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THE ROLE OF DEFECTIVE MISMATCH REPAIR IN THE DEVELOPMENT OF RESISTANCE TO DOXORUBICIN

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ABSTRACT

Resistance to chemotherapy is a major clinical problem in the treatment of cancer. Doxorubicin is one of the most frequently used cytotoxic chemotherapeutic drugs, especially for the treatment of breast tumours. Doxorubicin is a topoisomerase II inhibitor, but also induces a variety of different types of damage in DNA. Emerging data has suggested a potential role for DNA mismatch repair (MMR) in sensitivity of cells to certain DNA damaging agents. The most clearly understood function of DNA mismatch repair is the correction of mismatches occurring during DNA replication and recombination. However, loss of MMR is now also associated with resistance in mammalian cells to an ever increasing range of clinically important chemotherapy agents.

In this present study we have examined the role of MMR in doxorubicin induced cell death in yeast and human tumour cell lines. In addition, we have explored the clinical relevance of loss of MMR protein expression in locally advanced breast cancer.

Doxorubicin sensitivity was examined in matched cell lines of defined MMR status derived from A2780, a human ovarian cancer cell line. The cisplatin resistant derivative A2780/CP70 has lost MMR activity due to loss of expression of the mismatch repair protein MLH1 and is cross-resistant to doxorubicin. The *hMLH1* gene is located on chromosome 3. Microcell-mediated transfer of a normal chromosome 3 into A2780/CP70 restores MLH1 expression and significantly increases doxorubicin sensitivity, as assessed by clonogenic assay ($P<0.05$). Whole chromosome transfer introduces multiple genes and thus mechanisms not associated with MMR could be influencing doxorubicin resistance in these chromosome transferrants. For instance, the gene encoding topoisomerase II â on chromosome 3. However no alteration in topoisomerase II activity was observed following chromosome 3 transfer into A2780/CP70.

In order to examine doxorubicin sensitivity in cells with only MMR genes inactivated, the genetically tractable organism *Saccaromyces cerevisiae* was used. A significant 1.3-6 fold increase ($p<0.05$) in clonogenic resistance was seen following doxorubicin exposure in isogenic, haploid, strains with individual disruptions in the MMR genes *MSH2*, *MSH3*, *MSH6*, and *MLH1* compared to the wild type strain. Furthermore, re-introduction of the *MLH1* gene into the *mlh1* mutant, using a high copy yeast expression vector, restored doxorubicin sensitivity to wild type levels. Together these observations in yeast and human tumour cell lines are consistent with a role for MMR in sensitivity to doxorubicin.

To determine if doxorubicin exposure frequently results in loss of MMR in human tumour cell lines twenty cell lines were independently derived by repeated selection with increasing doses of doxorubicin from A2780 and, the human breast cancer cell line, MCF7. Resistance was confirmed by colony forming assay. All derived cell lines exhibited a significant increase ($P<0.05$) in clonogenic resistance to 24-hour exposures of 50nM (MCF7) and 15nM (A2780) doxorubicin. However, complete loss of expression of hMSH2, hPMS2 or hMLH1 was not seen on Western immunoblot or immunohistochemistry and no loss of MMR was observed as defined by acquisition of

microsatellite instability (MSI). This suggests that loss of MMR is not frequently observed in these cell line models using these selection and assay conditions

In order to examine the potential clinical relevance of MMR protein expression in breast cancer, we examined the expression of MLH1 by immunohistochemistry in 29 women with locally advanced breast cancer before and after primary (neoadjuvant) chemotherapy. MLH1 expression was correlated to disease free survival and clinical information was obtained retrospectively from patient notes. Primary chemotherapy results in a significant reduction in MLH1 expression as assessed by both intensity ($p=0.02$) and percentage ($p=0.01$) cell staining. In addition, loss of MLH1 expression is strongly associated with poor disease free survival for both percent ($p=0.003$) and intensity ($p=0.016$) of staining. In multivariate analysis using statistically significant clinical features percentage staining following chemotherapy is identified as an independent prognostic factor ($p = 0.019$). P53 expression, assessed by immunohistochemistry, was not significantly altered by primary chemotherapy and did not predict disease free survival. There was no association between p53 and MLH1 expression ($p = 0.41$).

In conclusion, we have demonstrated the clinical relevance of loss of MLH1 in locally advanced breast carcinoma as an independent predictor of poor DFS. Large prospective studies are now necessary to validate these clinical observations.

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DECLARATION

I, Helen J Mackay, declare that all the results presented in this thesis are my own work and that I have composed this thesis in it's entirety.

I have not presented this thesis for any other degree, diploma or professional qualification.

Helen J Mackay

FOR ALEXANDER

ABBREVIATIONS

A,T, C, G	Adenine, Thymine, Cytosine, Guanine.
CR	Complete Response
DFS	Disease Free Survival
DNA	Deoxyribonucleic acid
ER	Oestrogen Receptor
GSH	Glutathione
HNPCC	Hereditary non-polyposis colorectal carcinoma
IHC	Immunohistochemistry score (Intensity + %)
IC50	Concentration of drug inducing a 50% reduction in the Surviving Fraction
IC90	Concentration of drug inducing a 90% reduction in the Surviving Fraction
kDa	Kilodalton
kDNA	kinetoplast DNA
Kb	Kilobases
MDR	Multi-drug resistance phenotype
MSI	Microsatellite Instability
MIN+	Microsatellite instability phenotype
MLH	MutL Homologue
MMR	Mismatch repair
MSH	MutS Homologue
PBS	Phosphate Buffered Saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase Chain Reaction
PD	Progressive Disease
P-gp	P-glycoprotein
PI	Propidium Iodide
PR	Partial Response
RPMI	Rosswell Park Memorial Institute
RF	Resistance Factor
S. cerevisiae	*Saccharomyces cerevisiae*
SD	Stable Disease

SF	Surviving Fraction
WT	Wild Type
YPD	Yeast extract Peptone Dextrose

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CHAPTER 1

INTRODUCTION AND AIMS

1. INTRODUCTION AND AIMS

Why study drug resistance in malignancy?

Cancer is a vast medical problem. It is diagnosed in one in 250 men and one in every 300 women each year in the UK (Souhami and Tobias, 1998). Relative success has been achieved in terms of local control of disease using surgery and radiotherapy. Whilst this is a worthwhile and important aim it has not greatly improved prognosis. The most important cause of death remains visceral spread of disease. Despite over 50 years experience in the treatment of malignancy with chemotherapeutic agents the majority of patients who develop cancer at the beginning of the 21st century will eventually die from their disease (Kramer and Klausner,1997). As yet, we cannot determine the subgroups of people who will do well or badly. Treatment failure is a major problem. Many tumours appear inherently resistant to drug therapy, whilst others display initial sensitivity followed by resistance often, not only to the initial agents, but to a broad range of anticancer drugs. The study of how and why resistance to chemotherapy develops is essential to our understanding of tumour biology and imperative if we are to improve the outlook for cancer patients in the next century.

Anticancer drug resistance can be conceptualised into 3 general areas of study:

- drug delivery
- drug:target interactions
- post-target cellular responses.

This thesis focuses on one area of drug:target interaction the recognition of DNA damage and the engagement of apoptosis.

Several studies have suggested a role for DNA mismatch repair (MMR) deficiency in the development of resistance to cisplatin and the monofunctioning alkylating agents (Anthoney et al. 1996, Drummond et al. 1996, Fink et al. 1998 a and b, Karran and Marinus, 1982). The anthracycline antibiotic doxorubicin is an important antineoplastic agent which has a number of mechanisms of action including DNA topoisomerase II inhibition, DNA intercalation and the generation of free radicals (Doroshow, 1996). Cell lines selected for resistance to cisplatin and which have lost MMR protein expression have been shown to be cross-resistant to doxorubicin. Translation of laboratory findings into the clinic is the key to improving patient prognosis in the future.

This thesis aims to:

- Explore the relationship between mismatch repair deficiency and resistance to doxorubicin.
- Characterise DNA mismatch repair in independently derived doxorubicin resistant cell lines.
- Assess the impact *in vivo* of chemotherapy on the expression of the mismatch repair protein MLH1 in breast cancer.
- Assess the impact *in vivo* of chemotherapy on the expression of the tumour suppressor gene product p53 and its association with the mismatch repair protein MLH1 in breast cancer.

1.1 DOXORUBICIN

1.1.1. Background

The anthracycline antibiotic doxorubicin (also known as adriamycin) was discovered over 30 years ago and remains one of the most widely used antineoplastic agents in current clinical practice (Di Marco et al. 1969). It is active over a wide range of doses and in a variety of administration schedules(Legha et al. 1982, Jones et al. 1987) Its broad anti-tumour activity and lack of antagonism with other chemotherapeutic agents, makes it a valuable agent for use in drug combinations (Bielack et al. 1996). Doxorubicin-containing combination chemotherapy protocols have become standard therapy for cancers of the breast, bone and soft tissue sarcomas, haematological malignancies and many childhood solid tumours (Doroshow, 1996).

In order to understand how mismatch repair could have a role in doxorubicin induced cell death, it is first necessary to consider the intracellular interactions and proposed mechanisms of action of doxorubicin.

1.1.2. Doxorubicin: chemistry and mode of cytotoxicity

Doxorubicin entry into the cell is by free diffusion of the unionized drug across the cell membrane (Peterson and Trouet, 1978). Extracellular and intracellular pH have a significant impact on doxorubicin uptake, as the drug becomes protonated within the physiologic pH range. All nucleated cells have the ability to accumulate the drug, due to DNA binding, rapid association with cell membranes and storage

in several different intracellular compartments. The bulk of intracellular doxorubicin is located within the nucleus (Doroshow, 1996).

There remains considerable controversy over the mechanism of action of the anthracyclines and thus over the relative importance of various intracellular targets. The major theories are discussed below.

1.1.2.1 Topoisomerase II

The most generally accepted mechanism of doxorubicin cytotoxicity involves the interaction between doxorubicin and topoisomerase II. The DNA topoisomerases (I and II) are involved in altering the tertiary structure of DNA to allow processes such as transcription and replication to occur. The topoisomerases alter DNA topology by generating breaks in the phosphodiester backbone which they stabilise by the formation of transient covalent intermediates with the free ends of the DNA, thus allowing passage of the strands through the gaps and the release of torsional strain. Doxorubicin covalently binds to form a ternary drug-DNA-enzyme "cleavable complex" halting the catalytic reaction prior to re-ligation and preventing progression of the enzyme complexes involved in replication and transcription (Liu, 1989). Initially, intercalation leading to alterations in DNA topology was believed to be the key factor in topoisomerase II inhibition. Subsequently, it was found that:

- Topoisomerase II-associated DNA cleavage occurs at doxorubicin concentrations below the dissociation constant for DNA intercalation (Potsmesil et al. 1983).
- The sugar and side chain of doxorubicin, responsible for minor groove binding, is at least as important for drug activity as the planar intercalating moiety (Capranico et al. 1997).
- Doxorubicin binds to and inhibits the purified topoisomerase II enzyme (Doroshow, 1996).

DNA topoisomerase poisons are invariably DNA-sequence specific. The consensus sequence for highest doxorubicin affinity is 5'-TCA (Trist and Phillips, 1989). This specificity appears to be determined by the drug enzyme interaction (Capranico et al. 1997). Could MMR be recognising the drug DNA enzyme complex thus leading to cell death? Theoretical models for the MMR pathway are discussed in the next section.

In comparison with other topoisomerase II inhibitors doxorubicin is known to exhibit more cytotoxicity than expected per DNA break (Doroshow,1996). This means that either doxorubicin associated DNA breaks are qualitatively different from those produced by other anthracyclines or that other mechanisms of action are operating in parallel. The presence of alternative mechanisms of action is supported by the development of drug resistant cell lines, which fail to demonstrate drug DNA enzyme complex formation (Bronner et al. 1994).

1.1.2.2 Additional potential mechanisms of cytotoxicity

Doxorubicin is known to bind to DNA. Several theories have been proposed as to how DNA intercalation might mediate cell death, the best documented being the inhibition of RNA and DNA polymerases (Zunino et al. 1975). Achievable intracellular concentrations of doxorubicin *in vivo*, however, do not appear to cause intercalation mediated inhibition of RNA or DNA synthesis (Siegfried et al. 1983). Doxorubicin participates in redox cycling reactions that produce DNA damage including crosslinks (Skladanowski and Konopa, 1994). MMR is known to recognise cross links induced by cisplatin (Drummond et al. 1996). In addition, doxorubicin acts as a DNA minor groove binder (Capranico et al.1997). Is MMR acting as a sensor of doxorubicin induced DNA damage and cross-links?

Cellular metabolism of doxorubicin results in the generation of semi qiunone free radicals which may result in DNA damage and cell death (De Graffe et al. 1994). This model is consistent with reports of cytotoxic potentiation by glutathione depletion and of cytotoxic protection by endogenous free radical scavengers. These studies, however, have largely been undertaken using MCF7 cell lines which are characterised by unusually abundant activity of microsomal and pentose phosphate pathway enzymes, thus providing an optimal substrate for free radical generation. Studies using other cell lines have failed to implicate radical-mediated damage as the predominant mechanism of doxorubicin action (Epstein review, 1990).

Doxorubicin has been shown to alter the fluidity of tumour cell plasma membranes and cardiac mitochondria, bind to phospholipids and up-regulate epidermal growth factor receptor expression (Benchekroun et al. 1993; Dickstein et al. 1993, Zuckier and Tritton, 1983). At high concentrations doxorubicin can inhibit protein kinase C. At lower, clinically relevant doses, cell line data has shown a two-fold increase in cytosolic protein kinase C activity (Posada et al. 1989). A further study has suggested that doxorubicin may be exerting a direct effect on transcription by downregulating a sub-unit of RNA polymerase II (Fanciulli et al. 1996). These observations *in vitro* are of uncertain significance *in vivo* .

Doxorubicin produces the morphological changes associated with apoptosis in a range of cell lines (Doroshow, 1996). This apoptosis is modulated by the interplay between the expression of the *bcl-2* and *p53* genes (Haldar et al. 1994).

In summary, the evidence suggests that DNA (rather than for example the cell membrane) is the critical target for doxorubicin cytotoxicity. Speculation as to links between MMR and doxorubicin induced cell death are purely hypothetical. There

have been no published studies looking at recognition of doxorubicin-induced DNA lesions by components of the MMR system.

1.1.3. Doxorubicin: mechanisms of resistance

Many mechanisms have been proposed to account for the development of cellular resistance to the effects of doxorubicin.

