be the result of the low specificity of the antibody for human protein. Therefore we did not have any means of showing the presence of the XRCC3 protein in the transfected and absence of it in the non-transfected XRCC3 deficient cells. However results from RT-PCR of XRCC3 mRNA clearly show that the human XRCC3 mRNA is not being made in the untransfected irs1SF. Therefore it is extremely unlikely that XRCC3 band in irs1SF detected on the western blotting have stemmed from any human source, and all transfected variants are expressed well at the RNA level (Fig. 5)

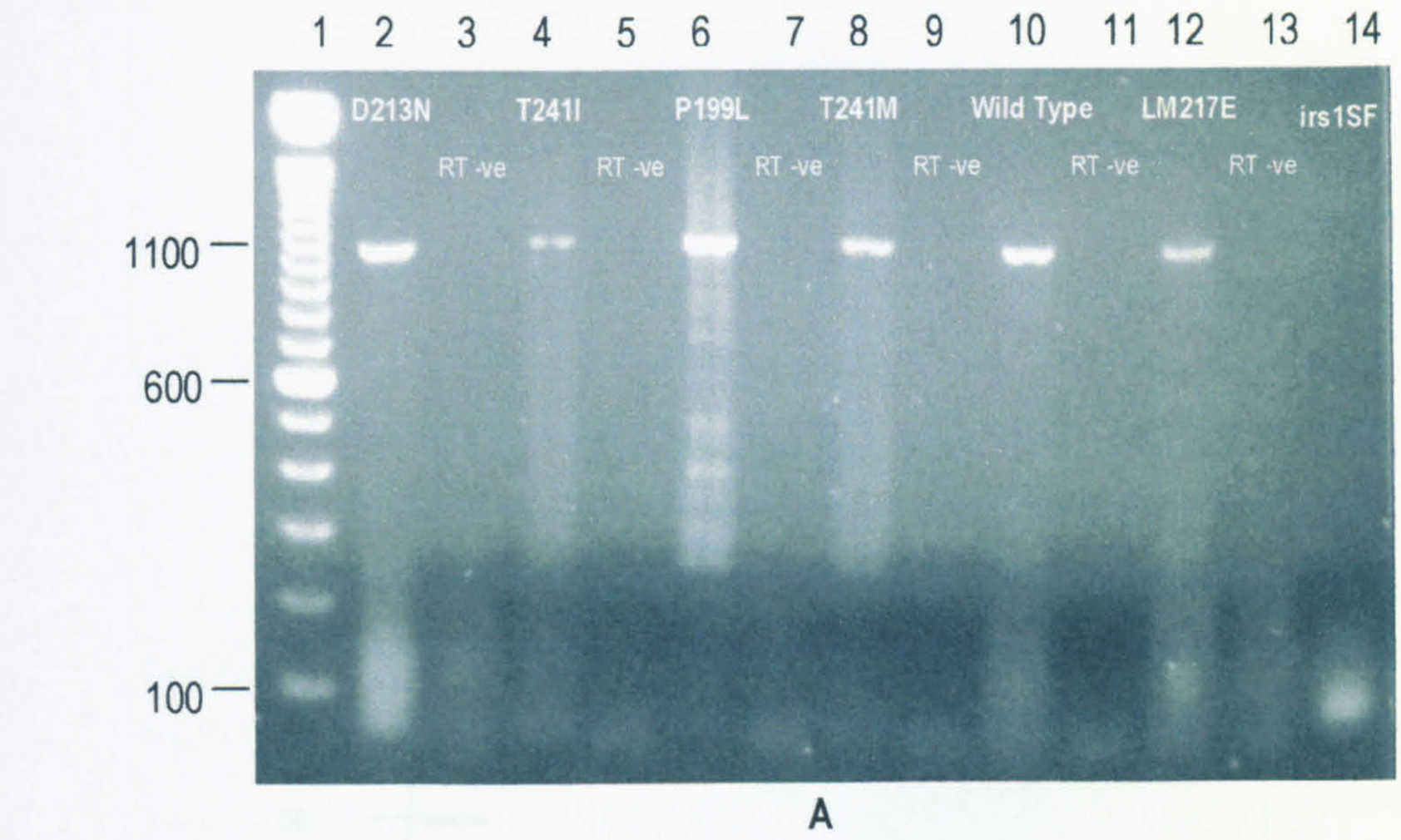


Figure 5: Transfected clones express variant and wild-type XRCC3

A: To confirm expression of the transfected wild-type or mutant genes, polyA+ mRNA was extracted from each transfected irs1SF clone and subjected to RT-PCR. The resulting RT-PCR gel image for one representative clone from each transfection is shown (lanes 2, 4, 6, 8, 10), together with RT-PCR products from the LM 217E human cell line as a positive control (lane 12) and non-transfected irs1SF as a negative control (lane 14). Negative controls for each RT-PCR reaction, lacking reverse transcriptase, were also included (lanes 3, 5, 7, 9, 11, and 13). Lane 1 contains a DNA size marker.

B: DNA sequence chromatograms derived from sequencing the 4 mutant RT-PCR products shown in A. The RT-PCR products were sequenced completely in both directions, to confirm the expression of the correct human allele and the absence of extraneous mutations. The position of the relevant base change is indicated with an arrow.

Figure 6: Western blot image of XRCC3 protein

XRCC3 protein bands (~ 37 KD) in irs1SF and transfected cell lines were visualized by exposing to goat polyclonal anti XRCC3 antibody. Human XRCC3 protein from LM217E cell line was used as positive control.

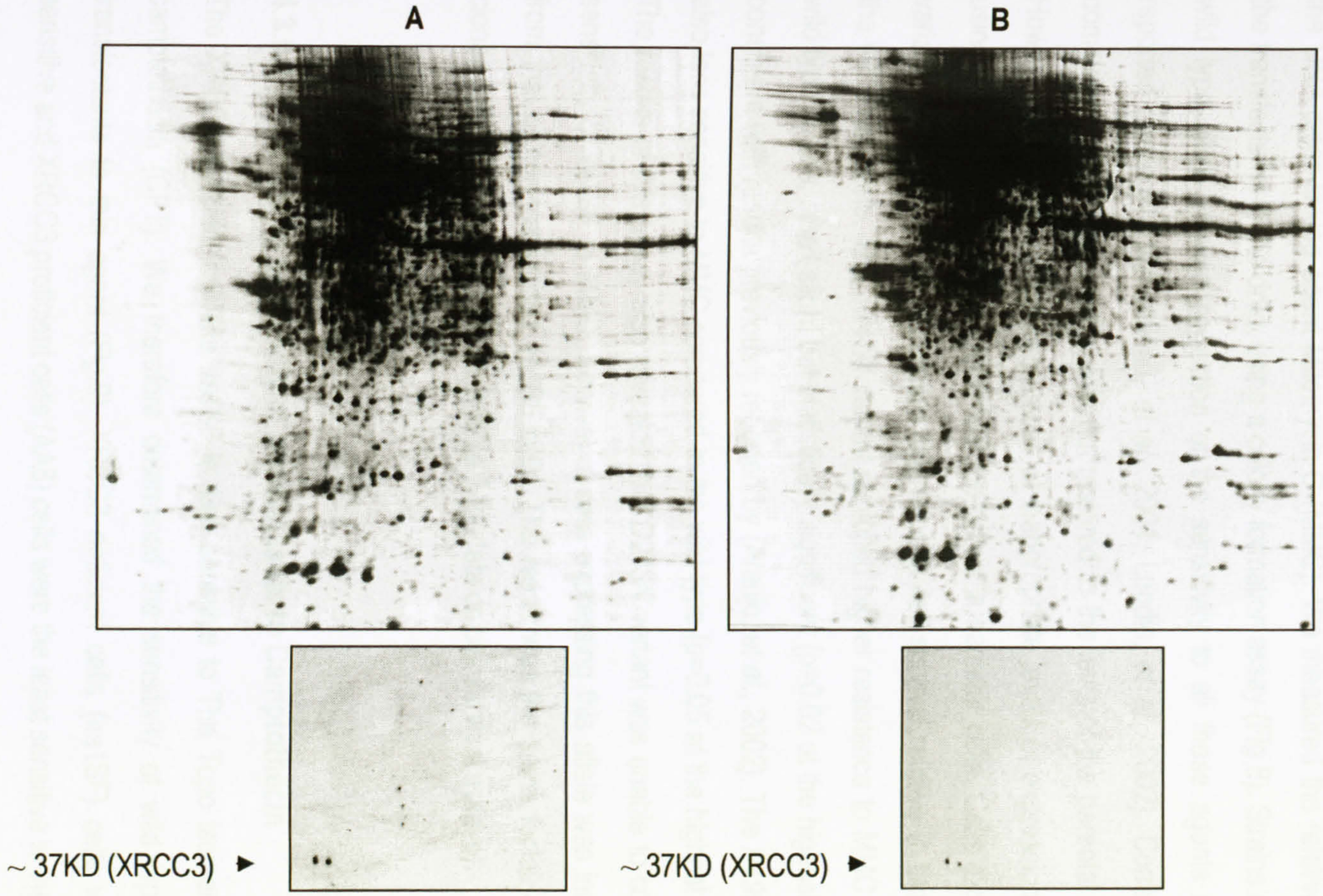


Figure 7: 2-D gel electrophoresis image of irs1SF cell lines.

Whole protein 2-D gel electrophoresis of irs1SF transfected with the human XRCC3 D213N variant (A) and irs1SF without transfection (B). subsequent western blotting of the same gels using the XRCC3 antibody did not show any difference between the XRCC3 protein of transfected and untransfected cells. Arrows show the location of the XRCC3 protein.

4.3.6. The XRCC3 D213N variant is not functional

4.3.6.1. Determining sensitivity of transfectants to Mitomycin-C

To assess whether the variant alleles retained XRCC3 function, we determined the ability of the expression constructs to correct the sensitivity of the XRCC3 deficient cells, irs1SF, to the DNA cross-linking agent Mitomycin-C (MMC). We measured the relative sensitivity of the transfectants to the drug, using a colony formation assay (Fig.8). Strains expressing the wild type allele showed correction of the sensitivity to all these agents similar to that reported previously (Arnaudeau, *et al.*, 2001, Lundin, *et al.*, 2002). Correction was not complete and the cell survival was not restored to the level of the parental cell line, AA8. However this could be a consequence of inappropriate levels of expression or a result of using the human allele to complement the defect in hamster cells. Cells expressing *T241I* variant did not show any significant difference in cell survival relative to those expressing the wild type allele. The *T241M* variant exhibited higher resistance to MMC relative to the wild type strains, albeit slight but statistically significant ($p=0.02$ at the highest dose of drug) consistent with results previously reported by (Araujo, *et al.*, 2002). The *P199L* variant was also less sensitive to MMC compared to the wild type ($p=0.05$ at the highest dose of drug). The results of this assay also revealed that *D213N* variant was unable to complement the sensitivity of irs1SF to MMC. Survival of cells expressing this allele was indistinguishable from that of non-transfected irs1SF cells. The result was the same for all 4 independent clones isolated for this variant (See table 2 for data on cell survival assays).

4.3.6.2. Determining sensitivity of transfectants to Camptothecin

The XRCC3 deficient cell line irs1SF is also sensitive to The Topo isomerase I inhibitor camptothecin (CPT). We therefore determined the sensitivity of wild type and variant transfectants to this agent (Fig.9). XRCC3 deficient cells (irs1SF) cells were the most sensitive and XRCC3 proficient cells (AA8) cells were the least sensitive to the drug.

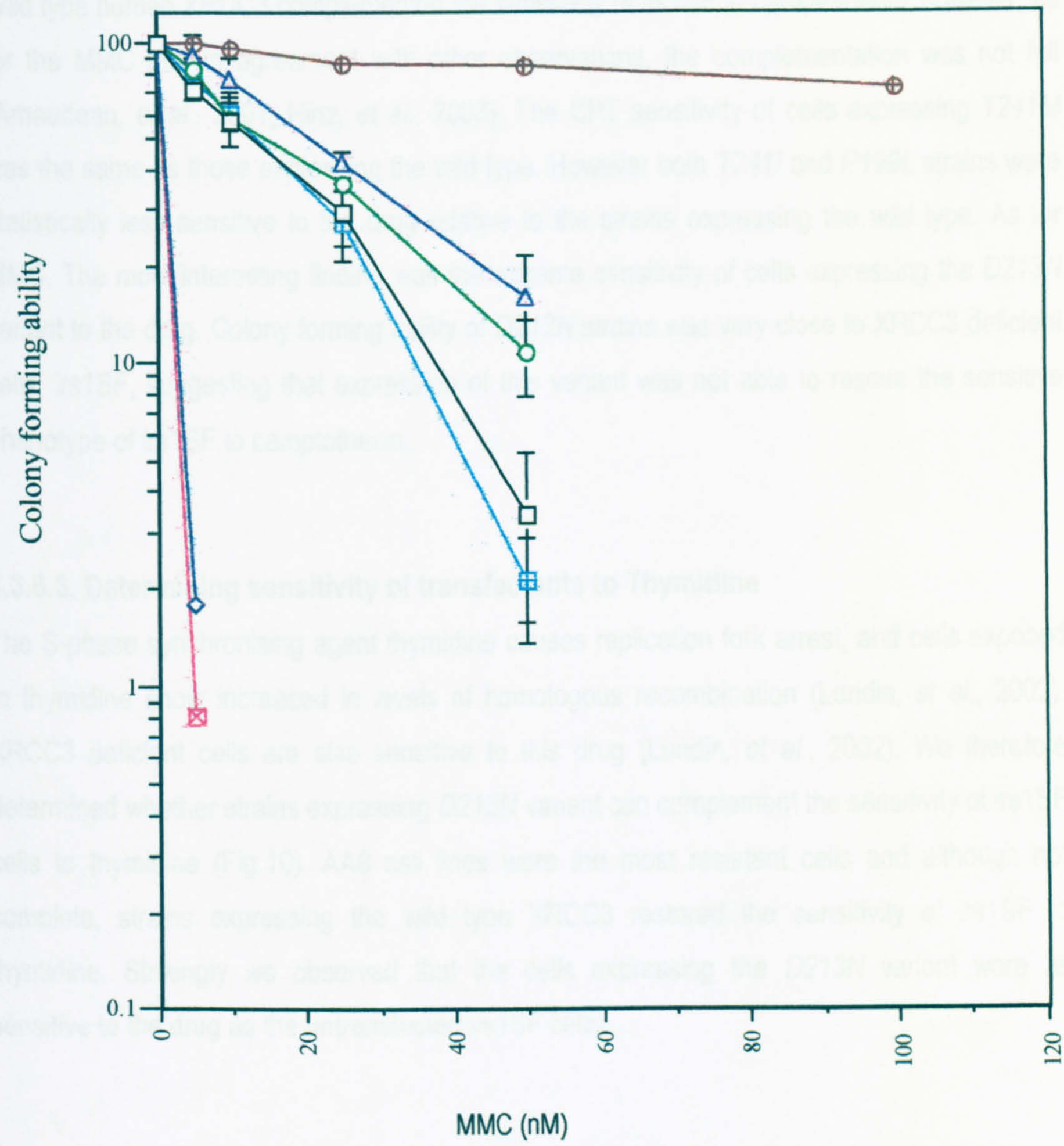


Figure 1: the effect of sequence variants on XRCC3 on cell survival following exposure to Mitomycin-C.

Mitomycin-C survival curves for XRCC3-deficient irs1SF cells, irs1SF cells stably transfected with wild-type or variant (*D213N, T241M, T241I, P199L*) *XRCC3* cDNA, and parental AA8 cells. Data are shown as mean and standard deviation of 12 experiments (3 experiments on each of 4 independent cloned transfectants) for *D213N* and 6 experiments (3 experiments on each of 2 independent clones) for all other transfectants. For some points the standard deviation is very low and thus error bars are not visible. Plot symbols: ⊕- AA8, □- wild type XRCC3, ⊠ - irs1SF, ◊ - D213N, O - T241M, △ - P199L, ⧾ - T241I

Wild type human XRCC3 complemented the sensitivity of irs1SF to camptothecin, however, as for the MMC and in agreement with other observations, the complementation was not full (Arnaudeau, *et al.*, 2001, Hinz, *et al.*, 2003). The CPT sensitivity of cells expressing *T241M* was the same as those expressing the wild type. However both *T241I* and *P199L* strains were statistically less sensitive to the drug relative to the strains expressing the wild type. As for MMC, The most interesting finding was the extreme sensitivity of cells expressing the *D213N* variant to the drug. Colony forming ability of *D213N* strains was very close to XRCC3 deficient cells, irs1SF, suggesting that expression of this variant was not able to rescue the sensitive phenotype of irs1SF to camptothecin.