1.1.3.1. Enhanced drug efflux

The multi-drug resistance (MDR) phenotype is perhaps the most thoroughly characterised mechanism of drug resistance (Kaye, 1988). Cell lines which display this phenotype can be derived by selection, *in vitro*, with drugs including doxorubicin. The *MDR1* gene has been shown to code for a 170kDa transmembrane glycoprotein called P-glycoprotein (Kartner et al. 1985) which is an energy dependent unidirectional drug efflux pump (Willingham et al. 1986). The role of this protein in experimental drug resistance has been well established. There is a good correlation between the presence of this protein and a pattern of broad spectrum drug resistance including to doxorubicin, the other anthracyclines and vinca alkaloids (Endicott and Ling,1989). Transfer of the MDR1 gene into cell lines results in the demonstration of the full drug resistant phenotype (Sugimoto and Tsuruo,1987). Reversal of resistance *in vitro* can be achieved by a range of compounds that block drug efflux by binding to P-glycoprotein (P-gp) (Kang and Perry, 1993; Hamada et al. 1987). Despite clear *in vitro* evidence the significance of p-glycoprotein expression in tumours is less clear. Tumours such as kidney and pancreas, which display a high level of expression of the gene, are often intrinsically resistant to a range of anticancer drugs (Fojo et al. 1987). Examination of other tumour types, however,

produces variable results. P-gp is rarely found at significant levels before or after drug therapy in small cell carcinoma of the lung, but expression is clearly increased in patients failing primary therapy for leukaemia, lymphoma or myeloma (Chabner and Fojo, 1989). In breast cancer results have been conflicting (Trock et al. 1997), although coexistent p53 and P-gp expression has been reported to be an independent prognostic factor for short disease free survival (Honkoop et al. 1998). Sequential samples taken from patients undergoing chemotherapy for breast cancer have failed to demonstrate a change in the level of P-gp expression as a result of exposure to chemotherapy (Linn et al. 1997).

P-glycoprotein is not the only cell membrane associated protein involved in the development of multidrug resistance. MRP, so-called MDR related protein is another ATP binding efflux pump implicated in the development of resistance to a range of drugs including doxorubicin (Cole et al. 1992). In addition to acting as an efflux pump from the cell this protein may also sequester drug in cytoplasmic vesicles which are subsequently removed by exocytosis. Once again the clinical implication of this protein remains unclear (Broxterman et al. 1995).

1.1.3.2. Intracellular drug metabolism

Glutathione (GSH) is one of the most prevalent intracellular thiols involved in cellular detoxification. The formation of GSH-drug conjugates catalysed by a family of Glutathione -S–transferase enzymes results in reduced toxicity and excretion of the drug. Some evidence exists to suggest that doxorubicin-GSH conjugates maybe excreted from the cell via an ATP dependent glutathione s-conjugate export pump (Ishikawa et al. 1995). *In vivo* evidence, however is conflicting with reports of both an association between glutathione S-transferase levels and acquired doxorubicin

resistance (Kramer et al. 1988) and no role for this mechanism (Mestdagh et al. 1994). Once again clinical significance remains unclear.

1.1.3.3. Topoisomerase II activity

The main target protein for doxorubicin is topoisomerase II. Doxorubicin resistance in a number of cell lines (including the breast cancer cell line MCF7) has been associated with reduced topoisomerase II activity and drug induced cleavage (de Jong et al. 1990; Son et al. 1998). Furthermore, it is relatively common for tumour cells selected with an anthracycline to exhibit altered topoisomerase II levels together with increased p-glycoprotein expression (Friche et al. 1991). Mutation of the topoisomerase II α gene has been demonstrated in cell lines selected with etoposide. These are cross resistant to doxorubicin and drug sensitivity is partially restored by transfection of the topoisomerase II α gene (Asano et al. 1996). The importance of alterations in topoisomerase II levels has failed to be demonstrated clinically. The activity of topoisomerase II in AML cells varies over more than a 20-fold range with significant cell to cell heterogeneity and no relationship between enzyme levels and drug sensitivity (Kaufmann et al. 1994). In breast cancer, Linn et al failed to demonstrate any predictive value for overall survival or response to chemotherapy for topoisomerase II (Linn et al. 1997).

1.1.3.4 Signal transduction pathways

The role of signal transduction pathways in cell transformation has been extensively studied. Cytotoxic agents disrupt these pathways and this may contribute to the development of drug resistant clones (Brunton and Workman, 1993). Inhibition of different isoforms of protein kinase C (PKC) results in alterations in doxorubicin resistance, intracellular accumulation and p-glycoprotein expression (Ahn et al. 1996; Budworth et al. 1997). Others have shown that the rate of efflux through p-

glycoprotein can be controlled by phophorylation of specific amino acids within the protein (Chin et al. 1992) suggesting a role for PKC in P-gp mediated drug resistance.

There is evidence to suggest that transcriptional regulation of the MDR gene may occur as a result of binding a complex consisting of activated c-fos and c-jun (Volm, 1993; Ransone and Verma, 1990). Other oncogenes involved in signal transduction pathways have also been implicated in drug resistant cell lines *in vitro* (Kellen, 1994). Some studies have suggested that early expression of the oncogene *c-fos* prepares cells for over-expression of other genes for example *MDR3* that contribute to the multidrug-resistant phenotype (Bhushan et al. 1996).

1.1.3.5. P53 and related pathways

The p53 tumour suppressor is involved in a number of key biological activities including cell-cycle checkpoints apoptosis, senescence, maintenance of genomic integrity and control of angiogenesis. Dysfunctional p53 correlates with decreased sensitivity to a broad range of anticancer agents *in vitro* (Weinstein et al. 1997). Multidrug resistant cell lines derived by exposure to doxorubicin have been shown to have mutations within the *p53* gene (Ogretmen and Safa, 1997). Furthermore, wild-type p53 is necessary for induction of apoptosis in mouse thymocytes by topoisomerase II inhibitors (Clarke et al. 1993). Lowe et al demonstrated the effect of mutated *p53*, *in vivo*, on doxorubicin sensitivity in fibroblastic tumours developed in mice (Lowe et al.1994). Some clinical studies have supported a role for mutant p53 in doxorubicin resistance (Aas et al, 1996) whilst others have failed to find a correlation (Makris et al. 1997; Allred et al. 1993). Debate continues over the best way to measure functional p53 and this will be discussed elsewhere in this thesis.

In addition to apoptosis two other aspects of p53 biology may contribute to drug resistance. First, mutant p53 can upregulate expression of P-gp thereby promoting drug resistance *in vitro* (Chin et al. 1992). Clinical data, however, is conflicting with reports of a correlation between mutant p53 and P-gp (Linn et al. 1997) and no association (Schneider et al, 1994). Interestingly, co-existing p53 and P-gp expression has failed to predict tumour responsiveness to chemotherapy *in vivo*. Secondly, *p53* mutations relax genomic integrity potentially leading to secondary mutations producing drug resistance.

1.1.4. A role for defective DNA mismatch repair in doxorubicin resistance?

Given the extensive literature examining mechanisms of resistance associated with doxorubicin, is there any point in pursuing further potential mechanisms? Doxorubicin is a very important clinical agent and with the development of new drug delivery systems such as the introduction of liposomal doxorubicin and the use of macromolecules such as PK1 (Duncan et al. 1988) it is likely to remain so. Despite intensive research , as can be seen from the discussions above, clinically significant factors in the development of resistance have remained elusive. Furthermore, loss of mismatch repair is potentially a common mechanism of resistance to a number of drugs which, given our poly-pharmacy approach to most types of malignancy, requires further investigation. The limited amount of experimental evidence for the involvement of MMR in doxorubicin sensitivity is discussed at the beginning of **Chapter 3.**

1.2. DNA MISMATCH REPAIR

1.2.1 Introduction

DNA mismatch repair (MMR) plays an important role in maintaining the integrity of the genome by recognising and allowing repair of errors made during DNA replication and recombination. In addition, increasing evidence suggests an association between expression of MMR proteins and sensitivity to a range of clinically important anticancer drugs including cisplatin/carboplatin, doxorubicin and temozolomide (Aebi et al. 1997). Loss of MMR leads to reduced ability of cells to engage drug induced apoptosis (Anthoney et al. 1996).

Mutations in MMR genes occurs in the cancer susceptibility syndrome hereditary nonpolyposis colorectal carcinoma (HNPCC) which results in an increased incidence of colorectal carcinoma as well as other tumours including adenocarcinomas of the ovary, stomach and pancreas (Lynch, 1993). Mutations in the MMR genes *MLH1*, *MSH2*, *PMS2* and *PMS1* have all been found in HNPCC with the vast majority of mutations effecting *MLH1* and *MSH2* (Liu et al. 1996). An increased rate of mutation occurs in simple repetitive base sequences (microsatellites) within cells taken from HNPCC related tumours, so-called microsatellite instability (MSI) or MIN+ (Aaltonen et al. 1993). The MIN+ phenotype is increasingly being observed in a variety of sporadic tumour types including breast, ovary, pancreas and small cell lung carcinomas (Paulson et al. 1996; Eshleman and Markowitz, 1995), suggesting that these tumours are also MMR-defective (Liu et al, 1995). It should be stated that loss of mismatch repair is only one cause of a mutator phentoype, other causes include: loss of genes involved in DNA repair, replication or chromosomal segregation, for example germline p53 mutations in Li-fraumeni syndrome (Loeb, 1994); however, not all of these

will give rise to an increased rate of frameshift mutations. It can also be envisaged that other, as yet unidentified, mechanisms give rise to a higher mutation rate in the genome.

1.2.2. Mechanism of strand specific mismatch repair

The most clearly understood function of MMR is the post replicative recognition of DNA polymerase insertion errors which have escaped proof-reading (Modrich, 1997). The proposed model for post replication MMR is based on work with the DNA adenine methylation-instructed MMR pathway of *Escherichia coli* (Modrich and Lahue,1996). The MutS protein binds directly to mispaired nucleotides that result from misincorporation errors. MutL protein is then recruited to form a recognition complex. MutS recognises all single mispairs, except possibly C-C (Su et al. 1988). The MutS:MutL complex then associates with another protein MutH. MutH is an endonuclease which incises specifically at hemi-methylated GATC sequences within the newly synthesised DNA. This allows discrimination between the strands and incision to occur only on the daughter strand containing the mispaired bases. It is this nick which directs DNA repair (Au et al. 1992). Following incision a single-stranded DNA exonuclease (Exo I, Exo VII or the RecJ protein) removes the DNA between the MutH incision site and up to 100 bases past the mismatch (Cooper et al. 1993). A single stranded DNA binding protein, SSB and helicase II (UvrD) proteins are also required during this process. After removal, DNA polymerase III synthesizes the correct strand which is subsequently ligated into place.

MMR in eukaryotes, as in prokaryotes, appears to be bi-directional and strand specific although the signal for strand-discrimination *in vivo* is unknown. Repair *in vitro* (in both humans and yeast) can be directed to one strand by a nick in the

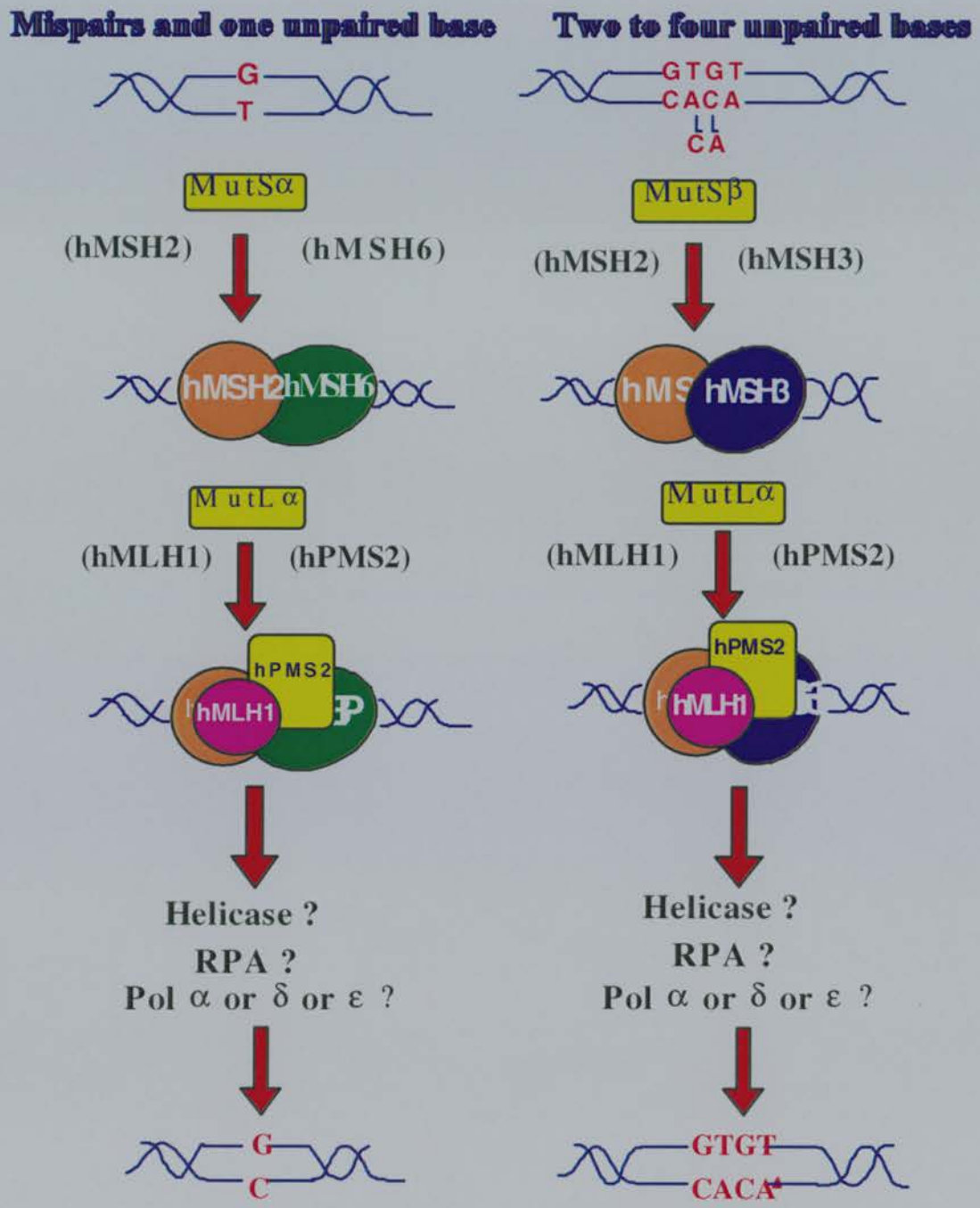

Figure 1. Schematic diagram of eukaryotic mismatch repair.

DNA substrate (Holmes et al. 1990). MutS and MutL homologues in mammalian cells exist primarily as heterodimeric proteins (illustrated in figure 1.). MSH2 protein associates with MSH3 and MSH6. These complexes are referred to as MutSα (MSH2/MSH6) and MutSβ (MSH2/MSH3). There appears to be some redundancy in the system as both complexes recognise short insertion/deletion loops, but only MutSα (MSH2/MSH6) recognises base-base mismatches. MutSα and MutSβ may act in a complementary fashion differentially recognising insertion/deletion heterologies depending on size and sequence context. Adenine nucleotide binding and hydrolysis by MutSα has been suggested to act as a molecular switch that determines downstream MMR events (Gradia et al. 1997). In this model MSH proteins survey DNA in their ADP-bound form. When DNA mismatch is detected, it provokes ADP→ATP exchange by the MSH subunits, which causes conformational change, leading to the formation of a hydrolysis-independent sliding clamp in which the MSH proteins encircle the DNA (Gradia et al. 1999). This ATP bound sliding clamp is then able to move along the DNA strand and signal downstream effectors.