4.3.6.3. Determining sensitivity of transfectants to Thymidine

The S-phase synchronising agent thymidine causes replication fork arrest, and cells exposed to thymidine show increased in levels of homologous recombination (Lundin, *et al.*, 2002). XRCC3 deficient cells are also sensitive to this drug (Lundin, *et al.*, 2002). We therefore determined whether strains expressing *D213N* variant can complement the sensitivity of irs1SF cells to thymidine (Fig.10). AA8 cell lines were the most resistant cells and although not complete, strains expressing the wild type XRCC3 restored the sensitivity of irs1SF to thymidine. Strikingly we observed that the cells expressing the *D213N* variant were as sensitive to the drug as the untransfected irs1SF cells.

Drug dose	AA8	irs1SF	irs1SF+ WT	irs1SF+ D213N	irs1SF+ T241M	irs1SF+ T241I	irs1SF+ P199L
MMC							
0	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)
5	100.1 (0.469)	0.82 (0.084)	71.24 (6.617)	1.81 (0.0019)	84.17 (6.385)	78 (11.174)	91.32 (14.437)
10	96.75 (1.558)	0	57.72 (5.531)	0	58.7 (11.053)	60.2 (7.48)	77.21 (12.47)
25	86.16 (7.87)	0	29.87 (6.589)	0	36.32 (9.699)	27.5 (6.928)	42.35 (4.832)
50	85.21 (7.311)	0	3.39 (1.813)	0	10.64 (2.82)	2.11 (0.763)	15.98 (5.44)
100	73.01 (4.1)	0	0	0	0		
CPT							
0	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)
5	99.125 (1.2374)	1 (0)	73.45 (11.407)	2.25 (0.037)	72.8 (3.741)	89 (7.211)	96.25 (13.585)
10	98.8 (0.420)	0 (0)	42.675 (13.154)	0	44.3 (1.453)	84.41 (6.531)	77.58 (8.703)
25	83.4 (4.242)	0 (0)	17.85 (5.36)	0	14.46 (0.012)	33.29 (5.907)	38.26 (9.402)
50	71.675 (1.944)	0 (0)	0.94 (0.301)	0	0.81 (0.213)	8.1 (2.851)	21.37 (3.107)
75	29.68 (3.365)	0	0	0	0	0	0
Thymidine							
0	100 (0)	100 (0)	100 (0)	100 (0)	-	-	-
0.5	97.45 (2.192)	35.62 (6.187)	92.08 (0.123)	41.5 (13.152)	-	-	-
1	82.75 (10.253)	6.25 (1.06)	82.25 (3.889)	10.25 (1.033)	-	-	-
2	66.75 (8.738)	0	63 (24.041)	0	-	-	-
5	16.63 (3.667)	0	10.03 (4.442)	0	-	-	-
10	6.45 (2.020)	0	1.48 0.452)	0	-	-	-

Table 2: Raw data for MMC, CPT and thymidne sensitivity of AA8 and irs1SF transfectant.

Average of colony survival % (Standard deviation).

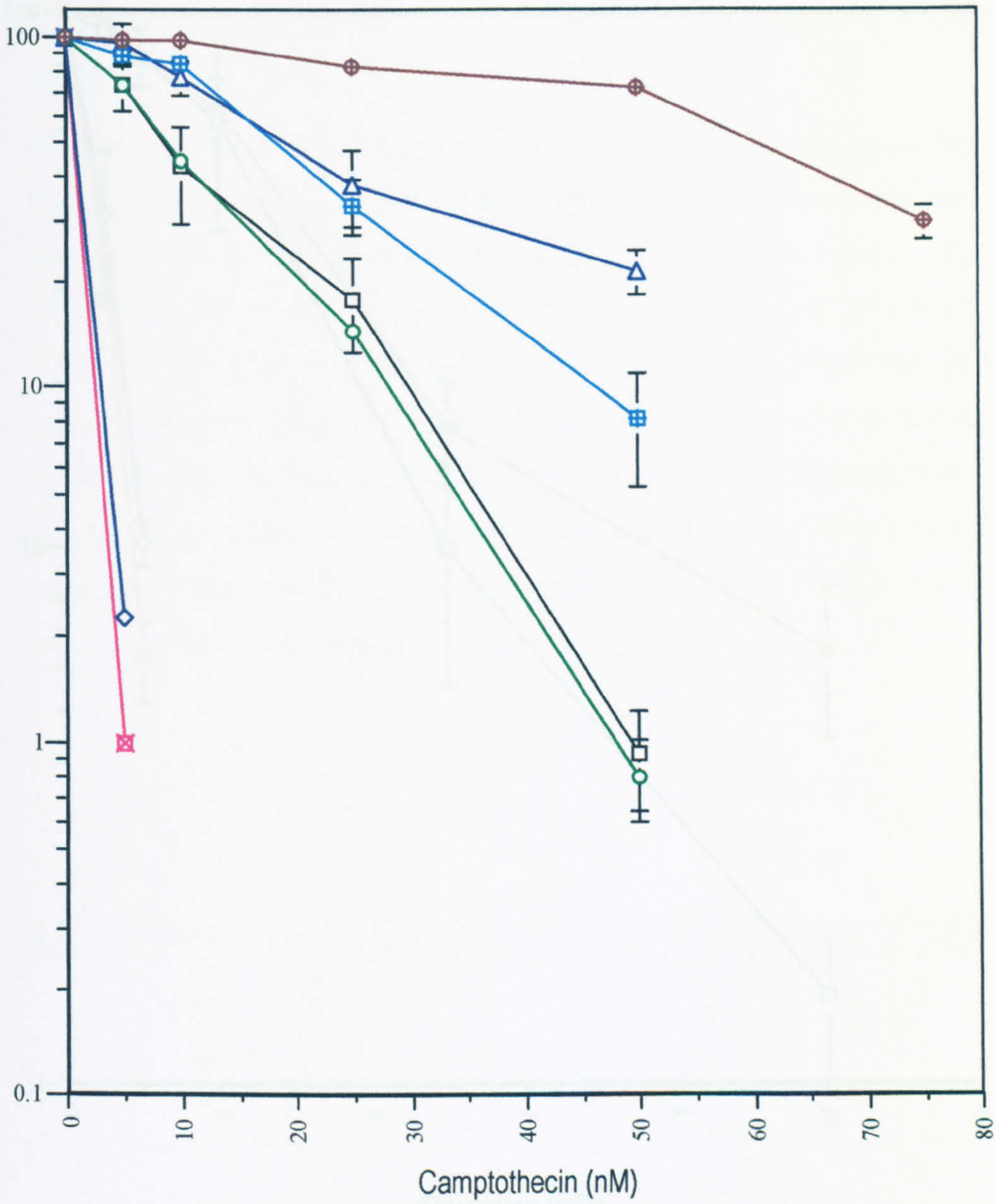


Figure 9: the effect of sequence variants on XRCC3 on cell survival following exposure to camptothecin.

Colony outgrowth assay in CHO-based irs1SFcell line, irs1SF transfected with wild type and variant human XRCC3 (*D213N, T241M, T241I, P199L*) and parental CHO XRCC3 proficient AA8 cell lines following CPT treatment. Data are shown as mean and standard deviation of 12 experiments (3 experiments on each of 4 independent cloned transfectants) for *D213N* and 6 experiments (3 experiments on each of 2 independent clones) for all other transfectants. For some points the standard deviation is very low and thus error bars are not visible. Plot symbols: ⊕- AA8, □- wild type XRCC3, ⊠ - irs1SF, ◊ - D213N, O - T241M, △ - P199L, ⌗ - T241I.

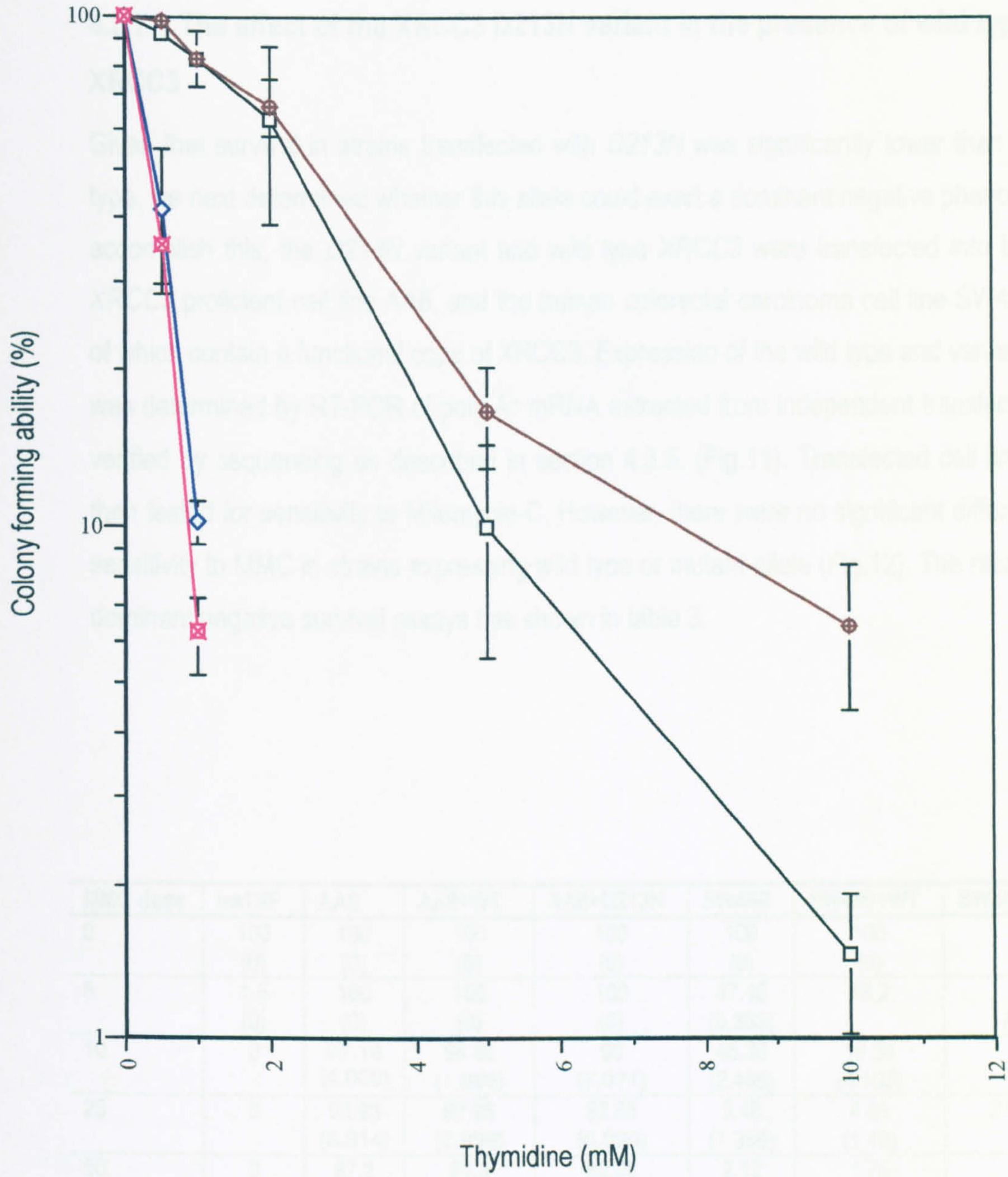


Figure 10: the effect of sequence variants on XRCC3 on cell survival following exposure to Thymidine.

Thymidine survival curves for XRCC3-deficient irs1SF cells, irs1SF cells stably transfected with wild-type or mutant (*D213N*) *XRCC3* cDNA, and parental AA8 cells. Data are shown as mean and standard deviation of 6 experiments (3 experiments on each of 2 independent cloned transfectants) for *D213N.* Plot symbols: ⊕- AA8, □- wild type XRCC3, ☒ - irs1SF, ◊ - D213N.

4.3.7. The effect of the XRCC3 D213N variant in the presence of wild type XRCC3

Given that survival in strains transfected with *D213N* was significantly lower than the wild type, we next determined whether this allele could exert a dominant negative phenotype. To accomplish this, the *D213N* variant and wild type *XRCC3* were transfected into the CHO *XRCC3* proficient cell line AA8, and the human colorectal carcinoma cell line SW480, both of which contain a functional copy of XRCC3. Expression of the wild type and variant alleles was determined by RT-PCR of poly-A^+ mRNA extracted from independent transfectant and verified by sequencing as described in section 4.3.5. (Fig.11). Transfected cell lines were then tested for sensitivity to Mitomycin-C. However, there were no significant differences in sensitivity to MMC in strains expressing wild type or mutant allele (Fig.12). The raw data for dominant negative survival assays has shown in table 3.

MMC dose	irs1SF	AA8	AA8+WT	AA8+D213N	SW480	SW480+WT	SW480+D213N
0	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)
5	1.5 (0)	100 (0)	100 (0)	100 (0)	47.40 (3.333)	48.2	58.33 (10.75)
10	0	97.16 (4.009)	98.85 (1.626)	90 (7.071)	45.30 (2.465)	19.34 (4.103)	52.85 (9.27)
25	0	93.93 (6.314)	97.95 (2.899)	82.65 (5.020)	3.48 (1.366)	4.61 (1.40)	3.55(1.12)
50	0	87.2 (4.242)	81.3 (7.071)	69.95 (2.333)	2.12 (0.851)	1.79 (0.86)	1.25 (0.53)
100	0	43.09 (8.329)	25.82 (0.954)	26.49 (6.943)	0	0.62 (0.25)	0
150	0	3.83 (0.947)	0.4 (0.081)	0	0	0	0
200	0	0	0	0	0	0	0

Table 3: Raw data for MMC sensitivity of AA8 and SW480 transfectants.

Average of colony survival % (Standard deviation).

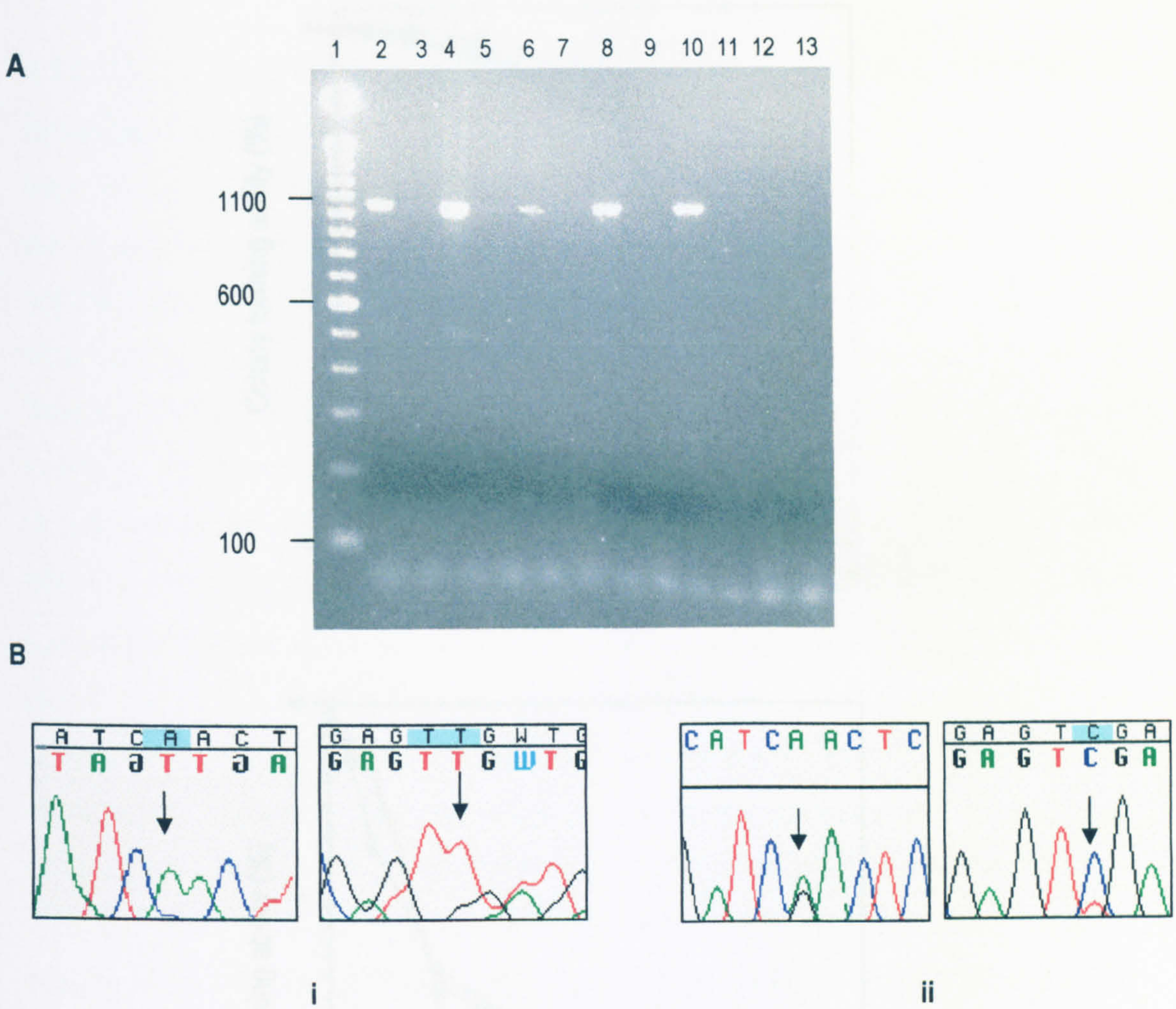


Figure 11: Transfected AA8 and SW480 clones express D213N variant and wild-type XRCC3

A: RT-PCR gel image following the extraction of polyA+ mRNA from the wild type and D213N transfected AA8 and SW480 cell lines. The result of RT-PCR of wild type and D213N transfection in AA8 (lanes 2 and 4) and SW480 (lanes 8 and 10) is shown, together with RT-PCR products from the non-transfected SW480 (lane 6) and non-transfected AA8 (lane 12) and irs1SF as a negative control (lane 13). Negative controls for each RT-PCR reaction, lacking reverse transcriptase, were also included (lanes 3, 5, 7, 9 and 11). DNA amplification occurred by using non-transfected SW480 mRNA due to presence of human *XRCC3* gene. Lane 1 contains a DNA size marker.

B: DNA sequence chromatograms derived from sequencing the D213N variant RT-PCR products shown in A. DNA sequence of D213N variant extracted from AA8 (i) cell line and from SW480 (ii). The RT-PCR products were sequenced completely in both directions, to confirm the expression of the correct human allele and the absence of extraneous mutations. The position of the relevant base change is indicated with an arrow. Heterozygous pattern is observed in samples extracted from SW480 transfected with XRCC3 D213N variant due to presence of human *XRCC3* in SW480 cell lines.

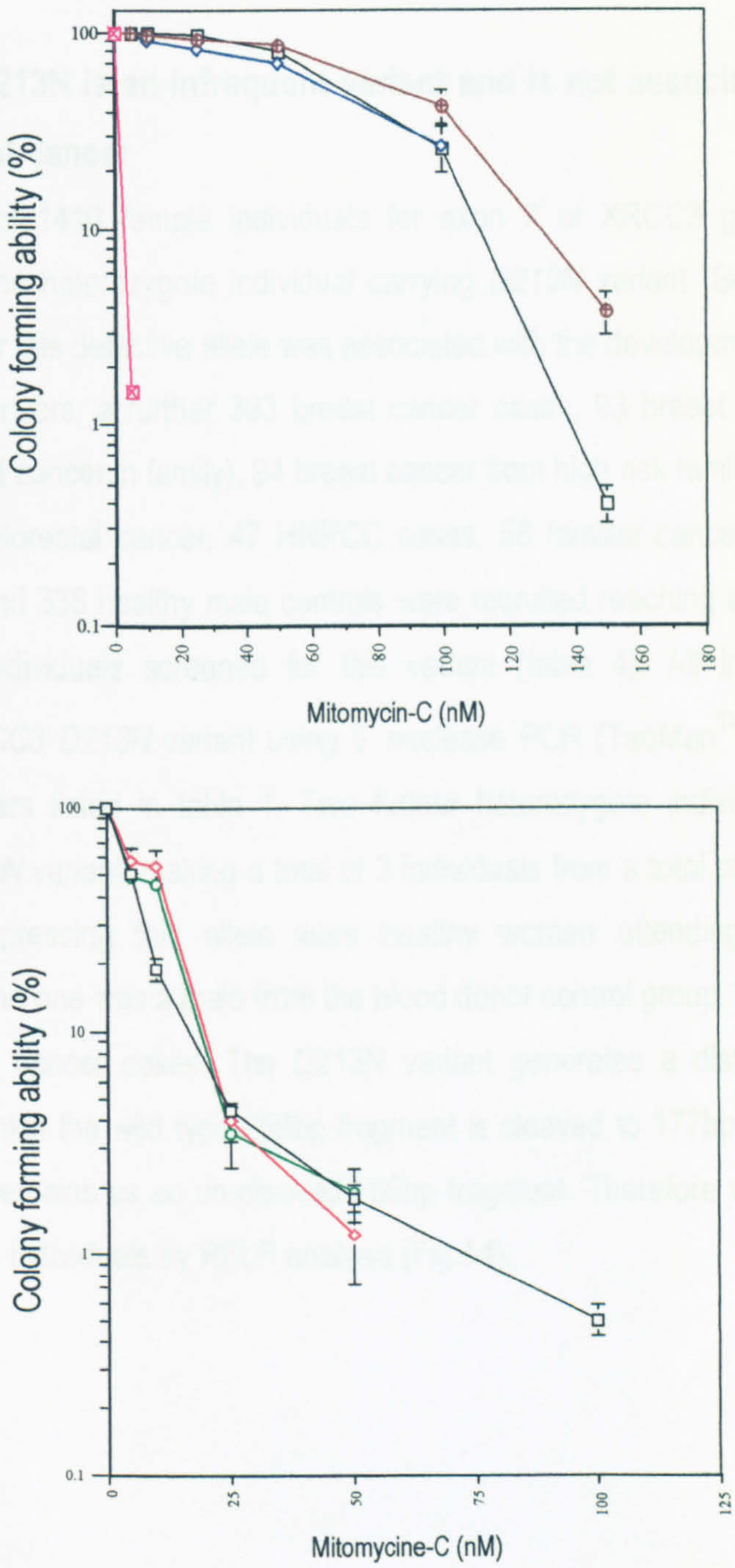
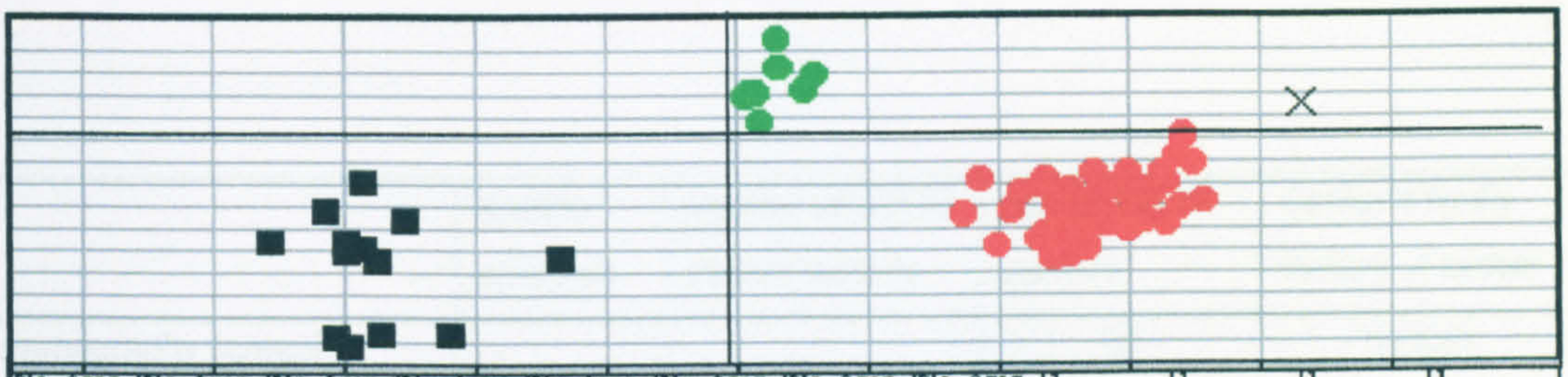


Figure 12: Determination of the dominant negative effect of D213N variant following exposure to MMC.

Mitomycin-C survival curves for the parental cell line (**A**) AA8 and (**B**) SW480, (containing endogenous functional XRCC3) and AA8 transfected with wild-type or mutant *XRCC3 D213N* cDNA. Data are shown as mean and standard deviation of 6 experiments (3 experiments on each of 2 independent cloned transfectants) for *D213N*. For some points the standard deviation is very low and thus error bars are not visible. Plot symbols: ⊕- AA8, □- wild type XRCC3, ☒ - irs1SF, ◊ - D213N.

4.3.8. XRCC3 D213N is an infrequent variant and is not associated with sporadic or familial breast cancer

Initial screening of 1416 female individuals for exon 7 of XRCC3 gene had resulted in identification of one heterozygote individual carrying *D213N* variant (See section 4.3.1). To determine whether this defective allele was associated with the development of breast or some other common cancers, a further 393 breast cancer cases, 93 breast cancer from low risk families (≤2 breast cancer in family), 94 breast cancer from high risk families (≥3 breast cancer in family), 131 colorectal cancer, 47 HNPCC cases, 58 familial cancer cases, 345 healthy female controls and 338 healthy male controls were recruited reaching a total of 2763 female and 338 male individuals screened for this variant (table 4). All individuals specifically screened for *XRCC3 D213N* variant using 5' nuclease PCR (TaqMan™) assay (Fig.13) and probes and primers listed in table 1. Two further heterozygote individuals were identified carrying the *D213N* variant, making a total of 3 individuals from a total of 3101 screened. Two of individuals expressing this allele were healthy women attending the mammography screening clinic and one was a male from the blood donor control group. The variant allele was not found in any cancer cases. The D213N variant generates a distinct Hin *f*I restriction enzyme site in which the wild type 259bp fragment is cleaved to 177bp and 82bp fragments, and the N allele remains as an un-cleaved 259bp fragment. Therefore we also confirmed the genotype of these individuals by RFLP analysis (Fig.14).



No Amp	No Amp	No Amp	No Amp	No Amp	No Amp	No Amp	No Amp	1	1	1	1
1	1	1	1	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2	1 and 2
1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	No Amp
1	1	No Amp	1	1	1	1	1	1	1	1	1
1	1	1	1	No Amp	1	1	1	1	1	1	? ? ?
1	1	1	1	1	1	No Amp	No Amp	1 and 2	1	1	1

Figure 13: Allelic discrimination result of D213N screening

Genomic DNA from cancer patients and healthy individuals were amplified in 96 well plates using primers and Fam and Tet-bounded probes. The level of fluorescent for each allele was measured for each sample by Sequence Detector 7200 allelic discrimination mode and compared with the level of fluorescent from control samples. Red dots (1) indicate samples with wild type sequence, green dots denote (1 and 2) samples with heterozygous sequence, black squares (No Amp) indicate samples that did not amplify and X (???) indicates samples with undetermined sequence. Arrow shows identification of one D213N heterozygous carrier.

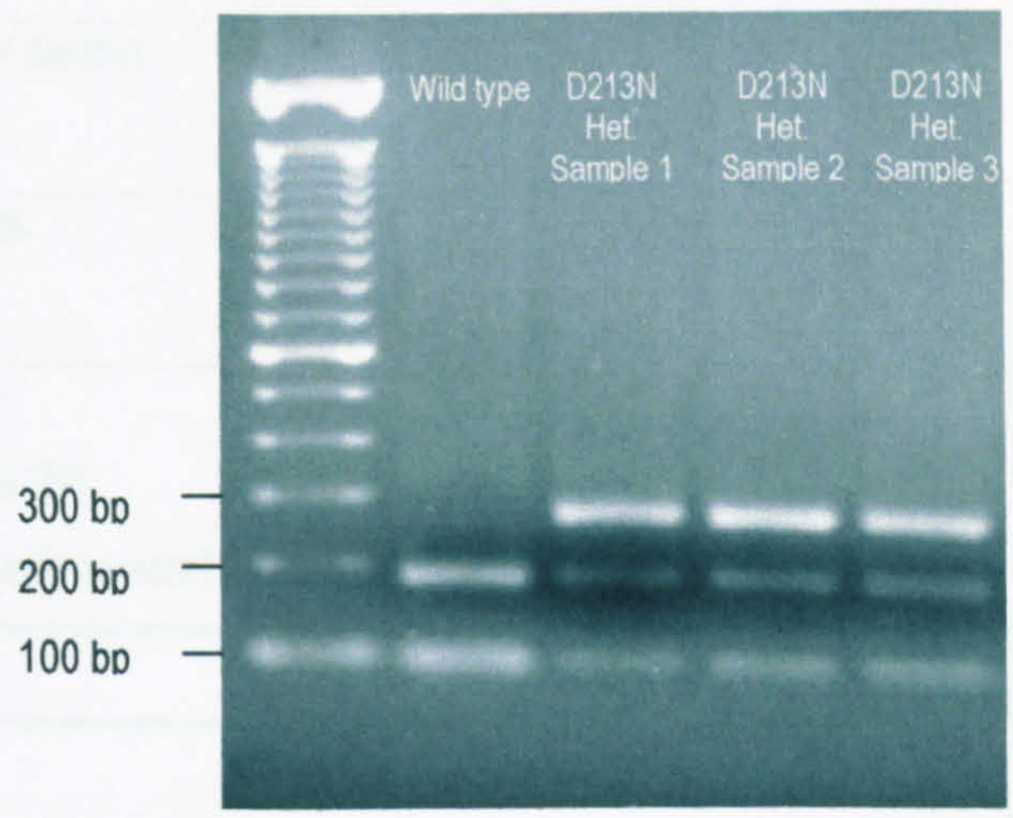


Figure 14: The result of *Hin f*I restriction digest of D213N heterozygous carries

The presence of the *D213N* variant in the 3 individuals was confirmed using a *Hin f*I PCR-restriction fragment length polymorphism assay. *Hin f*I cleaves the wild type allele but not the D213N allele. The cleavage results visualisation of three bands in the heterozygous samples and two bands in the wild type samples on 2% agarose gel. Samples 1 and 2 are from two females from mammography screening group and sample 3 is from an anonymous blood donor male.

	Number screened	Number of D213N variant carriers	Age of *D213N* carriers	Carrier's family history of cancer
Mammography screening control [a]	840	2	54 62	mother 2 x 2° relatives
Male blood donor control [a]	338	1	34	unknown
Female blood donor control [a]	399	-		
Unrelated breast cancer Cases [a]	915	-		
High risk breast cancer families (≥ 3x1st degree relatives with breast cancer)[b]	93	-		
Low risk breast cancer families (≤ 2x1st or 2nd degree relatives with breast cancer)[b]	94	-		
Unrelated colorectal cancer cases [a]	131	-		
Unrelated bladder cancer cases [a]	98	-		
Unrelated prostate cancer cases [a]	156	-		
Multi-case cancer families (≥ 3x1st or 2nd degree relatives with cancer) [a]	37	-		
Total	3101	3		

Table 4: The result of screening for D213N variant in cancer patients and normal controls. DNA extracted from blood lymphocytes of cancer patients and normal individuals was screened for *XRCC3 D213N* by 5' nuclease PCR. Three individuals carrying *D213N* variant were identified amongst the normal individuals. [a] Individuals from South Yorkshire, UK; [b] individuals from Sweden. Relevant ethical committee approval was obtained for each cohort, and informed consent was obtained from all subjects.