The MutL homologues also form hetero dimers. hMLH1 forms a complex with hPMS2 (yeast homologue sPMS1), hPMS1 (yeast homologue sMLH3) and the recently discovered hMLH3 (Lipkin et al. 2000). So far, in humans, hMLH1/hPMS2 (MutLα), and hMLH1/hMLH3 have been shown to participate in MMR. In yeast the homologue of hPMS1 sMLH3 in combination with sMLH1 plays a role in the repair of insertion/deletion mispairs by the MSH2/MSH3 pathway (Flores-Rozas and Kolodner, 1998). Defects in hMLH1 or hPMS2 lead to loss of mismatch correction at, or prior to, the excision stage of repair. It has been proposed that MutS homologues binding to mismatched DNA allows recruitment of MutL homologues which then allows MMR to proceed. How this occurs is unclear. In the MutSα

"sliding clamp" model MutL is proposed to act as an ATPase accelerating protein which induces hydrolysis of ATP and release of the MSH2/MSH6 heterodimer from the DNA thereby recycling the signalling switch (Fishel, 1999).

One of the important issues in MMR is the identification of other proteins required for the process. Studies so far have implicated the exonucleases Exonuclease 1 and FEN1 (RAD27 in yeast), DNA polymerases pol δ and ε, and DNA replication factors RPA and RFC (Kolodner, 1996, Kolodner and Marsischky, 1999). In the yeast *S. cerevisiae,* the replication factor, proliferating cell nuclear antigen (PCNA) has been suggested to participate in MMR (Johnson et al. 1996; Kolodner and Marsischky, 1999). PCNA is a required component of the eukaryotic replication apparatus forming a homotrimeric sliding clamp around the helix and increasing the processivity of DNA pol δ and ε. PCNA may be involved in assembling a complex of proteins at the mispair (or elsewhere on the DNA) which is required for initiating MMR (Gu et al. 1998). Proteins involved in strand discrimination and possibly mismatch incision could be part of the complex. Alternatively PCNA may couple MMR to the daughter strand directly as it is asymmetric and is loaded onto the DNA during replication with a polarity (Kolodner and Marsischky, 1999). These theories remain speculative.

The complexity of the emerging eukaryotic MMR system reflects roles beyond the initially identified correction of errors arising during replication and recombination. These roles may differ depending on the genetic background of the cell. MutL homologues have been implicated in meiotic recombination in mice (Jiricny, 2000). It has been shown that MSH2 and MSH2/MSH6 can bind types of DNA damage normally thought to be repaired by other repair pathways such as nucleotide excision repair (NER). Furthermore, MSH2 has been shown to interact, in yeast,

with RAD1-RAD10 and other NER proteins (Bertrand et al.1998) in addition to its emerging role in the detection of environmental and drug induced DNA damage.

1.2.3. MMR and the development of drug resistance

Loss of expression of MMR proteins has been correlated with resistance to the methylating agents procarbazine and temozolomide, the alkylating agent busulphan, the platinum containing drugs cisplatin and carboplatin, the antimetabolite 6-thioguanine, and the topoisomerase inhibitors etoposide and doxorubicin (Aebi et al. 1997; Drummond et al. 1996; Vaisman et al. 1998). Human colorectal and endometrial adenocarcinoma cell lines which have lost expression of MLH1 and MSH2 respectively are resistant to a range of drugs. Chromosome transfer, leading to re-expression of the MMR proteins, results in restoration of drug sensitivity (Aebi et al. 1997; Umar et al. 1997). Inactivation of *MSH2* in the mouse causes cellular resistance to methylating agents and cisplatin (De Wind et al. 1995). How does loss of MMR result in drug resistance? The answer is not clear. The observations above argue against the theory that loss of MMR results in increased mutations at other genes involved in drug resistance. They support a direct role for MMR proteins in sensitivity to drug induced DNA damage. Several theories exist as to how loss of MMR might lead to drug resistance and are discussed below.

Methylating agents form a variety of adducts in DNA including the cytotoxic lesion O^6-methylguanine. The MMR system does not recognise this lesion directly. It does, however, recognise the O^6 -methylguanine-thymine mismatch that occurs during the next cycle of DNA replication. Repair synthesis, which occurs in the newly synthesized strand opposite O^6-methylguanine, is doomed to failure because a

thymine will once again be incorporated. The resulting futile cycles of repair are proposed to lead to a double strand break that could trigger apoptosis (Fink et al. 1998a). This model predicts that loss of MMR would confer tolerance to the methylating agents by virtue of the fact that the cell does not recognise the mismatch and therefore would not attempt repair.

The above model is applicable to one type of DNA damage and certainly would not be applicable to the lesions produced by doxorubicin. The discovery that loss of MMR correlated with the acquisition of resistance to a range of DNA damaging agents (and in particular to cisplatin) meant that a more inclusive model was necessary. Several studies have suggested a link between MMR proteins and G_2-M-phase cell cycle arrest in response to a number of anti cancer drugs and radiation (Brown et al. 1993; Davis et al. 1998). Davis et al have proposed that in a MMR proficient environment the MMR system binds and recognises certain lesions resulting in DNA breaks. These breaks produce a signal that results in G_2-M-phase cell cycle arrest and a loss of survival. Thus, in the absence of MMR these lesions are not recognised, no strand breaks occur and G_2-M-phase arrest is unable to be induced (Davis et al. 1998).

Cellular proliferation, and hence, presumably DNA replication is required for induction of apoptosis by cisplatin *in vitro* (Evans et al. 1994). Replicative bypass of platinum induced DNA crosslinks occurs in MMR deficient drug resistant human ovarian cells (Vaisman et al. 1998). The mechanisms leading to bypass are unknown. *In vitro* DNA polymerase δ and ε have been shown to bypass cisplatin induced adducts although, so far, no role for MMR in this process has been demonstrated (Hoffmann et al. 1995). Brown et al have proposed a model suggesting increased recombination-dependent replicative bypass leading to drug resistance in yeast and

ovarian cell lines (figure 2.). They have demonstrated a requirement of RAD52, a protein known to be required for recombination, in drug resistance mediated by MMR in *S. cerevisiae*. Furthermore, in ovarian tumour cells loss of MLH1 correlates with acquisition of cisplatin resistance and increased cisplatin-induced SCEs (sister chromatid exchanges) both of which are reversed by restoration of MLH1 (Durant et al. 1999).

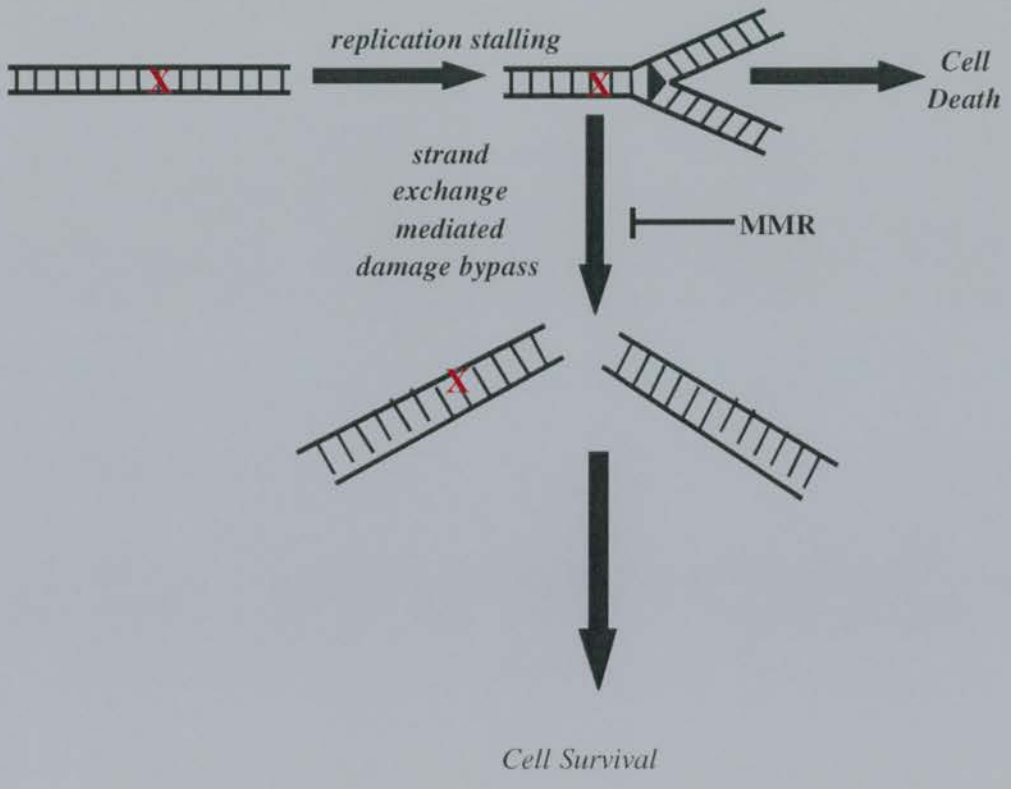

Figure 2. Model of recombination-dependent bypass.

These models imply that DNA damage within the cell could be processed in several different ways. First, the lesion may be repaired or may be cytotoxic and induce immediate death. Second, the lesion might persist and produce a signal

during replication that triggers a cell death pathway, perhaps as a result of replication stalling or arrest. Finally these lesions may be bypassed during replication allowing survival. Loss of MMR may result in tolerance by reducing the lethal signals being generated during replication by allowing increased bypass, possibly recombination dependent and cell survival (Brown, 1999). In the case of doxorubicin MMR could be recognising doxorubicin associated DNA cross links, intercalated DNA or the topoisomerase II-drug-DNA complex .

How MMR-generated signals lead to apoptosis is unclear. P53 has been implicated as part of one pathway linking MMR to apoptosis. It is a fundamental component of the DNA-damage-inducible G1 cell cycle check point and in the maintenance of G_2-M arrest following DNA damage (Kastan et al. 1992; Agarwal et al. 1995). The nature of the p53 response to DNA damage is governed, in part, by post-translational modification of the protein. The relationship between p53 and MMR is not fully understood. MutSα and MutLα-dependent activation of one or more protein kinases that phosphorylate p53 have been observed in response to DNA methylation damage *in vitro*. These result in phosphorylation of p53 at 2 serine residues. The identity of the kinases are unknown. If p53 activation does play a role in the response to methylation damage then alternate pathways must exist as p53 deficient cells are still subject to methylator induced death (Duckett et al. 1999). Hickman et al have demonstrated that MutSα is required for apoptosis induced by alkylating agents and that this is independent of p53 (Hickman and Samson, 1999). In mouse intestine, MSH2 dependent apoptosis in response to methylating agent damage is processed via p53. If however, p53 is absent a delayed p53 independent apoptotic response is engaged (Toft et al. 1999). P53 has been implicated in the apoptotic response to cisplatin (Gallagher et al. 1997). The absence of MMR in human ovarian tumour

models correlates with loss of p53 function and ability to undergo p53 dependent apoptosis (Anthoney et al. 1996). Restoration of MMR, although it restores cisplatin sensitivity, does not restore p53 function (Brown et al. 1998). Cisplatin, therefore, appears to be involved in inducing at least two pro-apoptotic pathways one of which involves p53 and the other signalled by the MMR pathway. This is supported by observations in a colorectal tumour model, that lack of MMR enhances the role of p53 in protecting the cells from cisplatin induced DNA damage (Vikhanskaya et al. 1999).

The c-Abl nonreceptor tyrosine kinase and the c-Jun NH_2 –terminal kinase are activated during the injury response to cisplatin. In MMR deficient endometrial and colorectal cell lines cisplatin activates JNK kinase less efficiently and the activation of c-Abl kinase is absent. This suggests that these kinases are part of the signal transduction pathway activated when cisplatin adducts are recognised by MMR proteins (Nehme et al. 1997). P73 is a structural and functional homologue of p53 and also induces apoptosis. Accumulation of p73 occurs following cisplatin exposure. This effect is dependent on a functional MMR system and c-Abl activation (Gong et al. 1999;Yuan et al. 1999). Cisplatin appears to be involved in one pro-apoptotic pathway involving p53 and a second involving p73 with only the latter dependent on a functional MMR pathway. It is not known if p73 is involved in inducing apoptosis in response to agents other than cisplatin. Furthermore, this pathway has been suggested in only one cell line and needs further investigation in other genetic backgrounds.

The inter-relationships between signalling pathways are likely to be complex and their relative contributions to the response to DNA-damaging agents may depend on

the cell type and agent examined. No published data exists examining MMR and the response to doxorubicin induced damage.

1.2.4. Is loss of mismatch repair relevant in human tumours?

The presence of a microsatellite instability phenotype, a marker for loss of MMR, has been shown to correlate with reduced survival and poor disease prognosis in breast cancer (Paulson et al. 1996). Conversely, a MIN+ phenotype correlates with a good prognosis in colon cancer (Bubb et al.1996; Elsaleh et al. 2000). These differences may reflect the different impact of a mutator phenotype on tumour progression versus drug sensitivity (this will be discussed more extensively in the **Discussion**). A study in ovarian cancer has demonstrated an increase in the proportion of MLH1 negative cells in tumour samples taken at second look laparotomy following chemotherapy (Brown et al. 1997).

Given that MMR status appears to have implications both for prognosis and for the development of multi-drug resistance, clinical studies are imperative.

1.3. BREAST CANCER

1.3.1. Introduction

Breast cancer is the most common malignancy affecting women in the Western world. It accounts for 20% of female cancer in the UK with 1 in 12 women developing breast cancer during their lifetime. In Britain the age standardised incidence and mortality are the highest in the world and the incidence is increasing (Cancer Research Campaign, 1996). Despite radical surgery, more than 50% of surgically treated patients eventually relapse. The introduction of adjuvant treatment (endocrine, chemo- and radiotherapy) has resulted in a reduction in mortality, with an 18-25% survival improvement at 10 year follow-up (Early Breast Cancer Trialists Collaborative Group, 1992). Nevertheless, the survival figures continue to make grim reading. For patients who develop distant metastases, the overall response rate to chemotherapy is 40 to 60% with a median time to disease progression of between 4 and 6 months (Porrka et al. 1994). Response rates decline with subsequent lines of treatment and all metastatic patients eventually die of their disease.

1.3.2. Doxorubicin in breast cancer

Doxorubicin is one of the most active agents in the treatment of breast cancer. Response rates in randomised studies are in the range of 50% in previously untreated patients with metastatic disease (Cancer: principles and practice of Oncology. 4th Edition 1993 Devita, Hellman and Rosenburg). Similar results are reported for combination regimens. Anthracyclines are also commonly included in both adjuvant and neoadjuvant chemotherapy protocols.

1.3.3. Prognostic factors in breast cancer

The identification of factors which predict the natural history of breast cancer and tumour response to chemotherapy is vital if we are to improve the prognosis for future patients. Currently, we are unable to fully identify sub groups of patients who are going to do well or badly. This means that for example, 70% of patients with lymph node negative disease will be cured by surgery alone, however we are unable to identify the 30% whose prognosis would be improved by adjuvant chemotherapy. This means that we are, undoubtedly, over treating some individuals and under treating others. The isolation of markers that predict drug resistance would allow us to select appropriate treatment regimens for individual patients and potentially could aid in the development of new treatment strategies and/or agents.

Traditionally, clinical features have been used to predict the natural history of breast cancer. The involvement of axillary lymph nodes by tumour remains the single most important indicator of the risk of recurrence and death, with tumour size and histological grade also of predictive value (Guidelines on the non-surgical management of breast cancer, 1999). Currently, the only tumour markers recommended for routine clinical use are oestrogen and progesterone receptor measurements made on the primary tumour. The data has been insufficient to recommend the routine use of proliferation indices, CA 15-3, CEA, P53 or cathepsin D. Over expression of c-*erbB-2* has been reported as an independent prognostic factor for short disease free and overall survival, although data are conflicting (American Society of Clinical Oncology, 1996). Mutation of the tumour suppressor gene p53 is a frequent genetic change in breast cancer (Elledge and Allred, 1994) and is generally viewed as an indicator of poor prognosis (Isola et al. 1992; Thor et

al. 1992). Data for the *MDR1* gene product P-glycoprotein (P-gp) are conflicting, although co-existent p53 and P-gp expression has been reported to be an independent prognostic factor for short disease free survival (Honkoop et al. 1998). Factors consistently predicting tumour response to chemotherapy have proved equally as elusive as those predicting survival. As discussed earlier in this chapter, mutant P53 and p-glycoprotein expression have produced variable results in predicting chemo-responsiveness in breast cancer. Other factors which have failed to predict tumour response include the proliferation indices Ki67 and SPF, the topoisomerases and MRP (Linn et al. 1997; Makris et al. 1997). Variable results have been obtained for expression of the apoptosis inhibitor *bcl2* and for over expression of c-*erbB-2* (Bonnetti et al. 1998; Makris et al. 1997; Rozan et al. 1998).

1.3.4. Microsatellite Instability in breast cancer

Microsatellite instability occurs in approximately 30% of sporadic breast carcinomas, although reported rates vary (Wooster et al. 1994; Yee et al. 1994; Paulson et al. 1996). This is largely due to the difficulty in defining the MIN+ phenotype, discussed in **Chapter 6**. Several studies have examined the association between clinico-pathological parameters and the presence of an MIN+ phenotype. There appears to be an association with features of poor clinical prognosis: including increased mitotic rate, aneuploidy, larger tumour size, positive lymph node status and higher pathological grade, most notably the histological subtype invasive lobular carcinoma (De Marchis et al. 1997; Aldaz et al. 1995; Paulson et al. 1996). Furthermore, Paulson et al have shown that the presence of an MIN+ phenotype is associated with reduced disease free and overall survival. No published data exists on the

impact of chemotherapy on the MIN+ phenotype or on the value of MIN+ as a predictor of response to chemotherapy in breast cancer. The expression of MMR proteins in sporadic breast cancer has not been evaluated. Further discussion on the importance of studying mismatch repair in breast cancer takes place in **Chapter 6**.

CHAPTER 2

MATERIALS AND METHODS

2.1. MATERIALS

The following section lists routinely used materials. Those less frequently used are described in the appropriate methods section

Chemicals

All chemicals were of the highest quality and were obtained from Gibco BRL, BDH Chemicals, Pharmicia LKB, Severn Biotech, Rathburn, Boehringer Mannheim and Sigma Chemicals

Radiochemicals

(α^{32}P)dCTP used for labelling of DNA was obtained from Amersham International plc,Little Chalfont, Bucks

2.1.1. EQUIPMENT

Routine equipment which would be an integral part of any laboratory is not listed.

Autoradiography film	Kodak, Cambridge. UK Fuji, Tokyo. Japan
Cell Counter	Coulter Electronics. Luton Beds
Confocal Microscope	Biorad. Hemel Hempstead. UK
UV Transilluminator and Digital Imaging System	Appligene, Chester-Le-Street, Co.Durham
Electrophoresis Tank	IBI Ltd. Flowgen.Lichfield. UK Life Technologies. Paisley
Milliblot SDE Electroblotter	Millipore, Watford, UK Biorad.Hemelhempstead
Film Processor	Kodak
Gel Drier	Biorad Hemel Hempstead.

Hybridisation Membranes

Hybond-N	Amersham International, Amersham UK
Immoblion-P	Millipore. Bedford,UK
Hybridisation Oven and Bottles	Hybaid Ltd, Middlesex,UK
PCR Thermal Cycler	Hybaid, Teddington,UK
UV Crosslinker/imager	Stratagene, Cambridge,UK

2.1.2. RESTRICTION ENDONUCLEASES AND OTHER ENZYMES

Hinf1 (10-15 units/μl)	
Proteinase K	Gibco BRL
Taq polymerase	Boehringer Mannheim

2.1.3. SIZE MARKERS

DNA

HindIII digested phage λ	IBI Cambridge
100bp DNA ladder	Gibco BRL

Protein

69-202 KDa pre-stained SDS page markers	Bio Rad

2.1.4. BUFFERS SOLUTIONS AND MEDIA

All solutions were made with double distilled water (ddH_20) and stored at room temperature except where indicated

Solutions for immunostaining

All tissue immunostaining carried out using Vectastain® *Elite* ABC Kit (Vector labs, Peterborough, UK)

0.1% Hydrogen peroxide

1ml 100 vol. hydrogen peroxide + 1litre of distilled ddH_20

Sodium tricitrate buffer

2.94mg sodium tricitrate in 1 litre ddH_20 pH to 6 with concentrated HCl

Blocking Solution

1 x Blotto
5% Marvel nonfat milk powder
0.01% Tween-20
1mM DTT

Blotto(10x)

500mM Tris-HCl (pH 7.5)
500mM NaCl
10mM EDTA

High Salt Lysis Buffer

500mM NaCl
1% NP-40
50mM Tris-HCl, pH7.5
Protease Inhibitors(1x)

Phosphate Buffered Saline (PBS)

0.8% NaCl
0.115% Na_2HPO_4
0.02% KCl
0.02% KH_2PO_4

Protease inhibitors (100x)

0.1mg/ml Aprotinin
0.1mg/ml Pepstatin
0.1mg/ml Chymostatin
0.05M Benzamidine
0.05M PMSF (phenylmethylsulfonylfluoride)
0.1mg/ml Leupeptin
(all stored at -20°C)

5 x Tank buffer

0.5M Tris base

0.5M Glycine
0.5% SDS

Transfer buffer

48mM Tris base
39mM Glycine
0.038% SDS
20% Methanol

RPMI medium

500ml RPMI 1640
13.3ml 7.5% $Na(CO_3)_2$
5ml 100mM Na pyruvate
5ml 200mM L-glutamine
1ml 1M NaOH
50ml Foetal calf serum
2.5ml Penicillin/Streptomycin

8% Running gel

8ml running gel buffer
8.5ml 30%Acrylamide,0.8%bis-acrylamide(Severn Biotech, Kidderminster)
3.2ml 1% Polyacrylamide
12.3ml ddH_20
120µl 10% Amonium persulphate
15µl Temed

4% Stacking gel

3ml spacer bel buffer
1.6ml 30%Acrylamide,0.8%bis-acrylamide
1.2ml 1% Polyacrylamide
4.8ml ddH_20
180µl 10% APS
15µl Temed

Running gel buffer

1.5M Tris-HCl, pH 8.9
4% SDS

Spacer gel buffer

0.5M Tris-HCl , pH 6.7
0.4% SDS

Nucleus Buffer

1mM KH_2OPO_4
5mM $MgCl_2.6H_2O$
1mM EDTA,pH6.4
2.5%SDS
PH to 6.4 with 2M KOH
Stored at 4°C.
Immediately prior to use protease inhibitors were added to the following final concentrations:
0.2mM DTT (from mM stock kept at -20°C)
1μM pepstatin
1mM PMSF

YPD media (Yeast, Peptone, Dextrose)

10g	Difco peptone
5g	Yeast extract
475ml	dd H_2O
50ml	40% dextrose
9g	Agar (if required for plates)

Dropout mixture

Adenine	800mg
arginine	800mg
aspartic acid	4000mg
histidine	800mg
leucine	800mg
lysine	1200mg
methionine	800mg
phenylalanine	2000mg
threonine	8000mg
trytophan	800mg
tyrosine	1200mg
isoleucine	1200mg

Add 870mg to 1l ddH_2O pH with NaOH to 5.5-6.0

Synthetic drop out yeast media

870mg/l	Drop out mixture
6.7g	yeast nitrogen base w/o amino acids
20g	glucose
20g	agar

TBE(1x)pH8

89mM Tris borate
89mM Boric acid
2.5mM EDTA

5 x Western loading dye

250mM Tris-HCl , pH8
10% SDS
10mM EDTA
50% Glycerol
0.25% w/v Bromophenol blue
0.25% w/v Xylene cyanol

Tris-EDTA buffer

10mM Tris-HCl, pH 7.5
1mM EDTA

TNE buffer

10mM Tris-HCl, pH8
100mM NaCl
1mM EDTA,pH8

TE buffer

10mM Tris-HCl, pH8
1mM EDTA

DNA loading buffer

30% v/v Glycerol
0.25%w/v Bromophenol blue
0.25% xylene cyanol FF

TAE buffer

40mM Tris-acetate, pH 8
1mM EDTA,pH 8

Denaturation solution

1.5M NaCl
0.5M NaOH

Neutralization solution

1.5M NaCl
0.5M Tris-HCl,pH8
1mM EDTA

X 20 SCC

3.0M NaCl
0.3M sodium citrate

Pre-hybridisation buffer

990ml 0.5M Na_2HPO_4, pH7.2
10ml 10% SDS

Hybridisation buffer

900ml Pre-hybridisation buffer
100ml Membrane blocking reagent - prepare a 10% w/v solution of casein hammarsten(BDH) in wash solution 2 by heating at 50-70°C for 1 hour Autoclave and store at -20°C.

Wash solution 1

160ml 0.5M Na_2HPO_4, pH7.2
10ml 10% SDS

Wash solution 2

13.8g Maleic acid
8.7g Sodium chloride
900ml Sterile water
pH adjusted to 7.5 with NaOH

2.1.5. CELL LINES

A2780

Human ovarian adenocarcinoma cell line derived from omental metastasis from an untreated patient (Eva et al. 1982). Received from R.F. Ozols and T.C. Hamilton, Fox Chase Cancer Centre, Philadelphia.

A2780AD

Doxorubicin resistant derivative of A2780, isolated by repeated exposures to doxorubicin (Rogan et al, 1984). This cell line is known to expresses high levels of p-glycoprotein (Sugawara et al, 1988).

A2780/CP70

Cisplatin resistant derivative of A2780 selected for resistance by exposure to increasing concentrations of cisplatin (Behrens et al, 1987).

A2780/CP70/chromosome 3

A2780/CP70 cell line transfected with chromosome 3 (Illand and Brown, 1998).

A2780/CP70/chromosome 2

A2780/CP70 cell line transfected with chromosome 2 (Illand and Brown, 1998).

MCF7

Human breast adenocarcinoma cell line established from the pleural effusion of an untreated patient (Soule et al, 1973).

MCF7/AD

Doxorubicin resistant derivative of MCF7 isolated by selection against increasing concentrations of drug (Sinah et al, 1986).

LoVo

Human colorectal adenocarcinoma cell line established from an untreated patient. (Drewinko et al. 1979).

2.1.6. *S. CEREVISIAE* MUTANT STRAINS

Haploid isogenic strains were obtained from Dr. R Borts, Yeast Genetics, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, UK. All strains are derivatives of RHB2096 (Hunter and Borts 1997)

Isogenic strain	MMR disruption
RHB2096	Wildtype
RBT311	Mlh1
RHB2344-1B	Msh2
RHB2345-1B	Msh3
NHT173	Msh6

Table 1. Phenotypes of *S. cerevisiae* MMR mutant strains

The strains were created by insertion of *LEU2* into coding sequences of cloned versions of the relevant genes. Plasmids were cut in sequences flanking the disruptions and transformed into yeast using the LiAc transformation protocol. Disruptants were selected on plates lacking leucine, screened for mutator phenotype/uv sensitivity where possible and were confirmed by Southern analysis. The kanmx disruption of *Mlh1* was made by PCR. Oligonucleotides homologous to the 5' and 3' flanking region of *Mlh1* were made which also had sequences homologous to the *Kan* gene. This was used to make a *Kan* containing plasmid to get PCR product which had "tails" of homology to the flanking regions of *Mlh1* surrounding the entire *Kan* gene. Transformation with this and selection for G418 resistance replaces the entire *Mlh1* reading frame for the *Kan* gene (Oliver, 1998). Transfected strains of RBT311, were obtained from S. Durant, PhD student Drug Resistance Group, Department of Medical Oncology, CRC Beatson Laboratories, Glasgow.

2.1.7. ANTIBODIES

The following are a list of antibodies used for western immunoblotting and immunocytochemistry

Antigen	Antibody	Isotype	Company
hMLH1	G168-15	Mouse monoclonal IgG_1	PharMingen
hMSH2	Ab-2 clone FE11	Mouse monoclonal IgG	Calbiochem
hPMS2	Ab-1 clone 9	Mouse monoclonal IgG_1	Calbiochem
p53	DO-1	Mouse monoclonal IgG_{2a}	Oncogene science
Vinculin	Vin-11-1	Mouse monoclonal IgG_1	Sigma

Table 2. Antibodies

Anti-mouse IgG horse radish peroxidase linked rabbit antibody (Amersham) and anti-

mouse IgG fluorescein 5-isocyanate conjugated (FITC) goat antibody(sigma)

2.1.8. PCR PRIMERS

The primers used for repeat sequences, chromosomal location, locus heterozygosity and size are shown in table 3. All repeats are CA (Dietrich et al. 1994).

Table 3. PCR primers

Locus	Chromosomal Location	Sequence of PCR primers	Heterozygosity	Size Range
D2S119	2p	CTTGGGGAACAGAGGTCATT GAGAATCCCTCAATTTCTTTGGA	0.8	214-232
D2S121	2q	GCTGATATTCTGGTGGGAAA GGCAAGAGCAAAACTCTGTC	0.81	156-184
D2S136	2p	AGCTTGAGACCTCTGTGTCC ATTCAGAAGAAACAGTGATGGT	0.74	91-111
D2S391	2p	ATGGAGCCAGTAGGTTACAGC GGTGAGAGGGTATGATGGAA	0.79	142-152
D3S1582	3p	CAGCAGGTACTATGAAAGCCTGT GGAACAGCCCTATGGTTCAC	0.8	154-178
D3S1606	3p	AAAGCCATATAAAATATCCCCT ACTGGCAGCCTTGACC	0.78	236-252
D3S1612	3p	TCTTTTAGTCAGCAGTTATGTC CCCATTAAGAAATGTTACTCTAC	0.69	206-226
D3S1619	3p	GTCCTGCAAGACTCATTG TTGCTAGGATGGTTGTTTTC	0.75	161-171
D17S796	17p	CAATGGAACCAAATGTGGTCAGT CCGATAATGCCAGGATG	0.8	144-174
D17S801	17q	CCTCAAACCGGACAACTATTT CAGAGAGCAAGATCCTACCTC	0.85	258-336

2.2. METHODS

2.2.1. TISSUE CULTURE TECHNIQUES

2.2.1.1. General cell culture methods

Aseptic manipulations were performed using sterile glassware in a class II microbiological safety cabinet with vertical airflow. Cell lines were grown and maintained as monolayer cultures in supplemented RPMI (Rosswell Park Memorial Institute) medium in the prescence of 5% CO_2. A2780/CP70 cells which had been transfected with chromosome 2 or 3 were grown in Hygromycin B containing media (200u/ml). Cell stocks were made by freezing $1x10^6$ cells in 1ml RPMI with 10% di-methyl sulphoxide (DMSO) at -70^o C in 1ml cryotubes(Nunc, UK). After 24 hours samples were transferred to liquid nitrogen. All cell lines were routinely tested for mycoplasma infection every 4 weeks.