4.4. Discussion

Four novel rare single base pair variants of *XRCC3* were identified using PCR-SSCP. All of them fall in exon 7. Three of these variants change the amino acid while the fourth is a synonymous single nucleotide polymorphism. We investigated the effects of the non-synonymous changes on XRCC3 function. In addition to these novel variants we also investigated the function of the previously known XRCC3 T241M polymorphism on XRCC3 function. This polymorphism has already reported to increase the risk of malignant melanoma, bladder and breast cancer and occurs at a frequency of about 30% in the population (Kuschel, *et al.*, 2002, Matullo, *et al.*, 2001, Stern, *et al.*, 2002a, Stern, *et al.*, 2002b, Winsey, *et al.*, 2000).

One of three non-synonymous novel variants, D213N is highly conserved between different species and is located in the putative ATP-binding domain of the protein, the Walker Box B. Little is known about the exact role of different regions of the XRCC3 protein due to lack of extensive functional and structural studies. *E. coli* RecA, human Rad51 and Rad51 paralogues share similar overall structure including two ATP-binding domains, the Walker A and B boxes which are highly conserved from bacteria to man. These motifs are believed to be required for ATP hydrolysis (Brocchieri and Karlin, 1998, Roca and Cox, 1997).The role of ATP binding and hydrolysis in recombination and repair is not well understood, however it has been shown in *E coli* that cells with mutated RecA walker box A or B proteins have reduced level of homologous recombination and increased sensitivity to ionising radiation (Konola, *et al.*, 1994). RecA homologues in yeast and human, hsRad51 and ScRad51, also exhibit ATP hydrolysis activity (Benson, *et al.*, 1994, Sung, 1994). Mutation in the Walker A motif of ScRad51 eliminates the strand exchange activity of the protein and results in sensitivity to methylmethane sulphonate (MMS) (Shinohara, *et al.*, 1992, Sung and Stratton, 1996). Mutation in a conserved residue in the Walker box A of ScRad55 also resulted in sensitivity to ionising radiation and defects in DNA repair (Johnson and Symington, 1995). In contrast, similar mutations of Rad57 Walker A motif do not display radiation sensitivity (Johnson and Symington, 1995). Mutations in both Walker A and B motifs of Rhp51 (Rad51 homologue in *Schizosacchromyces pombe*) also resulted in extreme cellular sensitivity to MMS and radiation which were indistinguishable from those found in a Rhp51 deficient cell

line (Kim, *et al.*, 2002). Interestingly the substitution that was used to study the effect of Walker B motif mutation of Rhp51 in the latter study (D244Q) is at the same position as the novel naturally occurring substitution in XRCC3 (D213N) in our study. There have been more limited studies of the effects of mutations of these domains in the human RecA paralogues. The mutant allele of the Rad51 paralogue XRCC2 carrying a site specific substitution of the Walker A box that eliminated ATP binding activity were able to correct the radiation and MMC sensitive phenotype of hamster XRCC2 deficient cells (O'Regan, *et al.*, 2001). Also, non viability of chicken DT40 B-cells deficient in Rad51 was rescued by over-expression of hsRad51 mutant in Walker A/B box that reduced ATP hydrolysis but not ATP binding (Morrison, *et al.*, 1999). In the present study, the D213N change substitutes the highly conserved acidic aspartate residue with the uncharged polar residue asparagine in the Walker B box, which could potentially disrupt the ATP-binding activity. This substitution results in a dramatic reduction in resistance to DNA damaging drugs that could be explained by deficient HRR as a result of loss of XRCC3 function. We could not observe any dominant negative phenotype for this mutation unlike the Walker A/B motif mutations in ScRad51 and Rhp51 (Donovan, *et al.*, 1994, Kim, *et al.*, 2002). It is known that not all mutations of Walker A/B motifs in RecA/Rad51 paralogues confer a dominant negative phenotype (Johnson and Symington, 1995). This may suggest that RAD51 paralogues may have different roles in HRR and response to DNA damage. Further biochemical studies are required to determine the effect of this substitution on ATP-binding and hydrolysis in HRR.

No adverse effect on the function of XRCC3 was observed for *P199L* and *T241I* substitutions. Cells carrying these variant alleles exhibited more resistance to MMC relative to those carrying the wild type allele. Since XRCC3 protein structure is not yet known, it is difficult to predict whether this slight increased resistance to MMC is related to better expression or stability of variant protein relative to the wild-type protein. Nevertheless these sites are less well conserved amongst RAD51 paralogues than the Walker Box regions and it is likely that they do not confer the same effect as those in highly conserved region. Different sensitivity to MMC and CPT for cells carrying the T241I variant is also not unexpected, since it has been shown that different cellular response to drugs could be the

result of differences in the genetic make of individuals as a result of genetic polymorphisms (Stoehlmacher, *et al.*, 2001).

The *T241M* variant did not have any adverse effect on the normal function of XRCC3 and corrected the sensitivity of irs1SF as well as the wild type *XRCC3*, consistent with the result of another report (Araujo, *et al.*, 2002). However association studies have shown that this allele is associated with increased risk of melanoma, bladder and breast cancer (Kuschel, *et al.*, 2002, Matullo, *et al.*, 2001, Stern, *et al.*, 2002b, Winsey, *et al.*, 2000).There are several possible reasons to explain why the results of association studies were not confirmed in the functional assays. Firstly, the original melanoma and bladder cancer associations have not been replicated (Duan, *et al.*, 2002). Secondly, the XRCC3 T241M itself may not be disease causing, but in linkage disequilibrium with other closely linked variants that cause disease. One study has shown that a rare haplotype containing the *XRCC3 T241M* variant was more strongly associated with breast cancer than *T241M* itself (Kuschel, *et al.*, 2002). Thirdly, the lack of any apparent functional effect may be due to subtle differences between species, since we have used the human gene to complement the deficiency in hamster cells. Alternatively, more sensitive assays might be needed to detect any subtle effect of this variant. More investigation at both the epidemiological level, using larger sample size studies and at the functional level using more accurate assays, are required to study the effect of this variant in cancer predisposition.

We screened a large number of cancer cases and high risk families; however we did not find any carriers of the D213N variant amongst individuals with cancer. All three individuals carrying this variant were from control populations. Two of them were women aged 54 and 62 from the breast cancer screening group with no mammographic evidence of breast cancer at the time of screening. The other individual was an anonymous 34 years old male blood donor. We do not have detailed family history data, but there is no evidence that these individuals come from high risk families. Although it is possible that they develop cancer later in their lifetime, our current data does not provide us enough evidence to classify *XRCC3* gene as tumour suppressor gene on the basis of its contribution to the HRR pathway.

CHAPTER FIVE

CHAPTER FIVE- MUTATION ANALYSIS OF *XRCC2* AND *XRCC3* GENES IN BREAST CANCER

List of tables and figures

Tables

Figures

5. Mutation analysis of XRCC2 and XRCC3 genes in breast tumours

5.1. Introduction

A large body of evidence indicates that cancers arise as a result of cumulative genetic changes in somatic cells (Vogelstein and Kinzler 2002). The accumulation of genetic changes in cells during cancer development and progression has most comprehensively been described for colorectal cancer (Kinzler and Vogelstein 1996). In colorectal cancer, inactivation of the tumour suppressor gene *APC* is subsequently followed by mutations in other tumour suppressor genes, such as *P53* leading to carcinoma. In tumours that have inactivation of the mismatch-repair genes the rate at which these mutations occur is accelerated by the genetic instability. Although for most other human tumours, such detailed knowledge of early genetic events is lacking, it is obvious that mutation in caretaker genes such as DNA repair genes can lead to high rate of mutations in the gatekeeper genes whose inactivation is required for carcinogenesis. Since one of the factors influencing somatic mutation rate is number of cell divisions, the rapid proliferation of breast epithelial cells during puberty and pregnancy offer the ideal opportunity for somatic mutation, including mutation in DNA repair genes. On the other hand, genomic instability is a common denominator in the vast majority of human cancers (Tlsty, Briot et al. 1995) and the connection between DNA damage and genomic instability in cancer cells has been well established.

The involvement of the *BRCA1* and *BRCA2* genes in complexes that activate double-strand break repair and initiate homologous recombination links the maintenance of genomic integrity to tumour suppression (Scully, Chen et al. 1997; Sharan, Morimatsu et al. 1997).

The connection between radiation-induced DNA damage and carcinogenesis is reinforced by studies that have shown homozygous mutation of most of the genes involved in repair of DSBs by HRR is often associated with embryonic lethality, or cancer predisposition where the knockout animals survive the loss of repair capacity (Hakem, de la Pompa et al. 1996; Lim and Hasty 1996; Liu, Flesken-Nikitin et al. 1996; Xu, Wagner et al. 1999; Deans, Griffin et al. 2000). There is also convincing evidence that show cells from breast cancer patients are more sensitive to irradiation and this "radiation hypersensitivity" is heritable amongst breast cancer families (Scott, Barber et al. 1999). Taken these with evidence of involvement of BRCA1/2 in HRR together, it seems plausible to assume that other genes that are involved in the processing of DNA damage and act in the same pathway as *BRCA1* and *BRCA2* could be among the list of candidate genes for breast cancer susceptibility.

More than 10 genes have been identified so far that participate in the repair of DSB by homologous recombination. It has shown that certain naturally occurring mutations in some of the genes of this pathway could abrogate the repair capacity of the cell following DNA damage (See chapter 4). It has also been shown that SNPs in *XRCC2* and *XRCC3* genes can act as low penetrance susceptibility alleles for breast cancer (Kuschel, Auranen et al. 2002; Rafii, O'Regan et al. 2002). To further investigate whether *XRCC2* and *XRCC3* genes play a role in carcinogenesis, we searched for mutations in these genes in breast cancer tumours.

XRCC3 (Accession number NM_005432) has been localized to 14q32.3, has 9 exons with exons 1, 2 and part of exons 3 and 9 being untranslated, and encodes a protein of 346 amino acids. It is a member of the *Rad51* DNA repair gene family and is required for assembly and stabilisation of Rad51 foci which accumulates following DNA damage. Cells lacking *XRCC3* have low levels of homologous recombination (Liu, Lamerdin et al. 1998; Pierce, Johnson et al. 1999). *XRCC2* (Accession number NM_005431) is located on 7q36.1, consists of 3 exons and encodes a protein of 280 amino acids. XRCC2 is also a

member of Rad51 family and intereacts with other members of this family Rad51B, Rad51C and Rad51D (Masson, Tarsounas et al. 2001). It is required for genetic stability and embryonic viability and its disruption results in early embryonic lethality and (Deans, Griffin et al. 2000). XRCC2 deficient cell lines also show increased sensitivity to ionising radiation and Mitomycin-C (Jones, Cox et al. 1987) and decreased in homologous recombination (Pierce, Johnson et al. 1999). We screened the coding exons of these two genes for mutations in breast tumour material.

5.2. Materials and methods

5.2.1. Subjects

Sixty seven paraffin-embedded breast tumour tissues from unrelated breast cancer patients were used for mutation analysis. Tumour specimens from patients who had undergone surgery for breast cancer at the Royal Hallamshire Hospital, Sheffield between 1996 and 1998 were retrieved from the histopathology archive of the Hospital. All tissues were from white Caucasian individuals and were collected without bias for age. H&E slides of all samples were reviewed by a histopathologist* to exclude contamination by normal tissue. In the cases for which tumour tissue formed less than 90% of whole specimen, the tumour borders were marked on slides which were subsequently used for tumour micro-dissection. Histopathologic data for all tumours were also collected for all samples. Ethical committee approval for the study was obtained from South Sheffield Research Ethics Committee and informed written consent was obtained from all subjects (See appendix 1).

5.2.2. DNA extraction

Paraffin embedded breast tumour material was provided as 2-3 sections of 5µm for each specimen or as 5-10 slides if less than 90% of the tissue comprised tumour. Paraffin was removed from tumour sections and the tumour area was excised from breast tissue slides and collected in eppendorf tubes as described in section 2.3.1.2. Samples prepared by these methods were used for DNA extraction using QIAamp® DNA Mini Kit (QIAGEN) according to the manufacturer's instructions as stated in section 2.3.1.2. The concentration of all extracted DNA was quantified as explained in section 2.3.2 and samples were kept at -20°C until use.

5.2.3. DNA amplification

PCR amplification of samples was performed in 20µl final reaction using 1.1X PCR mastermix as described in section 2.3.3. Primer sequences are shown in table 1 and primer concentrations are shown in table 2. Negative controls were included in each experiment in which the DNA template was replaced with 1µl of ddH_2O. Amplification

* See appendix for list of collaborators

conditions were for 40 cycles following a denaturing step of 95°C for 3 minutes (See table 2 for cycling conditions). A final extension step of 72°C for 5 minutes was also applied after the cycles were finished. To confirm amplification of the correctly-sized product, 5µl of PCR product was mixed with 1µl of loading dye, loaded on 1.5% agarose gel along with DNA size marker and electrophoresed at 70 volts for 30 minutes. Correct amplification of product was confirmed by comparison of the amplified DNA band and DNA size marker.

5.2.4. SSCP analysis

Mutation analysis of *XRCC2* and *XRCC3* by SSCP was carried out as described in section 2.3.5.1. A 24cm pre-prepared non-denaturing polyacrylamide gel (see table 2 for percentage, also see section 2.3.5.1) used for electrophoresis. Gel electrophoresis conditions are shown in table 2. DNA bands were then visualised by silver staining as described in section 2.3.5.1.

5.2.5. Sequencing

Exons to be sequenced were amplified in a similar fashion as described in section 5.2.3, except using 50µl reaction volumes. The PCR products were then electrophoresed on 1.5% agarose gel and isolated from the gel using the Qiagen gel extraction kit. Approximately 5ng of PCR products were then used in sequencing amplification reactions using fluorescent primers. Amplified reactions were then sequenced with an automated LI-COR sequencer as stated in section 2.3.6.2.

	Primers	Melting temperature (°C)
XRCC2		
Exon1	Forward: 5' GTT GGT GGC GGG AAA GTT 3'	56
	Reverse: 5' TCT CCC TCA CTC CCA ACC 3'	58
Exon 2	Forward: 5' TGA GTT TTC CTT CTC TCT TC 3'	56
	Reverse: 5' CTC TTG TGA GGA GTA TGT GT 3'	58
Exon 3-F1	Forward: 5' GAG CTA CTG CAT TTT GAC TG 3'	58
	Reverse: 5' CTT TGG GAT AGT TCT GTG CTC 3'	60
Exon 3-F2	Forward: 5' TGA TAT GCT CCG GCT AGT T 3'	56
	Reverse: 5' AGT TCA CAC TTT CTC CTC CA 3'	58
Exon 3-F3	Forward: 5' GGA TAG CCT GTC AGC TTT TT 3'	58
	Reverse: 5' GCC ATG CCT TAC AGA GAT AA 3'	58
Exon 3-F4	Forward: 5' TGC CTC TCG ACG ACT GTG 3'	64
	Reverse: 5' CGT AGT ACC CTG CAA AAG AC 3'	60
XRCC3		
Exon 3	Forward: 5' TGG GAA GTT ATT CAT CCT TGG T 3'	60
	Reverse: 5' GGT AGG CAA AGG AAG GAA GG 3'	62
Exon 4	Forward: 5' GCA CCT TTT GTC ACT GTG TTG 3'	62
	Reverse: 5' GGG ACC ATG ATG CTG GAG 3'	58
Exon 5	Forward: 5' TGA CAC TAT CCC TGC CCT TT 3'	60
	Reverse: 5' GCC CAG ACC CCA CGG AAC 3'	60
Exon 6	Forward: 5' GTT AGA AAT GGC GGG AGA CA 3'	60
	Reverse: 5' GGC TTC TCC CAC ACT CAC 3'	58
Exon 7	Forward: 5' GAC AGT CCA AAC GGG GTC T 3'	60
	Reverse: 5' TGC AAC CCT GCC TTG GTG 3'	58
Exon 8	Forward: 5' GCT GAC CTG GAC TGT GCT CT 3'	64
	Reverse: 5' CAG AAC CTG AGA AAC AGG AA 3'	62
Exon 9	Forward: 5' TGC CTG CTT CCT GTT TCT C 3'	58
	Reverse: 5' TCT CAG GCA GGG CTG TTG T 3'	60

Table 1: XRCC2 and XRCC3 PCR primers.

Forward and reverse primers used for amplification of different exons of *XRCC2* and *XRCC3* genes.

	Primer Conc. (pmol)	Denaturation (°C/ sec)	Annealing (°C/ sec)	Extension (°C/ sec)	PCR product size (bp)	SSCP gel%	Electrophoresis speed (V/H)
XRCC2							
Exon1	8	94/30	53/30	72/30	118	12	2500
Exon 2	8	94/30	50/35	72/35	204	12	4000
Exon 3-F1	6	94/30	54/35	72/35	228	10	3700
Exon 3-F2	6	94/30	52/35	72/35	242	10	3500
Exon 3-F3	6	94/30	54/35	72/35	251	10	4000
Exon 3-F4	6	94/30	57/35	72/35	247	10	3500
XRCC3							
Exon 3	6	94/30	56/35	72/35	200	12	3000
Exon 4	6	94/30	55/40	72/40	270	12	4000
Exon 5	6	94/30	55/35	72/60	267	12	4000
Exon 6	6	94/30	54/40	72/40	268	12	5000
Exon 7		94/30	52/35	72/35	312	12	5600
Exon 8	8	94/30	54/40	72/40	193	10	3500
Exon 9	8	94/30	58/35	72/35	275	10	4500

Table 2: XRCC2 and XRCC3 PCR and SSCP conditions

5.3. Results

5.3.1. Mutation analysis of XRCC2 in human breast tumours

All 3 coding exons of the *XRCC2* gene from 67 primary breast carcinomas were analyzed by the single-strand conformational polymorphism method using the primer pairs listed in Table1. The primers were designed so as the flanking introns could also be amplified. No band alterations suggestive of mutation were detected on SSCP analysis for samples which were amplified for exons 1 and 2 and fragments 1, 2 and 4 of exon 3 (Figure 1). However band alterations were observed in 11 (16.4%) samples for which fragment 3 of exon 3 had been amplified. Subsequent direct sequence analysis of the samples confirmed the presence of the G/A substitution in exon 3 of the *XRCC2* (Figure 2). The substitution is a non-synonymous coding polymorphism that changes arginine to histidine at position 188 of XRCC2 protein as described in chapter 3. The frequency of G/A genotype in the healthy population obtained from genotyping of 493 healthy female (see section 3.3.3) was 14.4%. The frequency of heterozygous genotype in the tumour samples is 16.4%. We therefore did not find any significant difference between the G/A genotype frequency in the tumour samples relative to healthy population (p=0.71).

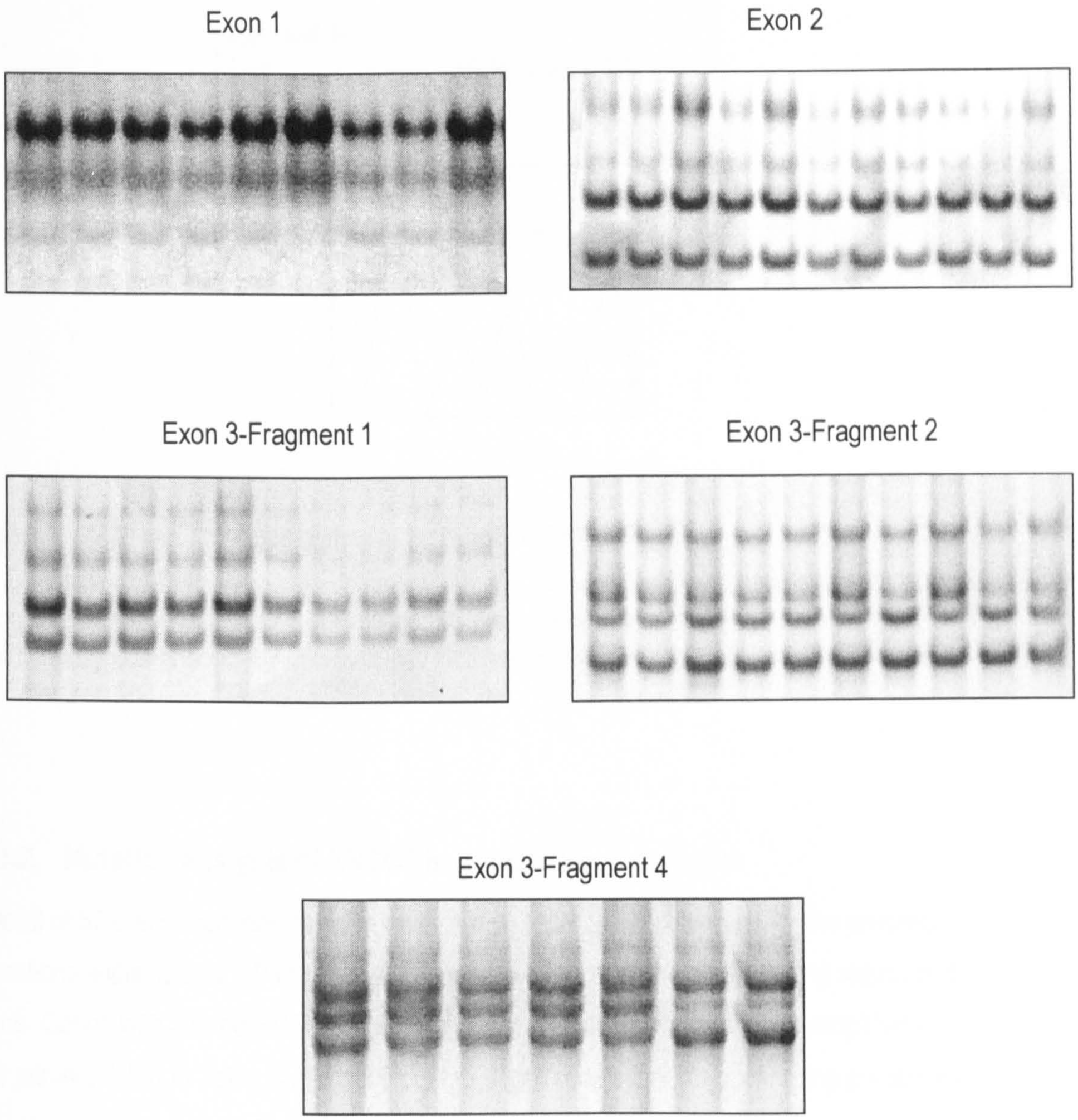


Figure 1: SSCP gel images for XRCC2 exons 1, 2 and 3.

SSCP gel images for XRCC2 exons 1 and 2 and fragments 1, 2 and 4 of exon 3. No band alteration is seen in SSCP analysis.

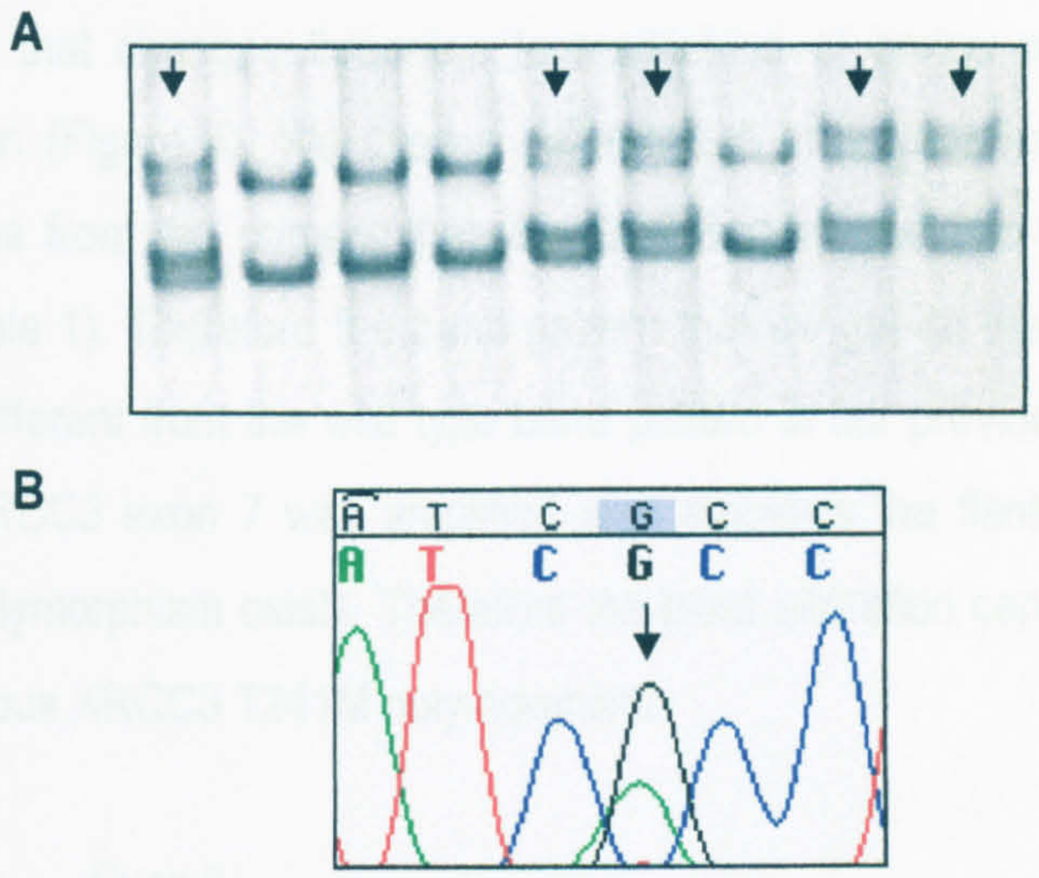


Figure 2: SSCP and sequencing images for exon3-3 of XRCC2 gene.

A; Gel image of SSCP analysis for fragment 3 of XRCC2 exon 3 showing band shift in number of samples. Arrows show altered SSCP bands. B; Direct sequencing of the PCR products shows the G/A heterozygous genotype. Arrow indicates the site of polymorphism

5.3.2. Mutation analysis of XRCC3 in human breast tumours

A total of 67 breast carcinomas were analyzed by PCR-SSCP to screen for the presence of mutations within exons 3-9 of the XRCC3 gene, which cover the entire coding region of the gene. Cases with altered SSCP profile suggestive of mutation were then re-amplified using the primers listed in Table 1 and subjected to direct sequencing to confirm the presence of any sequence alteration. No band pattern alterations suggestive of mutation were found in exons 3,5,6,8 and 9 (Figure 3). However we observed band alterations in 5 (7.5%) samples for which exon 4 and its flanking introns had been amplified. Direct sequencing of the samples confirmed the presence of a C/T polymorphism in intron V (Figure 4). This is known polymorphism and has already reported to the SNP database (GeneSNPs*). We

* See appendix for website address

also detected a band pattern alteration in 13 (19.4%) of samples for which exon 7 had been amplified. Direct sequencing of the samples showed a coding non-synonymous C/T polymorphism that changes threonine to methionine at amino acid position 241 of the XRCC3 protein (Figure 4). We used a different set of primers to amplify exon 7 and its flanking introns from the primers that we used to genotype our case-control study (See Chapter 3, table 1). Therefore the band pattern that we get on the SSCP for the wild type genotype is different from the wild type band pattern in our previous study. The primer set with which *XRCC3* exon 7 was amplified also amplifies the flanking intron 6 in which a known C/T polymorphism exists. Therefore the band alteration can not purely be related to the heterozygous XRCC3 T241M polymorphism.

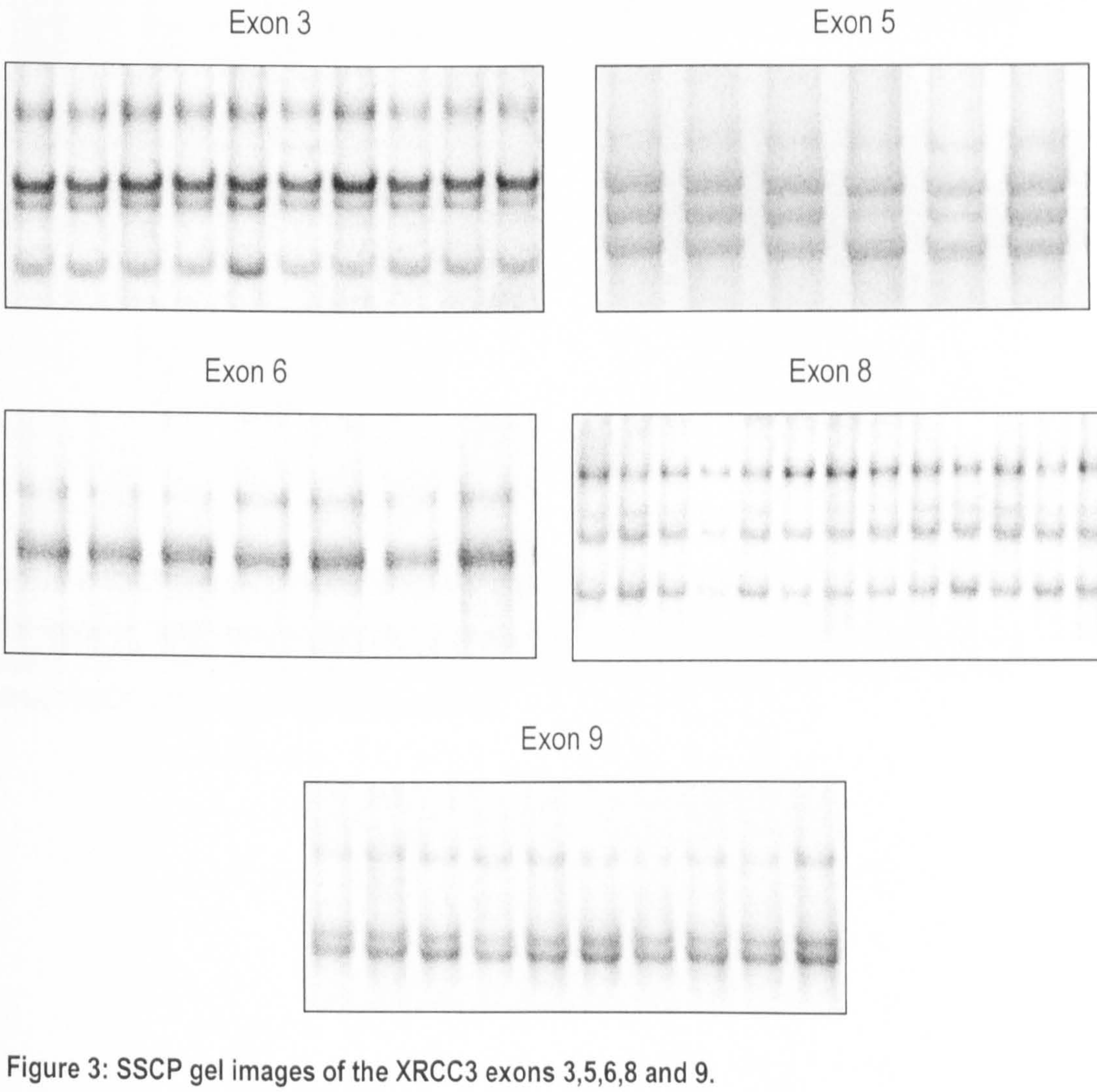


Figure 3: SSCP gel images of the XRCC3 exons 3,5,6,8 and 9.

No band alteration is seen in the SSCP analysis.