2.2.1.2. Selection of doxorubicin resistant cell lines

Ten doxorubicin resistant cell lines were selected by serial exposure to increasing concentrations of doxorubicin from parental A2780 and MCF7 cell lines. $1x10^6$ cells were grown in F75 tissue culture flasks (Bibby Sterlin, Aberbargoed.UK) for 36 hours to attain log phase growth. A stock solution of sterile doxorubicin was added to RPMI medium to obtain the desired final concentration. Cells were exposed to doxorubicin containing medium for 24 hours at which point it was replaced by fresh medium. Cells were allowed to grow for approximately 10 days with changes of medium as appropriate. Surviving cells were then harvested by trypsinisation and $1x10^6$ cells were placed

in a new F75 flask for the next round of selection. The concentrations used in the selection process were:

For A2780 10nM, 20nM, 30nM, 60nM, 90nM, 150nM

For MCF7 10nM, 20nM, 30nM, 60nM, 90nM, 150nM, 200nM, 300nM

2.2.1.3. Colony forming assay

1×10^3 cells were plated per 90mm diameter dish and after 24 hours doxorubicin was added in fresh medium. The cells were exposed to the drug for 24 hours. The doxorubicin containing medium was then removed and replaced by fresh medium. The cells acting as controls had changes of fresh, drug free, medium in parallel with the drug exposed cells. Colonies were grown for 10-14 days after doxorubicin exposure and then stained with 1x Geimsa stain for 10 minutes and rinsed with tap water. Colonies greater than 200 cells were counted. 5 plates were set up at each dose point per experiment. The experiments were repeated at least once, survival fractions in this thesis are reported as a mean of at least 10 plates (1×10^4 cells).

2.2.2. YEAST CULTURE TECHNIQUES

2.2.2.1. Preparation of YPD medium

Difco peptone, yeast extract and agar (if required for solid medium) were dissolved in ddH_2O and the pH adjusted to 5.8. The solution was autoclaved and allowed to cool to 55°C when 50ml of a filter sterilised 40% dextrose solution was added.

2.2.2.2. Yeast Clonogenic Assay

Cells were scraped from stocks stored at -70 °C. They were grown to saturation in 10ml YPD medium for approximately 48 hours in a 30 °C incubator undergoing constant agitation. Cells were counted using a haemocytometer (Weber, England) and 2 x 10^7 cells were added to 1ml of YPD medium containing a range of doxorubicin concentrations. These were incubated for 24 hours at 30 °C. 400 cells were then plated out on solid YPD culture medium and incubated at 30 °C. The surviving colonies were counted after 3 days and the surviving fraction calculated relative to the no drug control.

2.2.2.3. *ScMLH1* transfected yeast

These strains were obtained from S Durant.

The MLH1 deficient strain of *S. Cerevisiae* was transfected with wild -type sc*Mlh1* using low and high copy yeast expression vectors, pyx112 and pyx212 (R and D Systems). Cells were grown in selective Yeast Drop Out Medium (no uracil).

2.2.3. GEL SEPARATION AND IMMUNODETECTION OF PROTEINS

2.2.3.1. Extraction of protein

Cells, grown in monolayer culture, were washed with PBS, then lysed at 4°C for 5 min using high salt lysis buffer supplemented with 1 x protease inhibitors. Cell supernatant containing extracted proteins was collected by centrifugation of lysate in cooled Eppendorf tubes at 4°C (21000g/15min) and then stored at -70°C.

2.2.3.2. Determination of protein concentration

Estimation of protein concentration was performed using Bio-Rad Protein assay with standard curve constructed using Bovine serum albumin.

2.2.3.3. Sample preparation

75μg protein was mixed with 1/5 volume western loading dye and boiled for 3 min to facilitate protein denaturation.

2.2.3.4. Denaturing gel electrophoresis of protein

Protein samples and size markers were separated by SDS-polyacrylamide gel electrophoresis on an 8% running gel after passing through a preliminary 4% stacking gel, both suspended in 1 x tank buffer. Proteins were electrophoresed at 200V/40mA until the dye front had migrated through the gel.

2.2.3.5. Western transfer of protein

Electroblotting was performed using a semi-dry electroblotter. Immobilin-P membrane (Millipore, Bedford,MA) was immersed in methanol and then transfer buffer. Six sheets of 3mm Whatman filter paper were sandwiched adjacent to the anode and cathode with the membrane and gel layered in between. Transfer took place over 1 hour at 200mA. To assess the evenness of protein transfer the gel was subsequently stained in Coomassie stain (0.2% Coomassie Brilliant blue R250 in a 50:50:7 v/v ratio of methanol:H_20:glacial acetic acid) for 4 hr, then destained using 25:68:7 v/v ratio of methanol:H_20:glacial acetic acid overnight.

2.2.3.6. Immunological detection of protein

Membranes were incubated with blocking solution at 4°C for 4hr, probed overnight in the same buffer with primary antibody and washed with 0.1%Tween-20 in PBS. Blots were incubated in blocking solution with anti-mouse IgG HRP-linked rabbit antibody, then washed again in 0.1%Tween-20 in PBS, after which

bound complexes were visualised by enhanced chemiluminescence (Boehringer Mannheim). Membranes were exposed to X-ray film and these were developed to allow bound complexes to be visualised.

2.2.4. TOPOISOMERASE II ASSAY

2.2.4.1. Crude nuclear extraction

Subconfluent flasks of cells were washed with 10ml of ice cold PBS and cells scraped off the base of the flask using a disposable cell scraper. This solution was centrifuged at 500g for 10 minutes. The resulting cell pellet was resuspended in 200µl of nucleus buffer in a 1.5ml eppendorf and freeze thawed twice in a dry ice/ethanol bath. 3.5M NaCl was added to give a final concentration of 0.35M NaCl. The cell solution was vortexed briefly and incubated on ice for 1 hour with mixing taking place after 30 minutes. Cells were then spun at 21000g for 20 mins at 4°C. Protein estimation was performed using a Bio-Rad protein assay as described earlier. Nuclear extract was stored at -70°C.

2.2.4.2. Topoisomerase II Assay

This assay was performed according to the manufacturer's instructions (TopoGEN Inc., Ohio). Activity of topoisomerase II is observed through the enzyme's ability to decatenate kinetoplast DNA (kDNA). A known amount of nuclear extract was incubated with water, 10 x reaction buffer and kDNA at 37 °C for 20 minutes. Reactions were terminated by placing samples on ice and adding 1/5 volume of stop buffer/loading dye. Activity was resolved in 1% agarose/TBE gels containing 0.05% ethidium bromide. TBE was used as running buffer and gels were run at 150V/40mA for 45 minutes. Markers kDNA, linear

kDNA and Topo II decatenated kDNA were run on each gel. Electrophoresed DNA was visualised using a UV transilluminator and a record of the gel was made using a digital imaging system.

2.2.5. DNA EXTRACTION

Genomic DNA was extracted from monolayer cultures using the Nucleon I DNA extraction kit (Scotlab) following manufacturers protocol. DNA concentration were determined by Hoechst 33258 staining and spectrophotometric quantitation using standard curve human placental DNA.

2.2.6. AMPLIFICATION AND DETECTION OF DNA

2.2.6.1. Standard PCR

All PCR manipulations were carried out using dedicated micro-pipettes, aerosol resistant tips and plasticware that had been sterilised by brief exposure to UV irradiation. Water and other non-DNA containing reagents were also exposed to UV irradiation to reduce contamination of samples with extraneous DNA. PCR was carried out in a total reaction volume of 50μl in 0.5ml Eppendorf tubes (Nunc.) 2μl of sample (50 to 100ng DNA) was added to 2μl of each primer together with a master mix detailed below:

5μl	10 x PCR buffer + Mg
1.6μl	dNTP mix
0.2μl	α^{32}P dCTP
0.2μl	Taq polymerase

37µl sterile H_2O

Thermal cycler parameters were individualised for each primer template combination. Annealing temperatures were obtained, either from A Anthoney (PhD thesis), G Hirst (personal communication) or were calculated using:

Annealing temp = $2x(A + T) + 4 x (G + C)$

Where G,C,A,T are the number of each nucleotides in the primer. Standard melting and extension temperatures of 94 °C and 72 °C were used in each reaction unless otherwise stated. The number of cycles of amplification was generally 35. A technique of "Touchdown" PCR was used for reactions involving microsatellite repeats(Don et al, 1991).

2.2.6.2. Agarose gel electrophoresis

To check for a successful PCR, samples were run on a 1% TBE agarose minigel containing 0.05% ethidium bromide with 100bp DNA markers. Bands were detected using a UV transilluminator.

2.2.6.3. Polyacrylamide gel electrophoresis

Following successful PCR denaturing polyacrylamide gels were used to resolve individual microsatellite repeats. Gels were cast between pre-cleaned glass plates one of which was coated with a silicon solution (Repelcote,Sigma. UK). The glass plates were sealed to form a mould using adhesive tape and 30% Acrylamide/0.85% bis-Acrylamide/7M Urea (Easigel) pre mixed solution was used to make the gel:-

40ml Easigel
280µl 10% APS
14µl TEMED

Wells were formed using a comb and the gel allowed to set for 1 hour. Following this the combs were removed and the wells cleared of unpolymerised

polyacrylamide and urea using 1 x TBE buffer. The gel was pre run for 1 hour at 35mA/1800V. Samples were prepared for loading by adding 5µl of fromamide loading dye to 7µl PCR product and placing in a water bath at 92°C for 5 minutes. The reaction was terminated by placing the samples on ice. 8µl of sample was loaded per well and the gel was run at 35mA/1800V in 1 x TBE buffer until the xylene cyanol FF band was at the bottom of the gel (approximately 2-3 hours). The gel was blotted onto a sheet of 3mm chromatography paper (Whatmann,UK) and dried under vacuum at 80°C for 1 hour. It was then exposed to X-Ray film in a cassette with tungstate intensifying screens at room temperature.

2.2.7. DNA FINGERPRINTING

DNA fingerprinting was carried out using protocols supplied by the European Collection of Animal Cell Cultures, Centre for Applied Microbiology and Research, Porton Down, Salisbury.

2.2.7.1. Isolation of genomic DNA

DNA was extracted using a salt-chloroform method from approximately 10^7 cells as described by Mullenbach et al (Mullenbach et al. 1989). DNA concentration was determined as described earlier .

2.2.7.2. Restriction enzyme digestion of DNA

The DNA sample (equivalent to 40µg DNA) was added to 40µl x10 enzyme buffer in a 1.5ml microfuge tube and the volume made up to 390µl with sterile water. 10µl *Hinf1* (100-150 units) was added and mixed gently by vortex followed by pulse centrifugation to draw all the reaction mixture to the bottom

of the tube. This was placed in a water bath at 37°C for 4 hours, mixing every hour. The reaction was terminated by the addition of 10µl 0.5M EDTA, 50µl 5 M NaCl and 1 ml ethanol, mixed well and cooled at -20°C overnight. The samples were centrifuged 15000g at 4°C for 20 minutes by microcentifuge. The supernatant was removed and the pellet washed with 500µl ice cold ethanol. The sample was centrifuged at 15000g at 4°C for 2 minutes, the excess ethanol removed and the pellet was air dried. The resulting DNA pellet was dissolved in 40µl TE buffer.

2.2.7.3. Agarose gel electrophoresis

0.8% TAE agarose gel containing 0.05% ethidium bromide was prepared. 1µl of digested DNA sample and 1µl DNA loading buffer were mixed and loaded onto the gel together with DNA size markers. The gel was visualised using the uv transilluminator.

2.2.7.4. Southern blotting

The remaining DNA samples (39ìg) were mixed with 10µl of DNA loading buffer and run on a 0.8% TAE agarose gel. Approximately 10µg of DNA was loaded into each lane and the gel was run at 20V over 18 hours. The resulting DNA fragments were transferred to a nylon filter (Hybond N) by Southern blotting.

2.2.7.5. Hybridisation of the DNA fragments with a NICE™ labelled probe.

Nylon support mesh was cut to the appropriate size to separate the nylon filter membranes. The mesh and membranes were rolled and placed in a pre-warmed hybridisation bottle. 30ml of 1 x SCC, pre-warmed to 50°C, was added to the bottle for 5 minutes and then replaced by 30mls of, pre-warmed, pre-hybridisation buffer. This was left for 20 minutes at 50 °C on a rotisserie. 10 µl of Non-

Isotopic Chemiluminescent Enhanced (NICE™, ICI Cellmark Diagnostics) multi-locus probe (33.15) was added to 16μl hybridisation buffer. The pre-hybridisation buffer was removed, the above solution added and the bottle placed in the hybridisation oven for 20 minutes at 50°C. The solution was removed and 100ml of pre-warmed wash solution 1 was added to the hybridisation bottle for 10 minutes at 50°C. The membranes were then transferred to a plastic box containing 500ml of wash solution 1 and agitated for 10 minutes. 2 x washes in 500ml of wash solution 2 for 5 minutes at room temperature followed. Each membrane was transferred to a glass plate and transferred to a fume cabinet where 3-4mls of Lumi-Phos™ 530 (BDH) was added and spread out using a glass pipette. Each membrane was then sandwiched between two transparent acetate sheets and a ruler used to squeeze out any excess fluid. The membrane acetate sandwich was placed against an X-ray sensitive film in a lightproof cassette. This cassette was then placed in an incubator at 37°C for approximately 6 hours (the chemiluminescence continues for up to 5 days).

2.2.7.6. Dehybridisation

NICE™ probes can be removed from the membranes by agitating them for 15 minutes in 0.1% SDS at 80°C followed by a rinse in 1 x SSC. The membranes can then be stored.

2.2.8. IMMUNOCYTOCHEMISTRY

2.2.8.1. Immunohistochemistry of cultured cells

4 x 10^4 cells in 400μl of RPMI medium were added to each well of an 8 well chamber slide (Nunc, Naperville, Il. USA) and grown for 48 hours. The medium

was removed and the cells washed with PBS. The plastic gasket was removed, the slide dried in air and then, if not required, stored at -70°C wrapped in parafilm. Dried slides were fixed in methanol at -20°C for 20 minutes and then air dried for 40 minutes. Normal horse serum, diluted 1:10 with 0.1% BSA in PBS, was added to the cells as a blocking agent and left for 20 minutes at room temperature in a humid atmosphere. Excess liquid was removed and 50µl primary antibody diluted in 0.1% BSA in PBS, was added for 2 hours. Slides were then immersed in 0.1% Tween-20 in PBS for 2 x 10 minutes before incubation for 30 minutes with the secondary antibody. 50µl of the secondary antibody (2.4µl anti-mouse-FITC plus 397.6µl PBS/BSA) was added per well and followed by two further 10 minute washes in 0.1%PBS. The slides were then mounted using Vectashield (Vector Labs, Burlingham. Ca. USA) and the edges of the coverslip sealed with nail varnish. Slides were visualised and the image stored using confocal microscopy.