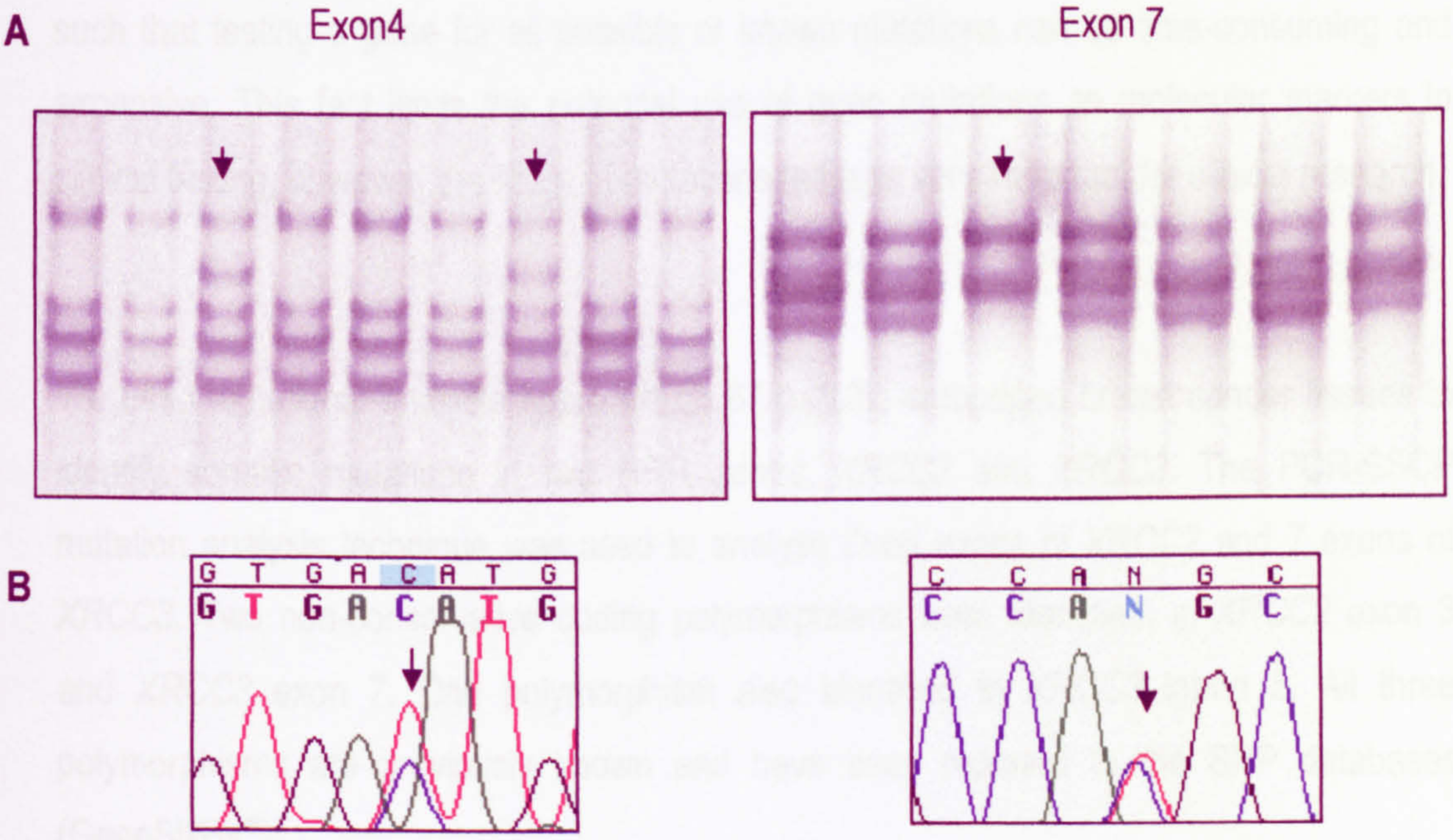


Figure 4: SSCP and sequencing imaged of the XRCC3 exons 5 and 7

Gel image of SSCP analysis for XRCC3 exons 4 and 7 showing band shift. Direct sequencing of altered SSCP bands shows the C/T heterozygous genotype in exon 7 and intron 5. Arrows show altered SSCP bands and site of polymorphism.

5.4. Discussion

DNA repair processes have evolved to defend cells against the deleterious effects of damage to their genetic material. Attempts to repair DNA damage can also cause many types of mutation, through inability to properly restore the DNA sequence. Mutations in DNA repair genes may also impair the fidelity with which the repair pathway can restore mutations. In human cancer, many different mutations can affect the function of a gene, such that testing a gene for all possible or known mutations can be time-consuming and expensive. This fact limits the potential use of gene mutations as molecular markers in clinical testing; however, the study of mutations remains very important for clinical research.

We used mutational analysis approach on 67 paraffin-embedded breast cancer tissues to identify somatic mutations in two HRR genes, *XRCC2* and *XRCC3*. The PCR-SSCP mutation analysis technique was used to analyse three exons of *XRCC2* and 7 exons of *XRCC3*. Two non-conservative coding polymorphisms were identified, in *XRCC2* exon 3 and *XRCC3* exon 7. One polymorphism also identified in *XRCC3* intron 5. All three polymorphisms are previously known and have been reported to the SNP databases (GeneSNPs*).

A number of mutational analysis approaches have been used to identify mutations in some of the genes of the HRR pathway in breast cancer. Germline mutation of *RAD51* was reported in two patients with bilateral breast cancer and one of them showed evidence of loss of heterozygosity (Kato, Yano et al. 2000). However no mutation was found in mutation analysis of 100 cases of early onset breast cancer (Bell, Wahrer et al. 1999). No somatic mutation in *RAD51* was also detected in mutation analysis of 47 and 15 breast tumours (Bell, Wahrer et al. 1999; Schmutte, Tombline et al. 1999). Although infrequent, somatic mutations have also been reported for *BRCA1* and *BRCA2* in sporadic breast and ovarian tumours (Merajver, Pham et al. 1995; Weber, Brohm et al. 1996). Mutation analysis of RAD52 and RAD54 in breast cancer patients also did not reveal any disease-associated mutations (Bell, Wahrer et al. 1999). In a recent study of 105 high risk non *BRCA1/2* breast/ovarian cancer families, no mutation was identified in the *XRCC2* gene (Rodriguez-

* See appendix for website address

Lopez, Osorio et al. 2003). No mutation in *XRCC2* and *XRCC3* genes were found in tumour samples from 106 colorectal cancer patients using the PCR-SSCP method (Lee J, unpublished data). Other genes involved in the processing of DNA damage such as cell cycle control genes, have also been analysed for mutation in tumours. In this regard a recently identified truncating germline mutation in *CHEK2* gene was found in 5.1% of non *BRCA1/2* breast cancer patients (The *CHEK2*- Breast Cancer Consortium, 2002).

The limiting factor in our study was the mutation detection technique. We used the PCR-SSCP in our study. This technique was also used for mutation detection in HRR genes in most of the above mentioned studies. Since the sensitivity of PCR-SSCP is about 70% there is always possibility that some mutations can not be detected by this method, particularly when small number of samples is used. Thus if a more sensitive technique and a larger number of samples are recruited the possibility of finding a mutation will be increased. However despite recent findings that the common R188H polymorphism in XRCC2 and T241M polymorphism in *XRCC3* are associated with increased risk of breast cancer (Kuschel, Auranen et al. 2002; Rafii, O'Regan et al. 2002), it seems unlikely from our data that either of these genes be a major tumour suppressor genes for breast cancer.

CHAPTER SIX

CHAPTER SIX- DISCUSSION

List of tables

6. Discussion

It is now widely accepted that cancer results from the interaction between an individual's genetic susceptibility and their environmental exposures. At the cellular level, cancer depends upon the balance between mutagen activation, detoxification and the effectiveness of mutagens at generating cellular damage as well as the ability of the cell to respond and repair potentially deleterious damage prior to cell division. Most environmental agents induce their effects through chemical modification of DNA. Therefore repair pathways that correct these types of DNA damage and prevent mutagenic events are directly related to cancer susceptibility (Lindahl and Wood, 1999). Some individual genetic variants that inactivate the gene product are associated with high individual risk of cancer and are known as high penetrance susceptibility alleles. However such alleles segregate in cancer families and contribute to only a small fraction of cancer cases. Familial history is a risk factor for most cancers independently of any environmental exposures, indicating that there is a genetic role for sporadic cancer as well. Thus identifying genetic susceptibility markers such as low penetrance polymorphic alleles that can be linked to disease proneness has become a target in recent years.

In breast cancer, for example, only a very small fraction of cases can be related to mutations in BRCA1/2 and some other infrequent high penetrance alleles. Although those cases of breast cancers that are not related to mutations in BRCA1/2 might have a genetic or an environmental origin, evidence from twin studies and the frequency of breast tumours in the contralateral breast of affected breast cancer patients, suggests a predominant role for genetic factors (Balmain, *et al.*, 2003). Identifying other genetic factors for breast cancer will not only help us to identify a population at higher risk, but will also reveal more detail about biological pathways that lead to breast cancer development.

Several studies have shown defects in DNA repair and increased chromosomal sensitivity to ionizing radiation in breast cancer patients and their healthy relatives (Helzlsouer, *et al.*, 1996, Kovacs and Almendral, 1987, Parshad, *et al.*, 1996, Patel, *et al.*, 1997, Roberts, *et al.*, 1999, Scott, *et al.*, 1999). Breast cancer is observed in some of the DNA repair deficiency syndromes such as Ataxia Telangectasia. BRCA1/2 deficient mice show higher sensitivity to ionizing radiation and are more susceptible to breast cancer (Moynahan, *et al.*, 1999, Moynahan, *et al.*, 2001, Ponnaiya, *et al.*, 1997). Even the high penetrance breast cancer susceptibility genes such as BRCA1/2 and ATM are now known to be part of the DNA damage signalling and processing pathway. This convincing evidence in the literature lead us to hypothesize that at least a proportion of breast cancer cases could be the result of polymorphisms in HRR genes that alter DNA repair capacity or fidelity.

In the present study we have tried to answer to two essential questions regarding single nucleotide variants of HRR genes and their possible role in breast cancer susceptibility. Firstly, could genetic polymorphisms of HRR pathway be propounded as low penetrance breast cancer susceptibility alleles? And secondly, what is the effect of rare single base substitutions in one of the genes of HRR pathway on DNA repair capacity and cancer susceptibility? In addition, we looked for mutation of HRR genes in tumours.

A genetic association study was used to investigate the role of four SNPs in four HRR genes in predisposition to breast cancer. We also cloned and expressed four naturally occurring sequence variants in the *XRCC3* gene to investigate their effect on DNA repair phenotype.

6.1. Methodological considerations in case-control studies

Case-control studies are the most commonly used method to seek potential associations between genetic polymorphisms and the risk of common diseases in the population, and interactions between genetic and environmental risk factors. To be able to detect moderate to large genetic effects (OR > 2.0) with an 80% power, at least 200 cases and 200 controls are usually needed (Garcia-Closas, *et al.*, 2000). The assessment of gene-gene or gene-environment interactions requires larger studies, especially when the factors under study are very rare or very common, or when the magnitude of the interaction is modest (Hwang, *et al.*, 1994). The power of our study to detect a moderate to large genetic effect was over

80% for all genotypes. This figure, however, only applies to the total study population and was not achieved in many of the subgroup analyses.

Concern has been raised that population stratification (*i.e.*, inclusion of individuals from a heterogeneous genetic background) may often explain the observed associations between genotypes and disease, rather than the physiological effect of the genetic variant (Altshuler, *et al.*, 1998). Any genetic (or environmental) factor, the distribution of which differs between ethnic groups, may falsely appear to be related to the disease, simply because the ethnic groups are distributed differently between cases and controls. Inclusion of only white Caucasian women, who have a genetically homogenous origin, is therefore considered to be a point of strength for the present study. All women participating in the study provided background information on reproductive factors, family history of breast cancer, previous cancers and smoking, etc. Tumour Histpathological data was also collected for breast cancer cases. This enabled us to assess the interaction of genotypes with these factors.

We used, as controls, women who attended mammography screening programme and did not have any mammographic evidence of breast lesion at the time of screening. The mammography screening controls were representative sample of the age matched population from which the cases arose. The uptake rate for our mammography screening controls living in the catchment area was 81.2%, however a possibility of some selection bias cannot be ruled out as highly educated women with a possibility of breast cancer in their family and friends may be more likely to volunteer for mammography screening.

It is nowadays acknowledged that the age distribution in controls should represent that of the cases; if controls are significantly younger than cases they may include several future cases (*i.e.* they will develop cancer at a later age) (Fryer and Jones, 1999). This could mask the possible associations. This potential source of bias can be overcome by matching the controls to the cases by age (Breslow and Day, 1980). In this study, mammography screening controls were used because they were better age-matched with the cases, but the study also included some controls (BDC) whose age range was younger than the cases.

At least one HRR gene polymorphisms has reported to increase the risk of breast cancer in mutation carriers of BRCA2 (Levy-Lahad, *et al.*, 2001, Wang, *et al.*, 2001). We did not have data on other susceptibility genes, such as *BRCA1* and *BRCA2* to study the effect of these polymorphisms as modifiers of breast cancer risk. However, in a recent study these mutations were shown to only account for a small number of hereditary breast cancer cases in British women (Peto, *et al.*, 1999). Therefore, the lack of information on these genes is not considered to have influenced the present results.

A potential weakness of our study is that the information on exposure to radiation and DNA damaging agent was unavailable for our patients and controls. Since ionizing radiation is a risk factor for breast cancer and a major cause of DSBs, analysis of our genotyping data in the presence of radiation exposure could have provided some result in favour of gene-environment interaction in susceptibility to breast cancer.

6.2. Polymorphisms in HRR genes and susceptibility to breast cancer

To answer the first question, regarding the association of genetic polymorphisms in HRR genes and breast cancer, we examined *XRCC2, XRCC3, BRCA2* and *RAD51* as candidate susceptibility genes for breast cancer in a population-based case-control study of white Caucasian women of North European descent. We found a suggestive association between XRCC2 188 His and increased susceptibility to breast cancer (OR: 1.32, 95% CI (0.98, 1.79)). Although the association fell out of statistical significance possibly due to the small number of homozygous individuals, we observed a dose effect for this variant allele as His/His genotypes (OR: 2.14, 95% CI (0.65, 7.06)) were more associated with breast cancer than Arg/His genotypes (OR: 1.29, 95% CI (0.95,1.76)).

No association was found for carriage of *XRCC3* (OR: 1.00, 95% CI (0.80, 1.25)) and *RAD51* (OR: 0.82, 95% CI (0.6, 1.13)) rare alleles and a weak statistically insignificant association was observed between BRCA2 His allele (OR: 1.21, 95% CI (0.80, 1.84)) and breast cancer. Consistent with this study, a positive association was reported for the XRCC2 His/His genotype and no effect was found for the *RAD51* C genotype in association with breast cancer in a bigger data set (Kuschel, *et al.*, 2002). In agreement

with the present study, a weak association has also been reported between BRCA2 rare allele and breast cancer in two independent studies (Healey, *et al.*, 2000, Spurdle, *et al.*, 2002). In contrast to our finding, however, Kuschel *et al* (2002) reported that the XRCC3 Met/Met genotype (and not the Thr/Met allele) was associated with increased risk of breast cancer (1.3 (1.1, 1.6)). Since the sample size in Kuschel *et al* study was bigger than the present study, they had more statistical power to detect even a small association (See section 3.3.3 for the power of present study). The studies investigating association between common cancers and SNPs of XRCC2, XRCC3, BRCA2 and RAD51 have been summarized in table 1.

Age and family history are two risk factors for breast cancer. However we did not observe any association between these polymorphisms and breast cancer in the age or family history subgroups. Although statistically insignificant due to small number of samples in subgroup analysis, we observed that carriage of the rare allele of *XRCC2*, *XRCC3* and *BRCA2* were more associated with breast cancer susceptibility when younger age breast cancer patients with positive history of breast cancer were compared to older age controls with no family history of breast cancer. This result suggests that other unknown genetic and environmental factors that are responsible for familial clustering of breast cancer may interact with these low penetrance genes.

Given the moderate increase in the risk associated with these low penetrance genes and the complex nature of breast cancer, studying the effects in relation to certain types of cancer is anticipated to be important. We therefore evaluated the effect of these polymorphisms in relation to tumour histopathologic characteristics such as tumour types, histology grades, vascular invasion and oestrogen receptor status. Despite the majority of breast tumours being of ductal type, we detected a significant positive association between the XRCC2 His allele and lobular breast carcinoma (p=0.001). This data needs confirmation in a bigger dataset but may indicate a relationship with cancer subtypes. However no association was detected between rare allele of either of the polymorphisms and the histology grades, vascular invasion and oestrogen receptor status. In a big association study Goode *et al* (2002) tried to identify whether rare alleles of DNA DSBs repair genes can be used as predictive biomarkers of survival after breast cancer. Rare homozygous allele of a silent polymorphism in codon 501 (D501D) of the Ligase IV gene

(LIG4) which is involved in NHEJ repair of DSBs was found to be associated with decreased survival in breast cancer patients (Goode, *et al.*, 2002).

A growing number of molecular epidemiological studies have tried to associate variations at a number of repair genes with cancer risk. For example, an additive effect of the *XRCC1* variants 194Trp and 399Gln has been associated with smoking-related squamous cell carcinoma of the head and neck (Sturgis, *et al.*, 1999). Other studies associate variations in *XRCC1*, *XPD*, *XRCC3*, *XPA*, excision repair cross-complementing rodent repair deficiency, complementation group 1 (*ERCC1*), and apurinic/apyrimidinic endonuclease 1 (*APE1*) with elevated risk of several cancers, including lung, colon, bladder, and breast (Butkiewicz, *et al.*, 2001, Chen, 2000, Divine, *et al.*, 2001, Duell, *et al.*, 2001, Ratnasinghe, *et al.*, 2001, Stern, *et al.*, 2002a, Stern, *et al.*, 2002b, Sturgis, *et al.*, 1999, Tomescu, *et al.*, 2001, Winsey, *et al.*, 2000). The odds ratios for the individual variants range from 1.3 to 2.0, and not all of the studies report consistent risk association.

Cancer	Cases	Controls	OR (95% CI)	Reference
XRCC2 R188H				
Breast cancer	2205	1826	2.60 (1.0, 6.7)	Kuschel et al, 2002
Breast cancer	519	891	1.30 (0.96,1.75)	Rafii et al, 2002
XRCC3 T241M				
Breast cancer	2205	1826	1.30 (1.1,1.6)	Kuschel et al, 2002
Breast cancer	162	302	0.95 (0.64,1.40)	Smith et al, 2003
Bladder cancer	124	85	2.77 (1.55,4.93)	Matullo et al, 2001
Bladder cancer	233	209	1.30 (0.9,1.9)	Stern et al, 2002
Lung cancer	331	687	0.92 (0.67,1.28)	David-Beabes et al,2001
Lung cancer	96	96	N/A [a]	Butkiewicz et al, 2001
Malignant Melanoma	125	211	2.36 (1.44,3.86)	Winsey et al, 2000
Malignant Melanoma	305	319	0.89 (0.65,1.24)	Duan et al, 2002
SCCHN	367	354	1.36 (0.89,2.08)	Shen et al, 2002
***BRCA2* N372H**				
Breast cancer	2775	2326	1.31 (1.07,1.61)	Healey et al, 2000
Breast cancer	1397	755	1.47 (1.05,2.07)	Spurdle et alB, 2002
Ovarian cancer	1121	2643	1.36 (1.04,1.77)	Auranen et al, 2003
***RAD51* C135G**				
Breast cancer	164	93	p= 0.07	Levy-Lahad eta al, 2000
Breast cancer	216	436	3.2 (1.4,40)	Wang et al, 2001
Breast cancer	2172	840	0.86 (0.68,1.08)	Kuschel et al, 2002

Table 1: Association between SNPs of HRR genes and common cancers

SCCHN; squamous cell carcinoma of head and neck, OR; odds ratio, CI; confidence interval

[a] No difference was observed in genotype frequencies between cases and controls

6.2.1. Gene-gene interaction and breast cancer risk

SNPs do not act in isolation, but against the background of thousands of other SNPs, and environmental factors. Therefore assessment of single polymorphic genotypes is not expected to be sufficient for evaluating individual susceptibility to the various endogenous

or exogenous harmful agents (Hirvonen, 1997). The study of interactions between different polymorphic genes should, however, be based on biologically plausible hypotheses. For instance simultaneous examination of genes for enzymes with overlapping substrate specificity for a given exposure or separate genes acting in sequence in the same metabolic pathway are anticipated to be well justified. Proteins in DNA repair pathways are often multifunctional, resulting in a variety of phenotypes. An example of this is the XPD protein, which has a role in both NER and basal transcription. Furthermore different repair pathways overlap with each other in repairing DNA damages. Therefore it is biologically plausible to assume that combinatory effects of polymorphisms resulting from interaction between different repair pathways could have an adverse effect on the capacity of the whole repair machinery.

In the present study, an interaction was found between the *XRCC2* and the *XRCC3* genotypes. Accordingly, the odds of breast cancer susceptibility for combination of XRCC2 His and XRCC3 Met alleles (1.50 (0.97, 2.23)) was more than the odds ratio of each of the *XRCC2* and *XRCC3* rare allele separately. Although the XRCC3 Met allele was not associated with breast cancer in its own, it was associated with increased risk of breast cancer at the presence of XRCC2 His allele (1.86 (0.97, 3.55)). Furthermore, the combinatory effect of XRCC3 Met and BRCA2 His alleles was more associated with increased breast cancer risk (1.35 (0.93, 1.96)). In contrast the presence of XRCC2 His allele together with BRCA2 His allele or RAD51 C genotype was suggestive of a protective rather than a predisposing role (See Table 10, Chapter 3).

Although in small numbers, the results from present study highly suggest that the combinatory effect of polymorphisms of genes of HRR pathway are more associated with susceptibility to breast cancer than the effect of a SNP in one gene. Some studies have already been carried out to examine combined effects of separate polymorphic oestrogen metabolising genes in relation to breast cancer (Curran, *et al.*, 2000, Helzlsouer, *et al.*, 1998, Matsui, *et al.*, 2000, Millikan, *et al.*, 2000, Millikan, 2000). However to investigate additive or combinatory effects of polymorphisms of genes of a pathway in association with a particular cancer, very large sample sizes are needed, which often requires multicentre or international collaborations. It should also be noted that when one considers the multi-dimensional implications of combinations of SNPs in profile, instead of one SNP at a time, the level of complexity increases dramatically. Therefore particular specialist analytical methods are required.

6.2.2. Gene-environment interaction

The general assumption in gene-environment interaction is that if the effect of genetic background and environmental exposure is greater than the effect of each individual factor then interaction between two factors is present (Brennan, 2002). Although it has been suggested that the role of environmental factors in breast cancer susceptibility is not as important as the role of genetic factors (Balmain, *et al.*, 2003), great effort has been dedicated to identifying the role of environmental exposure in breast cancer. Most of the environmental risk factors for breast cancer are classified in groups related to exposure to female sex hormones. In the present study no information on radiation exposure or known DNA damaging agents was available for either of study cohorts. We therefore were not able to study the effect of gene-environment interaction in association with breast cancer. The gene-environment interactions in susceptibility to cancer have been extensively studied. A statistically significant interaction has been reported between cumulative cigarette smoking and two polymorphisms in the *ERCC2* gene in lung cancer patients (Zhou, *et al.*, 2002). An interaction between smoking, alcohol drinking and XPD polymorphisms at codon 751 has also been suggested (Sturgis, *et al.*, 1999). One study also suggests an interaction between low dose X-Ray irradiation and hMSH3 polymorphism in childhood leukaemia (Infante-Rivard, *et al.*, 2000). Whilst the result of these studies should be interpreted with caution, the effect of polymorphisms in DNA repair genes in interaction with environmental exposure deserves further study. Refinement of cancer risk estimates associated with exposures will require epidemiological studies of large cohorts with well-documented exposures that also account for polymorphisms in the large number of genes determining individual susceptibility.

6.3. Investigating the effects of sequence variants of XRCC3 on cellular phenotype

Whilst several high frequency DNA repair genetic polymorphisms have been shown to associate with cancer risk (Goode, *et al.*, 2002, Kuschel, *et al.*, 2002), the contribution of DNA-repair genetic variants to repair function and human cancer risk is still unclear.

Two approaches have been used to study the effect of different genotypes of DNA-repair SNPs on the cellular phenotype. In one approach the associations between DNA repair

SNPs and damage/repair endpoints has been studied. Initial studies using lymphocyte-based assays associate several variants with altered DNA-repair capacity. (Spitz, *et al.*, 2001) reported that variant alleles at amino acid residues 312 and 751 of *XPD* reduced repair capacity in a lung cancer cohort. Being homozygous for a variant allele in either *XPC* or *XPD* is associated with reduced capacity to repair UV-induced DNA damage as assayed by the host-reactivation assay in a cohort of healthy subjects (Qiao, *et al.*, 2002). (Hu, *et al.*, 2001) report that, in cancer-free women, the *APE1* 148Glu allele is associated with prolonged mitotic delay in lymphocytes exposed to IR. In addition, women with at least three variant alleles of *APE1* and *XRCC1*, two genes of the BER pathway, have increased IR-induced cell cycle delay (Hu, *et al.*, 2001). Other studies relate repair genotypes to exposure biomarkers, such as DNA adducts and DNA damage. Some examples of this approach are the association of the variant allele of *XRCC1* Arg399Gln with increase rate of sister chromatid exchange (SCE), DNA adducts, and hypersensitivity to IR (Abdel-Rahman and El-Zein, 2000, Duell, *et al.*, 2000, Hu, *et al.*, 2001, Lunn, *et al.*, 1999, Matullo, *et al.*, 2001). The variant allele of *ERCC2/XPD* Asp312Asn was also found to be associated with increased UV-induced apoptosis (Seker, *et al.*, 2001). An association has also been reported between the variant allele of *ERCC2/XPD* Lys715Gln and higher DNA adducts or lower chromatid aberrations (Lunn, *et al.*, 2000, Palli, *et al.*, 2001). In one study DNA-repair activity measured by poly (ADP-ribose) polymerase (PARP) activity was lower in breast cancer cases than study controls. Also, carrying rare PARP variants were in association with PARP enzyme activity (Hu, *et al.*, 1997). Although results from most of the studies suggest potential functional roles of DNA-repair SNPs, association studies cannot provide direct evidence for a genotype/ phenotype relationship.

The second approach is to clone and express the protein from the wild-type and the variant allele and study the effect of the variant allele on the function of the protein. Only a limited numbers of studies have used this approach. In one study of this kind on seven polymorphisms of *APE1* gene, a gene critical to the BER pathway, functional assays revealed that four (L104R, E126D, R237A, and D283G) of seven amino acid substitution variants exhibited ~ 40–90% reductions in binding or incision activity (Hadi, *et al.*, 2000). Another study showed that cells expressing the *XRCC3* T241M variant allele were active for HRR of double-strand breaks and not more sensitive to the interstrand cross-linking agent mitomycin C (Araujo, *et al.*, 2002). The authors suggest that the increased cancer

risk associated with *XRCC3* Thr241 Met may not be due to an intrinsic homology-directed repair defect. Also our collaborators showed that cells expressing the *XRCC2* R188H allele only shows small, statistically insignificant sensitivity to MMC relative to the cells expressing the wild type allele (Rafii, *et al.*, 2002). In another approach, (Ellison, *et al.*, 2001) targeted yeast residues for alterations that would mimic variants in the human population in *MLH1* gene. Cells expressing variant (A41F, G64R, I65N, E99K, I104R, T114R, Q552L and R672P) MLH1 proteins, exhibited 62-130 fold higher mutation frequencies relative to the cell expressing the wild type allele (Ellison, *et al.*, 2001).

In the present study we investigated the functional effect of four naturally occurring variants (P199L, D213N, T241M, T241I) of *XRCC3* gene, all of them conserved between human and mouse. One of the variants (D213N) occurs in a highly conserved region of the protein, the Walker box B, and is conserved between 23 orthologues and paralogues from different species (SIFT online sequence alignment software). To determine the functional significance of these variants, first human *XRCC3* was cloned and then the variant bases were created using site-directed mutagenesis. The human *XRCC3* cDNA carrying the variant alleles were then transfected into CHO XRCC3 deficient cell line and the effect of variant alleles on XRCC3 mediated HRR was measured by evaluating resistance of transfected cell lines to DNA damaging agents, mitomycin-c, camptothecin and thymidine. Mitomycin-C induces DNA double-strand breaks (DSBs) (Natarajan, *et al.*, 1983) and extreme sensitivity of XRCC3 deficient cell lines (irs1SF) to MMC has already been established by several reports (Cui, *et al.*, 1999, Tebbs, *et al.*, 1995). Camptothecin is a plant alkaloid that can produce DNA DSBs by inhibition of topoisomerase I (Hsiang and Liu, 1988)(Trumberg D, 2000). Although thymidine does not generate any detectable DSBs, sensitivity of XRCC3 deficient cell lines to S-phase synchronising agent thymidine has been shown in previous reports (Lundin, *et al.*, 2002).

Clones expressing P199L, T241I and T241M variants did not show significant differences in the sensitivity to MMC or CPT relative to the clones expressing the wild type allele. Cells expressing the T241I variant were more sensitive to MMC and less sensitive to CPT. In contrast, cells expressing T241M were more sensitive to CPT and less sensitive to MMC. The differences in MMC and CPT sensitivity between T241I and T241M variants is not surprising as it has been known for a long time that the differences in genetic makeup can affect individual drug response. In this regard it has recently been shown that carrying XRCC1 399Gln variant allele was associated with increased resistance to 5-FU/oxaliplatin

chemotherapy in colorectal cancer patients (Stoehlmacher, *et al.*, 2001). In fact genetic variants can be used as markers of prediction of response to drug therapy in cancer patients if an association between a particular polymorphism and clinical response to a certain drug is established. To our knowledge the XRCC3 protein structure is not available on the database. We therefore could not study the effect of these polymorphisms on the protein structure. However it is possible that change in protein folding resulting from SNPs leads to different response to different drugs.

The most distinctive finding in this study was that all clones expressing the XRCC3 D213N variant showed extreme sensitivity to MMC, CPT and thymidine, which was hardly distinguishable from the sensitivity of the XRCC3 deficient cell lines. This finding is interesting because this variant occur in highly conserved putative ATP-binding domain of protein, Walker box B. Although a recent study has shown that the ATPase activity of RAD51 is necessary for HRR in mouse embryonic stem cells (Stark, *et al.*, 2002), it is not clear whether this ATPase is necessary for the function of all RAD51 paralogues in vivo. Also, not much is known about the function of the Walker boxes. However, various studies have investigated the functional significance of domains of HRR proteins by targeted mutation of Walker box residues that are conserved between different species. Mutations in the Walker A motif of ScRad51 and ScRad55 resulted in sensitivity to methylmethane sulphonate (MMS), ionising radiation and defect in DNA repair (Johnson and Symington, 1995, Shinohara, *et al.*, 1992, Sung and Stratton, 1996). An equivalent mutation to XRCC3 D213N variant in Walker B motif of Rhp51 (Rad51 homologue in *Schizosacchromyces pombe*) (D244Q) has been shown to produce extreme cellular sensitivity to MMS and radiation which is indistinguishable from the deficient cell line (Kim, *et al.*, 2002). Also mutation in Walker A motif of Rhp51 has produced the same result (Kim, *et al.*, 2002). However targeted mutations of conserved Walker box A of the *XRCC2* and *Rad57* only had small effects on mitomycin-c and radiation resistance of mutant cell lines (Johnson and Symington, 1995, O'Regan, *et al.*, 2001). To determine the effect of XRCC3 213N variant in the presence of wild type allele, we also carried out dominant negative assay by transfecting XRCC3 D213N allele into CHO cell line AA8 and human colorectal carcinoma cell line SW480 which both carry wild type copies of XRCC3. However we did not observe any significant difference in sensitivity to MMC in strains that carry D213N alleles relative to the strains transfected with the wild type copy of XRCC3. Lack of dominant negative effect has also been seen in other mutations of Walker A/B motifs in RecA/Rad51 paralogues

(Johnson and Symington, 1995). Although due to unavailability of proper XRCC3 antibody we could not prove that the level of expression of the variant allele is the same as the wild type allele, assuming that the variant allele was over-expressed under the viral promoter, the lack of dominant negative effect in the present study suggests that normal XRCC3 protein produced by the parental gene is sufficient for cell to repair MMC induced damage.
To determine whether the D213N variant, with such a drastic inactivation of the protein function could have a role in predisposition to cancer, we screened a large number of cancer cases and control groups for the presence of this variant. A total of 1524 patients with common cancer including breast, bladder, prostate and colorectal cancer and 1577 male and female healthy individuals were screened, two further heterozygous carriers of the D213N variant were identified, both of them from the control population. Although 2 of 3 D213N carriers in the control group had a weak history of cancer in their family, we did not identify any cancer patient carrying this variant allele. Therefore we did not find any evidence on the basis of the present study to propose that the XRCC3 gene could act as a tumour suppressor gene.
There is some evidence that suggests that the *XRCC2* and *XRCC3* genes are required for accurate chromosome segregation during replication (Griffin, *et al.*, 2000). We also know that correct duplication of cells depends on accurate replication of genome, repair of DNA damages prior to replication and precise chromosome segregation during mitosis. In this context, genes that are involved in repair of DNA damage, fidelity of chromosomal segregation and accurate replication are predicted to be of crucial importance as a suppressor of cancer development. Therefore it has been proposed that *XRCC2* and *XRCC3* genes could act as tumour suppressor genes on the basis of their participation in chromosome segregation (Griffin, *et al.*, 2000). Two other HRR genes, BRCA1 and BRCA2 have been identified as tumour suppressor genes that predispose to breast and ovarian cancer. A *RAD51* mutation was identified in a bilateral breast cancer patient with evidence of loss of heterozygosity (Kato, *et al.*, 2000). However no human cancer has been associated with mutation of *RAD51* paralogues so far.
As no data on knockout *XRCC3* in mice has been published, the effect of *XRCC3* disruption on cellular viability and carcinogenesis is not fully understood. However embryonic lethality has seen as a result of knockout of *XRCC2* and some other *RAD51* paralogues (Deans, *et al.*, 2000, Pittman and Schimenti, 2000, Shu, *et al.*, 1999). This indicates that cell death rather than proliferation may be the normal outcome of loss of

XRCC3 in vivo. Therefore, our data suggest that mutations in genes encoding proteins that are involved in the same repair pathway as BRCA1 and BRCA2 may not confer the same risk of cancer as do mutations in the genes for BRCA1 or 2 themselves.