2.2.8.2. Immunohistochemistry of paraffin embedded breast tissue

Immunohistochemistry was performed on histology sections taken from tru-cut biopsy and mastectomy specimens. All samples were formalin fixed and paraffin embedded. Mouse monoclonal antibodies G168-15 (1 in 100 dilution, PharMingen) and DO-1 (1 in 200 dilution, Oncogene Science) were used to detect hMLH1 and mutant p53 respectively using a peroxidase labelled streptavidin-biotin technique. Slides were deparaffinized in histoclear then rehydrated through graded alcohols and water. Removal of endogenous peroxidase activity was achieved by incubation in 0.1% hydrogen peroxide for 20 minutes. Sections were immersed in 10mM sodium tricitrate buffer pH6 and subject to heat-induced antigen retrieval by microwaving for 15 minutes then allowed to cool for 20 minutes.

The tissue section was isolated using a PAP-pen. agent The commercially available Vectastain® *Elite* ABC Kit (Vector labs, Peterborough, UK) was used to detect the antigen. Slides were placed in a humid atmosphere and Normal Horse serum (Vectastain® Yellow 3 drops in 10mls PBS) used as a blocking agent. This was left for 20 minutes, the excess liquid removed and the primary antibody added for 30 minutes. The slides were immersed in PBS for 5 minutes. They were returned to the humid box and biotinylated anti-mouse IgG (Vectastain® Blue 1 drop + 3 drops yellow in 10mls PBS) added for 30 minutes. ABC reagent (Vectastain® Grey 2 drops of A and B in 5mls PBS) was made while the slides were washed in PBS for a further 5 minutes. The slides were returned to the humid box and ABC was added to the slides for 30 minutes. The PBS wash was repeated. The slides were returned to the humid box and each tissue section was covered with 2–3 drops (100-200µl) of DAB diaminobenzidine tetrahydrochloride for 10 minutes. The PBS wash was repeated. Sections were then lightly counterstained with haematoxylin, dehydrated, placed in histoclear and a coverslip was placed on each slide. Internal controls were obtained for each slide by omitting the primary antibody. External controls included: for MLH1 A2780 cells (positive control for MLH1 and negative control for p53) and A2780/CP70 (negative control for MLH1 and positive control for p53). Two slides per patient sample were immunostained in separate runs. Both scoring and immunostaining were blinded to clinical outcome. Slides were scored by light microscopy by a consultant pathologist. A score of 0 to 3 for stain intensity was assigned: No staining = 0, weakly positive = 1, positive = 2, strong positive = 3. Percentage staining was assessed by: 0 = 0-5%, 1 = 5-20%, 2 = 20-80%, 3= 80-100% and the 2 scores combined to give a potential maximum score of 6 (the IHC score).

2.2.9. STATISTICAL METHODS

For statistical analysis of the patient samples involved in immunohistochemistry variables taken into account included ER status, age, menopausal status, size, grade and response to chemotherapy. All information was obtained retrospectively from patient records. The within patient comparison of MLH1 and p53 scores pre- and post chemotherapy was assessed using Wilcoxon signed rank sum test. The examination of an association between p53 and MLH1 score was made using Spearman's rank correlation coefficient. The univariate examination of the association between disease free survival and p53, MLH1 and clinical factors were made using the Cox regression analysis. The likelihood ratio P-value is quoted. The multivariate analysis of factors associated with disease free survival was also conducted using Cox (multiple) regression techniques and a forward selection procedure (P to enter = 0.05, P to remove = 0.10). Disease free survival was taken from the time of commencing chemotherapy to relapse.

CHAPTER 3

DOXORUBICIN RESISTANCE IN TWO MISMATCH REPAIR DEFICIENT OVARIAN AND BREAST CELL LINES

3.1. Introduction

The development of drug resistance is a major clinical problem in the fight against cancer. The identification of common pathways of drug resistance has a key role to play in enabling us to plan therapeutic strategies and design new approaches to cancer treatment. The circumstantial evidence for an association between loss of MLH1 expression and doxorubicin resistance at the start of this study included:

1. Enrichment of MLH1 deficient colonic tumour cells following exposure to doxorubicin (Fink et al. 1998a.).
2. Cross-resistance to doxorubicin in, *in vitro* derived, cisplatin resistant ovarian tumour cell lines which has lost MMR activity due to complete loss of MLH1 expression (A Anthoney, personal communication) .
3. One, *in vitro* derived, doxorubicin resistant human ovarian tumour cell line which has lost MMR activity due to complete loss of MLH1 expression. This cell line exhibits cross-resistance to cisplatin (Drummond et al. 1996).

How loss of MMR could lead to drug resistance is not understood. It is, however, important to establish if these findings represent direct involvement of MMR proteins in sensitivity to DNA damage or if loss of MMR results in increased mutation at other genes involved in drug resistance. This chapter examines 2 cell lines known to be resistant to doxorubicin and to have lost functional MMR activity in order to begin to address this issue.

The human breast cancer cell line MCF7 has functional MMR activity and expresses all mismatch repair proteins. The clonal doxorubicin resistant derivative MCF7AD is known to display microsatellite instability at 6 out of 13 loci

examined, suggesting loss of mismatch repair activity (Anthoney et al. 1996). MCF7AD had not been generated or characterised within our laboratory. It was necessary to establish the authenticity of the cell line and confirm that it was indeed derived from MCF7 before investigating it further.

A2780/CP70 a clonal derivative of the human ovarian adenocarcinoma cell line A2780 isolated by *in vitro* selection for cisplatin resistance, demonstrates cross-resistance to doxorubicin (Drummond et al. 1996). Furthermore, this cell-line has lost expression of the MMR proteins MLH1 and PMS2 and exhibits MSI at 7 out of 11 loci examined. The parental A2780 cell line has intact mismatch repair activity (Drummond et al. 1996; Anthoney et al. 1996). The gene encoding for MLH1 is located on chromosome 3 (Bronner et al. 1994). Microcell mediated transfer of chromosome 3 into A2780/CP70 results in re-expression of MLH1 (Illand and Brown,1998). If doxorubicin sensitivity could be restored by chromosome 3 transfer this would suggest that MLH1 has a direct role to play in the development of resistance to doxorubicin. A2780/CP70 cells containing chromosome 3 were used as single *MLH1* gene transfer had proved technically difficult to achieve. The disadvantages of whole chromosome transfer include the potential introduction of other genes involved in drug resistance. Topoisomerase II is known to be a target protein for doxorubicin and to be associated with doxorubicin resistance (Epstein, 1990). The gene for topoisomerase II β is located on chromosome 3. To ensure that any observed restoration of doxorubicin sensitivity was due to MLH1 expression topoisomerase II enzyme activity and expressed protein levels were compared between cell lines.

3.2 Aims of this chapter:

- To confirm the derivation of the doxorubicin resistant derivative MCF7AD from MCF7.

- To determine if loss of MLH1 plays a direct role in sensitivity to doxorubicin by comparing doxorubicin sensitivities between the cell lines A2780, A2780/CP70 and A2780/CP70chr3.

- To assess the impact on topoisomerase II activity of chromosome 3 transfer into A2780/CP70.

3.2. DNA fingerprinting of MCF7 and MCF7AD human breast cancer cell lines.

The genome of each individual has a unique set of repeated DNA sequences. These can be identified in a wide range of species by Southern analysis of *Hinf I* restriction enzyme digests of genomic DNA using the DNA probe 33.15 discovered by Alec Jeffreys. Visualisation of the unique pattern of sequences provides a DNA fingerprint (Jeffreys et al. 1985 a and b). The DNA fingerprint pattern of MCF7AD was compared to that obtained from parental MCF7 according to protocols obtained from the European Collection of Animal Cell Cultures and described in **Materials and Methods.** Southern analysis of *Hinf1* restriction enzyme digests of genomic DNA using the multi locus DNA probe 33.15 was used as this combination gives the most reliable differentiation of human minisatellite polymorphisms (Jeffreys et al. 1985b; Stacey et al. 1991).

Figure 3. shows that the MCF7AD cell line within the laboratory was not derived from parental MCF7. The Southern blot of the *hinf1* digest clearly demonstrating different bands in each cell line as indicated by the arrows. This implies that either during or prior to the time that MCF7AD was introduced into the laboratory the line was either mixed or cross-contaminated with a faster growing cell line. Investigation of this cell line was not pursued further.

A2780AD has been previously authenticated (McLaughlin PhD Thesis)

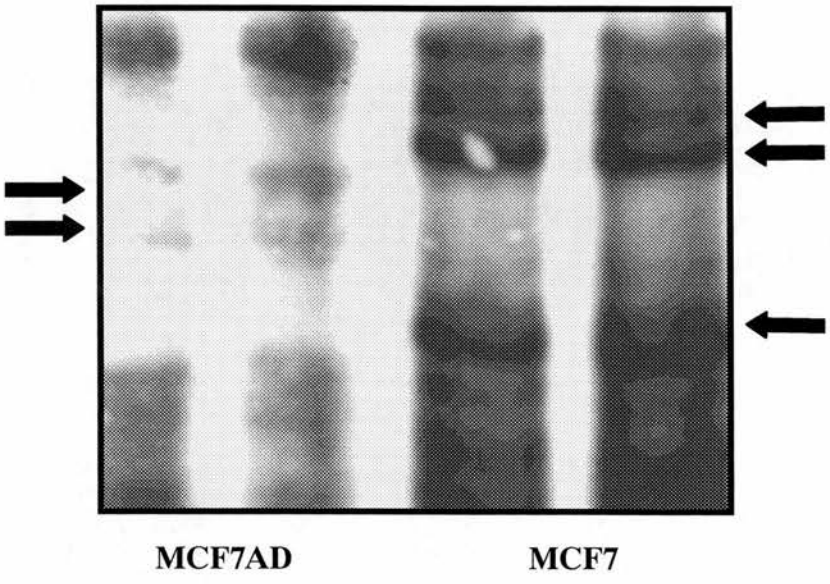

Figure 3. DNA fingerprinting of MCF7 and MCFAD using *hinf1* digest.

Genomic DNA was isolated from 10^7 MCF7 and MCF7AD cells. The extracted DNA was digested to completion using the restriction endonuclease *hinf1*. After electrophoresis the DNA fragments were southern blotted and probed using the multilocus probe 33.15. The digest patterns detected differ between the 2 cell lines suggesting that the MCF7AD cell line is not derived from the parental MCF7 line.

3.4. Doxorubicin resistance in the chromosome 3 transferred derivative of A2780/CP70

Doxorubicin sensitivity was compared between parental A2780, A2780/Cp70, the chromosome 3 transferred derivative A2780CP70/ch3 and the chromosome 2 transferred derivative A2780/Cp70/ch2. A2780/Cp70/ch2 was used as a control for the chromosome transfer process. A2780/CP70 derivatives which had undergone microcell mediated transfer of chromosome 3 and of chromosome 2 were kindly donated by M Illand (CRC Department of Medical Oncology, BOC, Glasgow). Doxorubicin resistance was assessed by performing colony forming assay over a range of doxorubicin concentrations. At least 15 plates of 10^3 cells were exposed to doxorubicin at each dose point. Mean survival fractions were calculated for each dose point by dividing the number of colonies formed following exposure to doxorubicin by the numbers of colonies formed by the controls (cells which were not exposed to doxorubicin). Doxorubicin dose/response curves are shown in figure 4, error bars represent +/- one standard error of the mean. Mean IC50 values and MLH1 status are shown in table 4.

Chromosome 3 transfer into A2780/CP70 resulted in restoration of doxorubicin sensitivity to parental A2780 levels, as shown in figure 4. A2780/CP70 and A2780/CP70ch3 are significantly more sensitive to doxorubicin than A2780/Cp70 and A2780/CP70ch2 as determined by two-tailed Students t-test ($p<0.01$) No significant difference in sensitivity was observed between the values obtained for A2780/CP70 and the chromosome 2 transferred derivative.

Doxorubicin resistance has previously been associated with alteration in DNA topoisomerase levels (de Jong et al. 1990). Since topoisomerase IIβ is located on

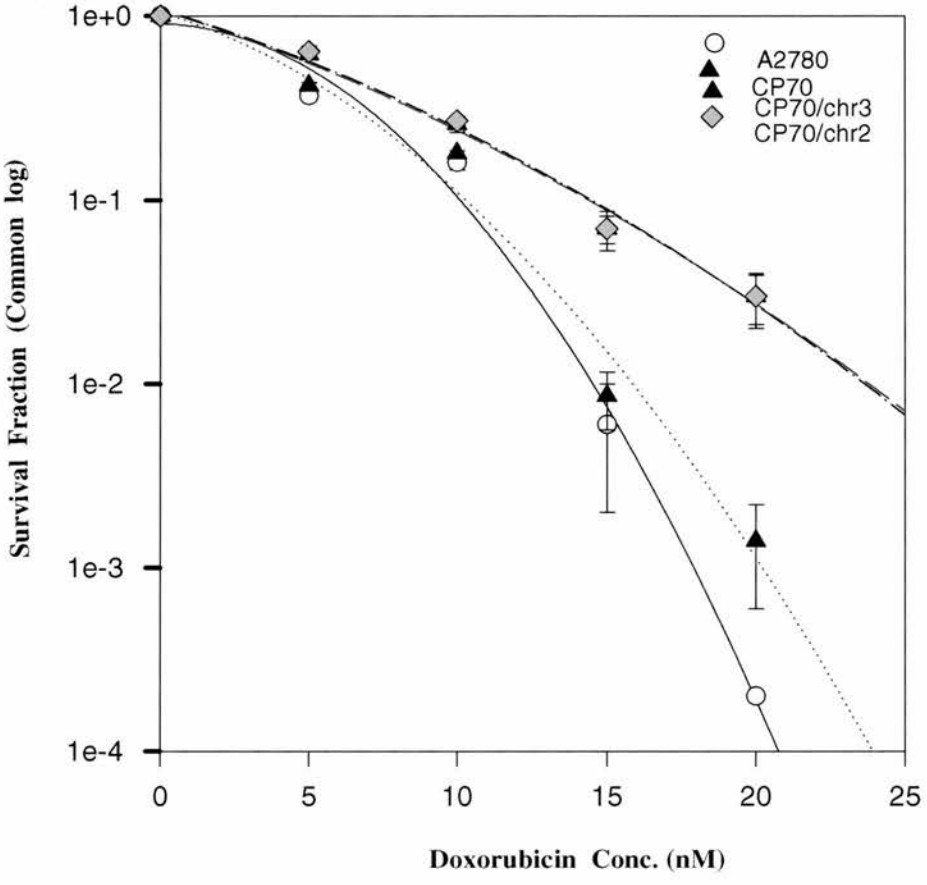

Figure 4. Doxorubicin dose/response curves for A2780, A2780/CP70/chromosome 3, A2780/CP70/chromosome 2 and paternal A2780.

Doxorubicin dose/response curves derived using clonogenic assay. At least 15 repeat colony forming assays of 10^3 cells were plated out (a total of at least 1.5 x10^4 cells) and exposed to doxorubicin at each dose point over 3 separate experiments. Mean survival fraction calculated by dividing mean number of surviving colonies on each plate by the mean number of colonies formed on the no drug control plates for each dose point. Error bars represent +/- one standard error of the mean

Introduction of chromosome 3 into A2780/CP70 restores doxorubicin sensitivity.