Another issue that should be addressed is that despite published positive association between DNA repair gene polymorphisms and increased risk of cancer, the result of these association studies often are not supported by the functional assays. In this context we found that carriage of the *XRCC2* R188H rare allele is associated with increased risk of breast cancer, however the result of mitomycin-c sensitivity of cells expressing this variant was weakly different from the wild type allele. Two independent studies have also showed that the XRCC3 Met allele is functional (Araujo, *et al.*, 2002, Rafii, *et al.*, 2003), despite reports on positive association of this allele with increased risk of breast, bladder and skin cancer (Kuschel, *et al.*, 2002, Matullo, *et al.*, 2001, Winsey, *et al.*, 2000). The reason for these discrepancies between the epidemiological and functional studies is not clear. However given the large variation and complexity of individual genotypes for each of the DNA-repair pathways, it is clear that the relationship among genotype, repair capacity, and, ultimately, individual risk will be complex. In the end, the integrated function of the genes of the relevant pathways, not the variation at any one gene, will determine susceptibility to an exposure. Therefore the relatively low impact on cancer risk associated with one variant allele compared to the reduced-capacity phenotype is not unexpected. The functional assays integrate the impact of a whole pathway, while molecular epidemiological studies genotype for only 1 or 2 from among lots of variants expected among the 10-20 genes in each repair pathway. The small adverse effect of one polymorphism on one protein of a repair pathway therefore can be compensated by other proteins of the pathway or the effect could be weak and undetectable by some assays. In addition, in the functional studies of *XRCC2* and *XRCC3* polymorphisms, due to unavailability of human XRCC2 and XRCC3 deficient cell lines, deficient CHO cell lines were used to study the effect of human XRCC2 and XRCC3 variant alleles. The interspecies differences, however small, can potentially mask any weak effect arising from a single base pair substitution. While we need more accurate assays to study the functional effect of single base substitutions, independent replication of association studies in bigger sample size groups is also required to ensure a real association between SNPs and cancer.

6.4. Concluding remarks and future directions

The results from this study and other independent studies suggest that variants of HRR genes that are involved in repair of DNA DSBs are weakly associated with increased risk of breast cancer (Healey, *et al.*, 2000, Kuschel, *et al.*, 2002, Rafii, *et al.*, 2002). Moreover when the combinatory effect of these variants is studied they may confer even greater risk of developing breast cancer. Although before any definite conclusion is taken, the result of these studies should be replicated by independent studies in different populations, more evidence now is in favour of participation of HRR genes as low penetrance cancer susceptibility genes. The present study also suggests that instead of looking at the one gene, one outcome hypothesis, studies should be designed to answer the question of how collections of genes, either alone or in combination, contribute to complex diseases like breast cancer. Although there are different repair pathways for repairing different types of DNA damage, there is no clear cut border between different repair pathways *in vivo*. Repair of DNA damage may be the result of interaction between many proteins and enzyme from different repair pathways. In this regard investigating pathways of genes in large association studies could provide invaluable information of biological pathways as well as estimation of penetrance or disease association. In other words, the search should not be confined to identifying more genetic markers, but also for the biological significance of the markers. No single study is definitive, each has its own limitations, and all results should be replicated in different population. Only the combined consideration of studies in different populations replicating similar data will result in the belief that a candidate gene polymorphism is indeed a cancer risk factor. The average sample size of most of current case-control studies for genetic polymorphisms is 150-350 cases and similar number of controls which is appropriate for detecting common (>5%) SNPs with OR of ~2 (Brennan, 2002). However this sample size does not provide enough power to detect smaller odds ratio or less common SNPs. Since collecting enough samples to generate big association studies may not be feasible particularly in less common cancer, multicentre collaborations will provide enough samples size and adequate power to detect weak effect that has been observed for these alleles. Also the results of all genetic association studies should be published in a format that allows pooling with similar studies. All SNPs should be publicly available and to avoid bias the result of negative finding should be published as well as positive associations. At this time positive results are more likely to be published than

negative findings because investigators only submit and journals are keener to accept positive findings.

Collecting accurate data on environmental exposure will also allow us not only to evaluate the effect of gene-environment interaction in cancer predisposition, but also to find subgroups who are genetically more sensitive to one special environmental exposure. Studies that concentrate on analysis of interaction between genetic effects and particular exposure require significantly larger sample size to have enough power to detect such interactions.

Although there is a crucial difference between looking at a SNP as a susceptibility factor in the general population and in a population at risk, identifying more genetic markers will hopefully allow medicine to specify population at risk and eventually create a profile of biomarkers for susceptibility to diseases. Genetic variation may even be used as a tool to select optimal therapy, tailor dosage regimens, and improve clinical outcomes.

The present study suggest that ATP binding domain Walker B box may be important for DNA repair activity of XRCC3. Therefore the effect of *XRCC3* D213N variant on the ATPase activity, RAD51 focus formation following DNA damage and HRR capacity may be considered as fields of future studies.

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Appendices

THE Central Sheffield University Hospitals

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(Please quote the Ethics Reference No in your reply)

Ref: PRJ/LT

31 July 1998

Ms I Azmy
Research Associate
Surgical & Anaesthetic Sciences
K Floor

Dear Ms Azmy,

SS 98/137 - The role of inflammatory cytokines and other candidate genes in breast cancer

Thank you for your letter of 23 July 1998 answering the queries raised by the Ethics Committee and enclosing a revised consent form and information sheet.
I can now confirm unreserved Ethics Committee approval for the above study subject to the following terms and conditions.

1. It is understood that approval of the investigation does not absolve you from total responsibility for the safety and well-being of the subjects.
2. If any substantial change is made to the protocol it will be necessary for you to obtain approval from the Ethics Committee.
3. That should any untoward event occur during the conduct of the study the Chairman of the Committee or failing this the Administrator should be informed immediately.
4. Reports of progress shall be submitted at yearly intervals. A follow-up form will be sent to you annually to keep the Ethics Committee informed of the progress of the project.
5. The Ethics Committee is to be advised if the project has not commenced within six months.
6. No deviations from or changes of the protocol will be initiated without prior written approval of an appropriate amendment, except when necessary to eliminate immediate hazards to the subjects or when the change(s) involve only logistical or administrative aspects of the trial.
7. That you should promptly report any changes increasing the risk to subjects; adverse drug reactions or new information that may affect adversely the safety of the subjects or conduct of the trial.
8. That you familiarise yourself with the ICH Guidelines laid down for the conduct of human experiments.
9. The Ethics Committee is to be advised when the project is completed.

Yours sincerely

Peter Jackson
Chairman

BREAST CANCER QUESTIONNAIRE

Hospital Number

Date Of Form

Name

Address

Post code

Date of Birth

Age at Diagnosis

Height

Weight

Ethnicity

Presenting Symptom

Smoking

Never

Past → *Stopped at yrs; for /day yrs*

Current → *for /day yrs*

Menopausal status

Age at Menarche

Age at Menopause

Hysterectomy

Age

why?

OCP yrs months

HRT yrs months

No of Preg

Children

Age at I preg

Breast Feeding

Surgery

Date

Axillary Surgery

Level

Additional Surgery

Radiotherapy

Chemotherapy

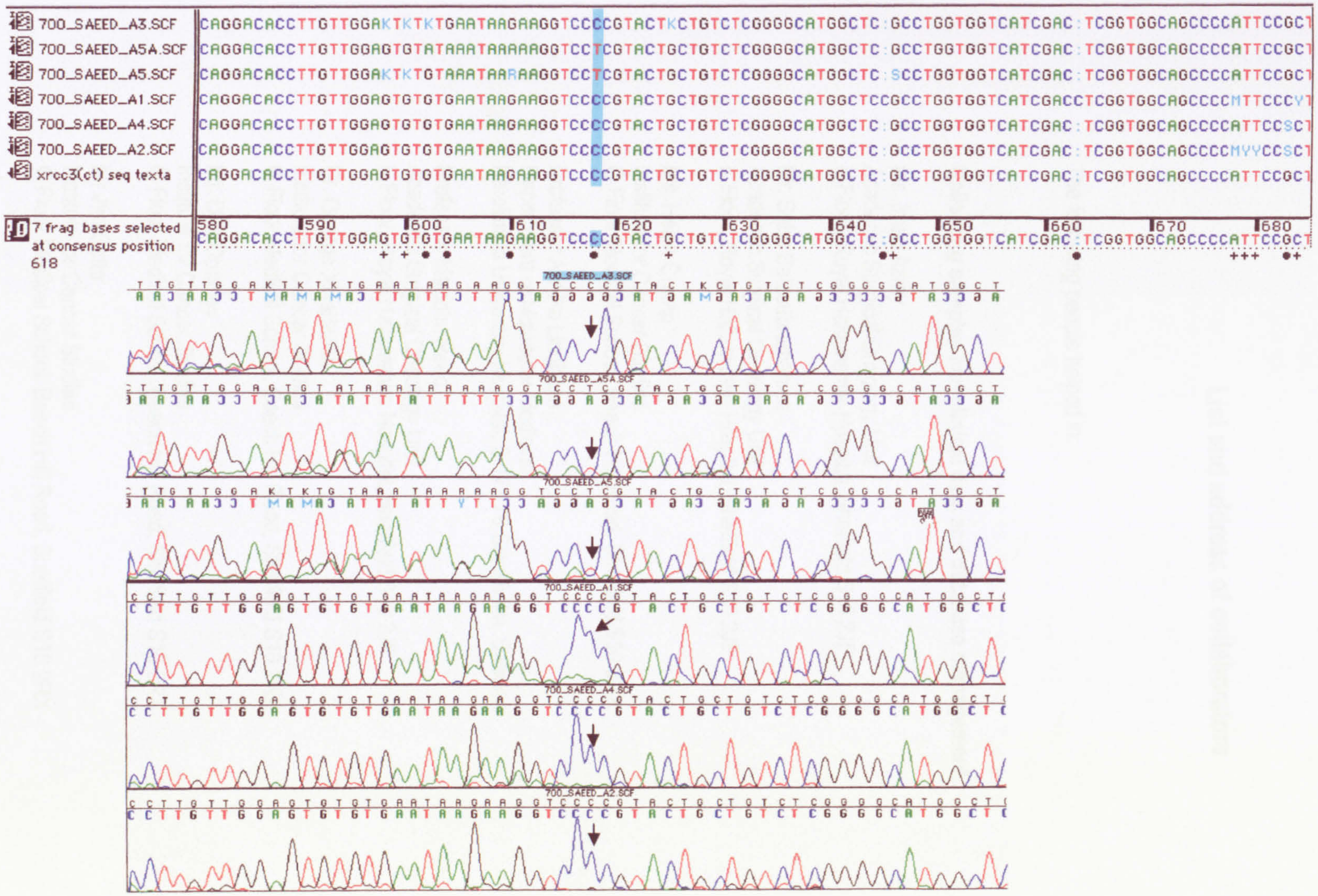
Hormonal Therapy

Past Medical History- (especially other tumours)

Family history of Breast cancer ☐ F/H Other cancers ☐

Relationship	Cancer	Age Diagnosed	Age Died	Details
Sister				
Brother				
Mother				
Father				
Mat/pat Aunt				
Mat/pat Uncle				
Mat/pat grandma				
Mat/pat grandpa				
Others				

	Histology Report-1	Histology Report-2
Main Report		
Lab No.		
Side		
Non-Invasive		
Micro-Invasive		
Invasive		
Whole Tumour size (including insitu)		
Max. diameter of Invasive tumour		
Insitu size	mm / ?? / M.focal	mm / ?? / M.focal
Nodes:Level involved		
No involved		
No taken		
Excision margins		
Grade		
Disease Extent		
Vascular Invasion		
Prognostic Index		
ER status		
Mets / Recurrence date, site & Rx)		



The result of sequence alignment of cloned XRCC3 exon 7. Two clones showed mutant P199L XRCC3 (C/T) and five showed Wild type XRCC3.

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Collecting samples and clinical data and database management.

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Identification and functional work on XRCC2 R188H polymorphism.

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Website addresses

National Centre for Biotechnology Information (NCBI)
http://www.ncbi.nlm.nih.gov

A public database that provides multiple software which are widely used in molecular biology.

Multiple Sequence Alignment, Baylor College of Medicine
http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html

Provides sequence alignment of given proteins.

GeneSNPs, University of Utah
http://www.genome.utah.edu/genesnps/

Thsis free to use website provide a collection of SNPs in DNA repair, cell cycle control, cell signaling, cell division, homeostasis and metabolism pathways.

Sorting Intolerant From Tolerant (SIFT)
http://blocks.fhcrc.org/~pauline/SIFT.html

This free online service predict that what amino acid substitutions will affect protein function.

Publications

Rafii, S., Lindblom, A., Reed, M., Meuth, M. and Cox, A., (2003). A naturally occurring mutation in an ATP-binding domain of the recombination repair gene XRCC3 ablates its function without causing cancer susceptibility. *Hum Mol Genet*, 12: 915-923

Rafii, S., O'Regan, P., Xinarianos, G., Azmy, I., Stephenson, T., Reed, M., Meuth, M., Thacker, J. and Cox, A., (2002). A potential role for the XRCC2 R188H polymorphic site in DNA-damage repair and breast cancer. *Hum Mol Genet*, 11: 1433-8

The results of this study also presented at the following conferences:

Rafii.S, Azmy I, Xinarianos G, Reed M, Meuth M, Cox A. An investigation of the role of XRCC2 and XRCC3 sequence variants in susceptibility to breast cancer. British Society for Human Genetics Conference, 2001, York.

Rafii.S, Azmy I, Xinarianos G, Reed M, Meuth M, Cox A. An investigation of the role of XRCC2 and XRCC3 sequence variants in susceptibility to breast cancer. Yorkshire Cancer Research Conference, 2001, Sheffield.

Rafii.S, O'Regan P, Azmy I, Xinarianos G, Reed M, Thacker J, Meuth M, Cox A. The role of homologous recombination repair gene polymorphisms in breast cancer susceptibility. American Association for Cancer Research, 2002, San Francisco, USA.

Saeed Rafii, Paul O'Regan, George Xinarainos, Gordon MacPherson, iman Azmy, Tim Stephenson, Malcom Reed, Mark Meuth, Paul O'Regan, John Thacker and Angela Cox. The role of polymorphic variation in DNA repairs genes in breast cancer. Yorkshire Cancer Research Conference, 2002, Hull.

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