Cell line	Mean IC 50 (nM)	SE mean	RF	MLH1 status
A2780	4	0.35		+
A2780/CP70	6.4	0.52	1.6	-
A2780/CP70 + chromosome 3	4.2	0.22	1.05	+
A2780/CP70 +chromosome 2	6.8	0.3	1.7	-

Table 4. Relative doxorubicin resistance of A2780/CP70, A2780/CP70/chromosome 3, A2780/CP70/chromosome 2 and parental A2780.

IC 50 values were derived from dose response curves obtained from 3 separate experiments in which a total of at least 15 repeat colony forming assays (1.5×10^4 cells) were performed at each dose point.

Chromosome 3 transfer into A2780/CP70 restores doxorubicin sensitivity.

chromosome 3 it could be argued that the restoration of doxorubicin sensitivity observed is due to altered topoisomerase II activity. Topoiomerase II activity was, therefore, compared between the cell lines. The topoisomerase II activity of crude nuclear extract was measured using an activity assay kit obtained from TopoGen Inc., Ohio (described in **Materials and Methods).** Activity of topoisomerase II is observed through the enzymes ability to decatenate kinetoplast DNA (kDNA) as shown overleaf. Kinetoplast DNA is mitochondrial DNA from Crithodia fasciculata and consists of networks of 2.5kb rings catenated together. Topoisomerase II can monomerise these rings via its catalytic, ATP-dependent cycle of cleavage, strand passage and religation. The amount of topoisomerase II within a cell will therefore determine how much of the kDNA is decatanated. This is shown in figure 5.

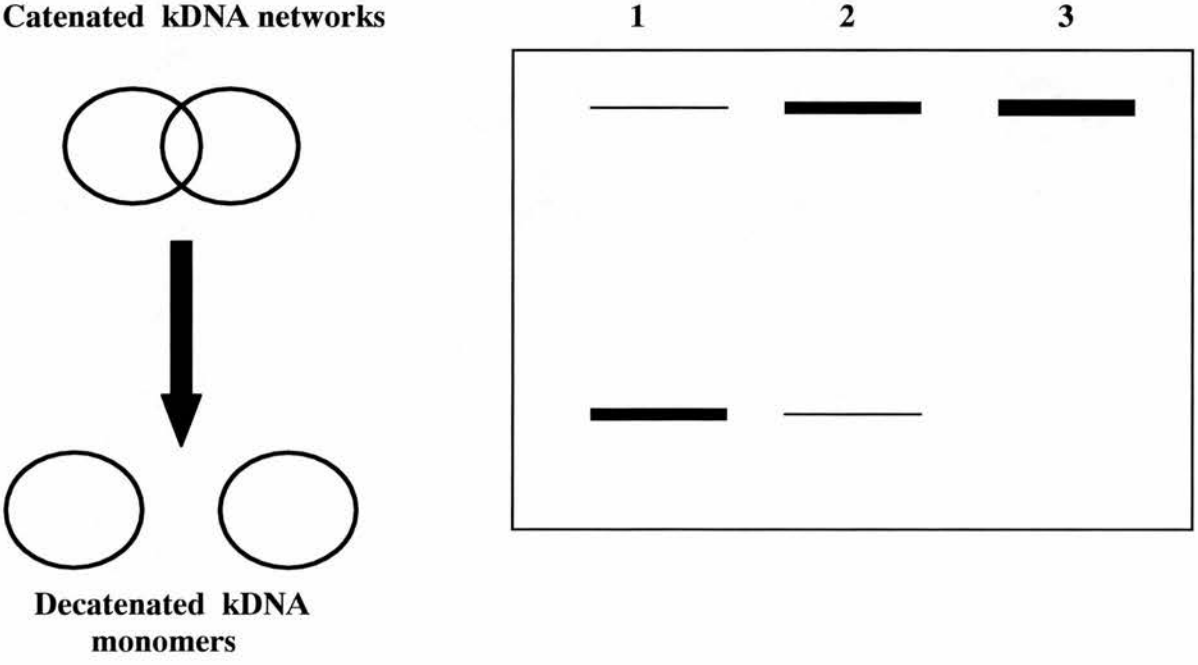

Figure 5. Schematic diagram of Topoisomerase II activity Assay

When reactions are resolved on an agarose gel, the monomeric circles of DNA migrate much faster than catenated circles and therefore move further within the gel. Any remaining networks of kDNA stay in the wells as they are not able to migrate. Three situations may arise: Lane **1** indicates the result if an extract containing high topoisomerase activity is used, almost all the kDNA will be monomerised. Low activity is shown in lane **2** where most of the kDNA remains catenated. An extract containing no activity would give lane **3**. Activity was expressed as the protein concentration (of nuclear extract) at which almost all kDNA was monomerised (as in lane **1**) and assessed over 3 separate experiments. Results are summarised in table 5 and figure 6. There was no difference in topoisomerase II activity between A2780/CP70, A2780/CP70chr2 and A2780/CP70chr3.

One must be careful not to over interpret results obtained from tumour cell lines. They represent a monoclonal population of cells with a common genetic background. This is not the case in a tumour, which may contain many different tumour cell clones. Furthermore, tumours derived from different tissues will have different genetic backgrounds and may engage different pathways leading to apoptosis following drug exposure. In addition, the contribution of the various mechanisms of doxorubicin resistance will differ between tumours, for example the level of expression of p-glycoprotein.

A2780

Protein conc. ug/ul	Topoisomerase activity
4.4	+ + +
2.2	+ + +
1.1	+ + -
0.55	- - -

A2780/CP70

Protein conc. ug/ul	Topoisomerase activity
3.9	+ + +
2.6	+ + -
1.3	- - -
0.7	- - -

A2780/CP70/ch3

Protein conc. ug/ul	Topoisomerase activity
6.4	+ + +
3.2	+ + +
1.6	+ - -
0.8	- - -

A2780/CP70/ch2

Protein conc. ug/ul	Topoisomerase activity
3.75	+ + +
2.5	+ + +
1.25	- - -
0.7	- - -

Table 5. Comparison of topoisomerase activity between A2780, A2780/CP70, and A2780/Cp70/ch3

Activity was expressed as the protein concentration (of nuclear extract) at which almost all kDNA was monomerised. Summary of 3 separate experiments

\+ = kDNA monomerised

\- = kDNA catenated

No change in topoisomerase II activity between the A2780/CP70 derived cell lines

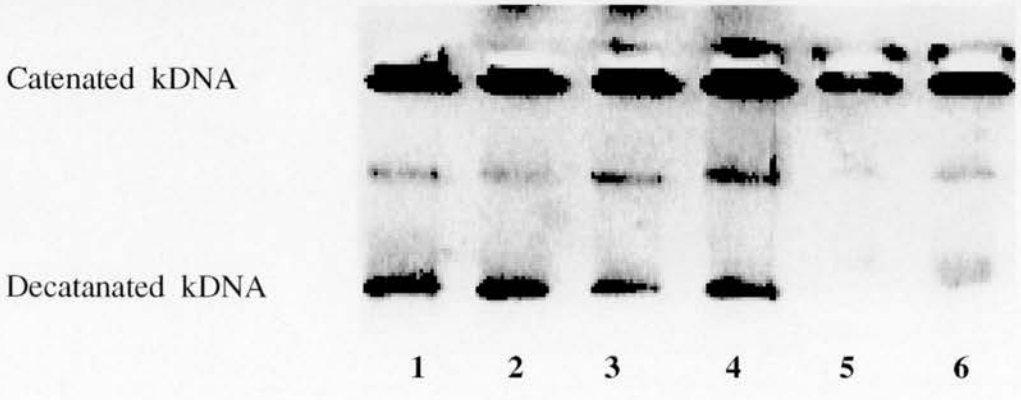

Figure 6. Topoisomerase activity in nuclear extracts of A2780/CP70

Lanes 1 to 6 represent topoisomerase II activity at 6.6 5.2 3.9 2.6 1.3 and 0.7 ug/ul nuclear extract

3.5. Conclusions

- The breast cell line MCF7AD was not genetically related to MCF7 and therefore not derived from it. Evidence for loss of MMR in acquired doxorubicin resistance rests with the solitary doxorubicin derived ovarian cell line A2780AD.

- Chromosome 3 transfer into the ovarian cell line A2780/CP70 results in re-expression of MLH1 and restoration of doxorubicin sensitivity. This appears to be independent of the chromosome transfer process since the chromosome 2 transformant did not show any difference in sensitivity from A2780/CP70. The observation that restoration of MMR activity in a MLH1 deficient cell line restored drug sensitivity supports a direct role for *MLH1* in doxorubicin induced cell death and argues against doxorubicin resistance arising due to increased mutation at drug resistance genes.

- Topoisomerase II activity is unaffected by chromosome 3 transfer into A2780/CP70 and is therefore unlikely to be responsible for restoration of sensitivity to doxorubicin in this cell line

CHAPTER 4

CHARACTERISATION OF DOXORUBICIN RESISTANCE IN MISMATCH REPAIR DEFICIENT STRAINS OF *S. CEREVISIAE*.

4.1. Introduction

The results obtained in **Chapter 3** support a direct role for loss of mismatch repair in the development of resistance to doxorubicin. They were, however, obtained from only one tumour cell line (A2780) and provide information on only one component of the MMR system, MLH1. The yeast *S. cerevisiae* has been extensively used as a model to investigate repair of mismatched DNA formed during replication and recombination (Hunter and Borts, 1997; Kolodner, 1996). Using a yeast model has several advantages:

1. The complete genome sequence of *S. cerevisiae* has been known since April 1996 (Goffeau et al. 1996). The small size of the genome (12 Mb) and the ease with which it can be manipulated using recombinant DNA techniques makes it possible to generate specific deletion mutants. By disabling a single gene the function of one and only one protein is eliminated and can be studied (Oliver, 1998).
2. Many of the genes that are frequently altered in tumours have structural or functional homologues in yeast. There is significant conservation of function between yeast and human components of the DNA mismatch repair and cell division pathways with a large body of published research. In *S. cerevisiae* 3 of the 6 MutS homologues (MSH2, 3 and 6) and two of the 4 MutL homologues (PMS1 and MLH1) have been shown to play a role in MMR (Kolodner and Marsischky, 1999; Prolla et al. 1994). This makes it a suitable model for studying MMR.

3. Screening drugs against yeast mutant panels is an established technique. Quantitative comparison can be made between mutant isogenic strains of *S. cerevisiae* to evaluate the effect of a given mutation on drug sensitivity (Hartwell et al. 1997).
4. Results from colony forming assays using *S. cerevisiae* are available within 6 days (see **Materials and Methods**) making this a rapid and convenient technique.

There are limitations to using a yeast model and the results obtained in yeast must be interpreted with caution. Results obtained from yeast are only relevant if analogous defects occur in human tumours. Drug sensitivities identified in yeast may not necessarily be extrapolated to tumours. Mammalian cells may have different damage response pathways not present in yeast (for example oncogene mediated apoptosis) or mechanisms of resistance such as the expression levels of p-glycoprotein (discussed in **Introduction**).

Isogenic haploid strains of *S. cerevisiae* which had disruption of specific MMR genes were examined for sensitivity to doxorubicin. These strains were obtained from Dr Rhona Borts, a description is provided in **Materials and Methods**. The mutants were used to determine if loss of expression of the known MMR proteins has a direct effect on sensitivity to doxorubicin. Copies of the *MLH1* gene were introduced into the *mlh1* mutant strain resulting in re-expression of MLH1. To determine if loss of MLH1 plays a direct role in sensitivity to doxorubicin, drug sensitivities were compared between the sc*mlh1* mutant and the sc*mlh1* mutant strain containing copies of the *MLH1* gene.

4.2. Aims of this chapter:

- To assess the effect on doxorubicin sensitivity, in a *S. cerevisiae* model of disrupting the genes for *MLH1, MSH2, MSH3* and *MSH6*.

- Using the *Mlh1* disrupted isogenic strain of *S. cerevisiae* to assess the impact on doxorubicin sensitivity of re-introducing a wild type *MLH1* gene.

4.3. Doxorubicin resistance in mismatch repair deficient strains of *S. cerevisiae*

Isogenic, haploid, strains of *S. cerevisiae* with disruption of the genes encoding for the mismatch repair proteins MLH1, MSH2, MSH3 and MSH6 were exposed to a range of doxorubicin concentrations for 24 hours. Doxorubicin dose/response curves were derived using colony forming assay. 5 repeat assays for each strain at each dose level were counted. The plating efficiency was calculated for each strain by dividing the number of colonies formed by the controls (which had no exposure to doxorubicin) by 400 (the number of cells plated out). For each strain the mean survival fraction was determined by comparing the number of colonies formed in replicates of doxorubicin treated cells compared to untreated controls. Error bars represent +/- one standard error of the mean. Figure 7. demonstrates that disruption of the mismatch repair genes *Mlh1, Msh2, Msh3* and *Msh6* results in increased resistance to doxorubicin. In addition results in table 6. demonstrate a significant difference in IC50 and IC90 values between the disrupted strains and parental *S. cerevisiae* ($p<0.05$). A two tailed Students t-test was used to compare the IC50 and IC90 values between mutant and wild type strains. These results are consistent with results obtained using other DNA damaging drugs cisplatin and carboplatin (Durant et al. 1999).

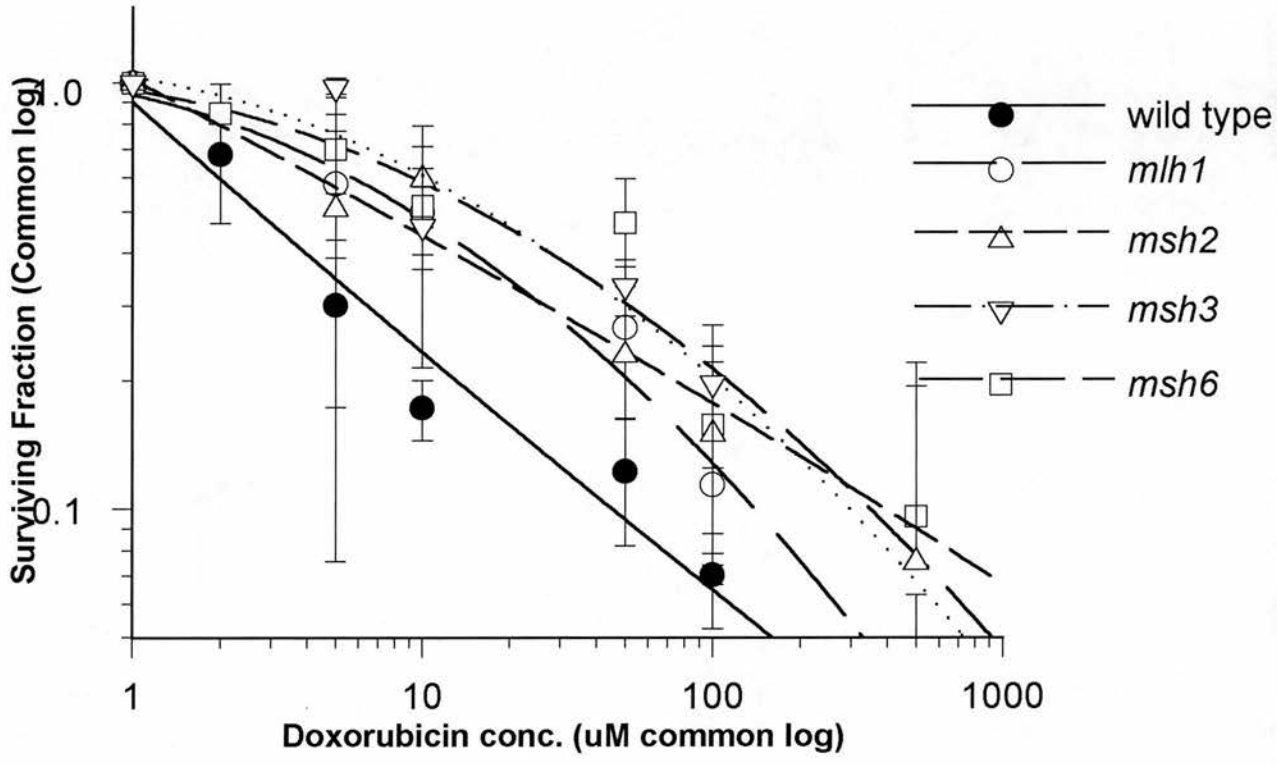

Figure 7. Doxorubicin dose/response curves for wild type and MMR mutant strains of *S. cerevisiae*

Doxorubicin dose response curves derived using colony forming assay.
Mean survival fraction calculated by dividing mean value of at least 5 repeat assays (a total of at least 2000 cells)at each drug concentration point by that for no drug control.
Curves through data points represent second order linear regression.
Error bars represent +/- one standard error of the mean
Mlh1, msh2, msh3 and *msh6* mutant strains exhibit less sensitivity to doxorubicin than wild type.

Genotype	IC50 (iM)	IC90 (iM)	RF	Plating efficiency (%)	P
Wild type	3.2 ± 0.4	70		42.5	
Mlh1	9.3 ± 0.8	150	2.9	31.2	0.001
Msh2	15 ±2.9	370	4.7	43	0.05
Msh3	4.2 ±0.2	310	1.3	91	0.03
Msh6	9.6 ±0.4	420	6	80	0.001
Mlh1 + vector	6.2 ±0.6	120	1.9	36.2	0.02
Mlh1 + *pMLH1*	3.2 ±0.5	30	1	55.8	0.9

Table 6. Sensitivities of MMR mutant strains of *S. cerevisiae* to doxorubicin.

Doxorubicin toxicity was measured by exposing exponentially growing cells in liquid culture for 24 hours to doxorubicin, plating out 400 cells onto YPD medium and allowing colony formation. Mean survival fraction calculated by dividing mean value of at least 5 repeat assays at each drug concentration by that for no drug control.

Mean IC 50 and IC 90 values (+/- 1 S.E.M.) were obtained from doxorubicin dose/response curves derived using colony forming assays.

The resistance factor (RF) is the resistance of a given strain at IC50 relative to the wild-type.

P, p-value for sensitivity of mutant compared to wild-type strain assessed by two tailed Students *t*-test at IC50.

Mlh1, Msh2, Msh3, and Msh6 mutant strains are less sensitive to doxorubicin than wild type.

Introduction of MLH1 to the Mlh1 mutant strain restores doxorubicin sensitivity to that of wild type.

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4.5. Doxorubicin resistance in *Mlh1* transfected strains of *S. cerevisiae.*

Using a high copy vector, s*cMLH1* was transfected into the *Mlh1* mutant strain of *S. cerevisiae* (Durant et al. 1999). Sensitivity to doxorubicin was compared between wild-type *S. cerevisiae*, *Mlh1* mutant and the *Mlh1* mutant strain containing copies of the *scMLH1* gene (*mlh1* + *pMLH1*). *Mlh1* mutant containing the vector alone was used as a control (*Mlh1* + vector). All transfected strains were obtained from S. Durant, PhD student within the group.

As detailed above, doxorubicin dose/response curves were derived using colony forming assay. 5 repeats for each strain at each dose level were counted and mean survival fractions determined by comparing treated and no drug controls. Figure 8 confirms that *Mlh1* mutant is more resistant to doxorubicin than wild-type *S. cerevisiae*. More importantly it shows that transfection with *Mlh1* results in restoration of doxorubicin sensitivity to wild type levels. Mean IC50 (+/- 1 S.E.M.) values are shown in Table 6. Comparison of IC50 values between *Mlh1* mutant and the *Mlh1* + vector strain using a two tailed Students t-test shows a significant increase in sensitivity ($p<0.05$) to doxorubicin. This implies that the transfection process alone, or clonal variation, may be effecting drug sensitivity. Transformation of the *MLH1* gene into the *Mlh1* mutant strain resulted in restoration of doxorubicin sensitivity to wild-type levels. Furthermore, there was a significant difference between IC50 values obtained from *Mlh1*+ vector compared to the Mlh1 + *pMLH1* ($p<0.05$) suggesting that the increased sensitivity was not entirely due to the transfection process. This study provides further confirmation of a direct role for MLH1 in doxorubicin induced cell death.

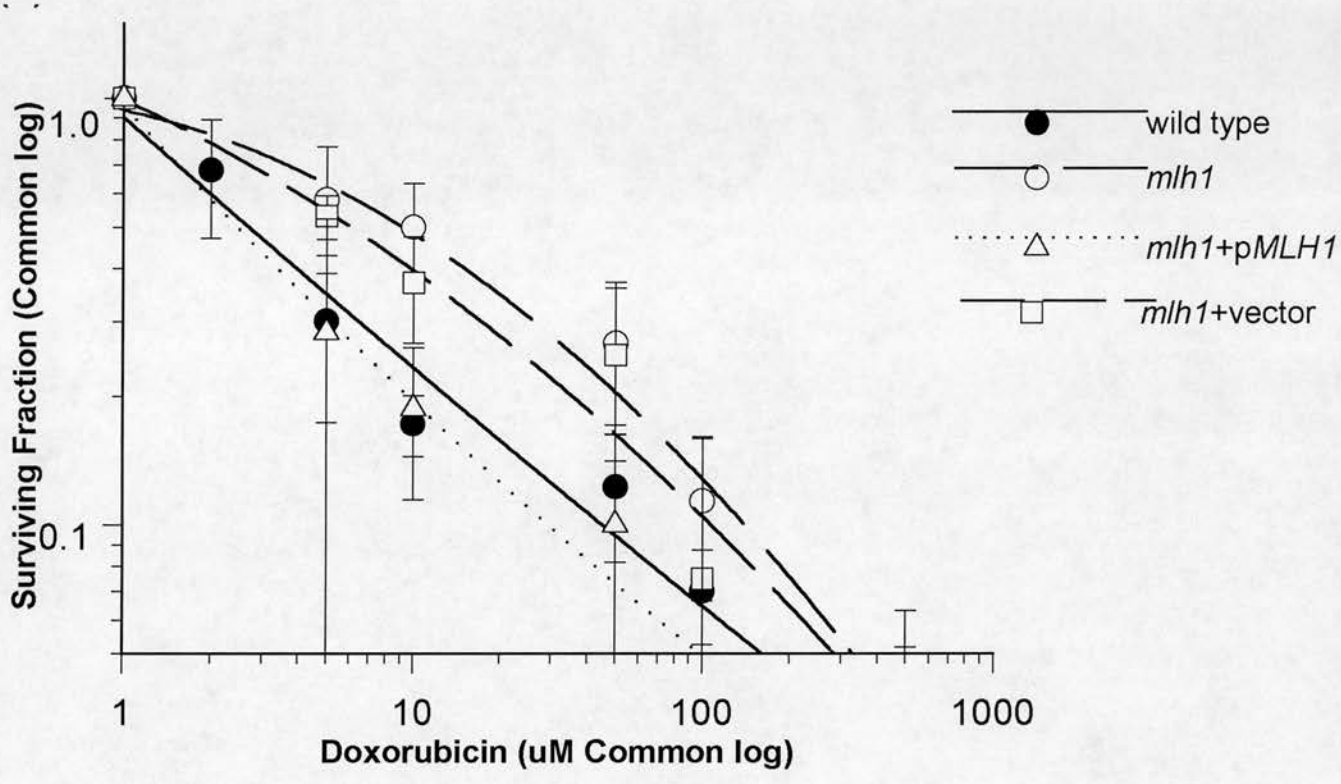


Figure 8. Doxorubicin dose/response curves for wild type and *mlh1* mutant strains of *S. cerevisiae*.

Doxorubicin dose response curves derived using colony forming assay.
Mean survival fraction calculated by dividing mean value of at least 5 repeat assays (a total of at least 2000 cells) at each drug concentration point by that for no drug control.
Curves through data points represent second order linear regression.
Error bars represent +/- one standard error of the mean.
Introduction of *MLH1* increases sensitivity to doxorubicin in the *mlh1* mutant strain.

4.6. Conclusions

- Genetic inactivation of the MMR genes *MLH1*, *MSH2*, *MSH3* and *MSH6* in isogenic strains of *S. cerevisiae* leads to decreased sensitivity to doxorubicin. These results are in keeping with results obtained using other anticancer drugs cisplatin and carboplatin, thus supporting a role for loss of MMR activity in drug resistance.

- Sensitivity to doxorubicin is increased in *Mlh1* mutant strains by the reintroduction of the *MLH1* gene providing further confirmation of a direct role for MLH1 in doxorubicin induced cell death.

CHAPTER 5

CHARACTERISATION OF MISMATCH REPAIR IN DOXORUBICIN RESISTANT OVARIAN AND BREAST CARCINOMA CELL LINES

5.1. Introduction

The data presented in the preceding two chapters suggests that loss of MMR results in resistance to doxorubicin. The questions remain, however, does exposure to doxorubicin induce loss of MMR and how frequently does this occur?

Only one cell line has been shown to have lost MMR activity and MLH1 expression as a result of serial exposure to doxorubicin (Drummond et al. 1996). A2780AD was derived from the chemo-naïve, human ovarian carcinoma cell line A2780 by repeated exposure to doxorubicin (Rogan et al. 1984). In order to determine the frequency of loss of MMR activity as a result of doxorubicin exposure two new sets of doxorubicin resistant derivatives were developed. Cell lines were derived from clonal populations of A2780 and the breast carcinoma cell line MCF7.

5.2. Aims of this chapter:

- To independently derive 20 doxorubicin resistant cell lines by serial exposure of A2780 and MCF7 parental cell lines to increasing concentrations of doxorubicin.

- To determine the frequency of loss of MMR activity in the doxorubicin resistant cell lines.

5.3. Development of doxorubicin resistant A2780 and MCF7 cell lines against increasing concentrations of doxorubicin

Two sets of doxorubicin resistant cell lines were developed by serial exposure of A2780 and MCF7 cell lines to increasing concentrations of doxorubicin. To reduce the influence of genetic differences between cells on the resistant phenotype clonal populations were used. The concentration for the initial selection was based on a surviving fraction of 30%, derived from initial parental dose response curves. This was 10nM in both cases. Further selections against increasing doses of doxorubicin were performed as detailed in **Materials and Methods**. All drug selections were run in parallel. The derived cell lines were maintained in doxorubicin free medium, with frozen stocks suspended in 10% DMSO.

5.4. Measurement of the level of resistance to doxorubicin in A2780 and MCF7 derived cell lines.

Correlation of loss of MMR activity with selection for drug resistance first requires proof of the development of stable resistance to doxorubicin. To this end doxorubicin sensitivity was compared between the derived cell lines A1 to A10 and M1 to M10 and their respective parental cell lines A2780 and MCF7.

5.4.1. Clonogenic Assay: Single dose exposure

One method of determining the level of doxorubicin resistance of the derived A2780 and MCF7 cell lines is to assay cell survival after exposure to doxorubicin. This assay depends on the demonstration of the reproductive integrity of the surviving cells as evidenced by clonogenicity. Five plates of 10^3 cells per cell line were exposed to a single dose of doxorubicin. The doses of doxorubicin selected were 50nM for MCF7 derived cell lines and 15nM for A2780 derived cell lines (see **Materials and Methods**). Control plates of cells, which were not exposed to doxorubicin, were set up for each cell line. This experiment was repeated 3 times resulting in a total of 1.5×10^4 cells from each cell line being exposed to doxorubicin. In an attempt to demonstrate the stability of any doxorubicin resistance observed, the cells were maintained in doxorubicin free medium between each experiment, thus removing them from drug induced selection pressure.

The cumulative data on all clonogenic survival assays is presented in figures 9, 10 and table 7. Figures 9 and 10 display in diagrammatic form the fraction of cells surviving compared to untreated cells. Each column represents the mean of at least 15 plates (a total of 1.5×10^4 cells) per cell line. A2780 and MCF7 show the smallest surviving fractions (SF) indicating that they are more sensitive to doxorubicin than their derived cell lines $p < 0.001$, as assessed by a two-tailed Students t-test.

The mean surviving fractions ($\pm$ 1 standard error of the mean) for each cell line compared to their respective parental cell line are shown in Table 7 confirming the data in figures 9 and 10. The plating efficiency was calculated by dividing the

number of colonies formed by the controls (which had no exposure to doxorubicin) by 1000 (the number of cells plated out). The mean plating efficiency ranges between 25 to 75% for A2780 and 32 to 68% for MCF7 derived cell lines. This suggests that poor plating efficiency did not influence our results in any one cell line.

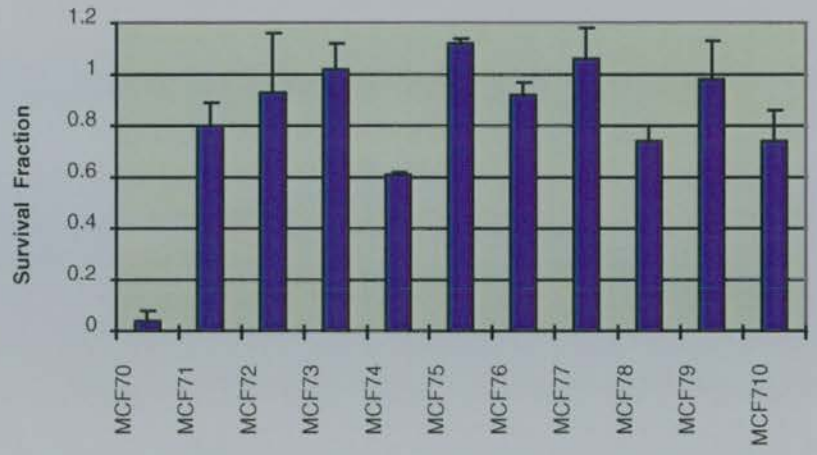


Figure 9. Clonogenic determination of doxorubicin resistance in MCF7 and derived cell lines

For each cell line the mean survival fraction was determined by comparing the number of colonies formed in replicates of doxorubicin treated cells (50nM over 24 hours compared to non-treated control. A total of 1.5 x 10^4 cells from each cell line were treated with doxorubicin. Error bars represent one standard error of the mean.
All derived cell lines are less sensitive to doxorubicin than parental MCF7.

Figure 10. Clonogenic determination of doxorubicin resistance in A2780 and derived cell lines

For each cell line the mean survival fraction was determined by comparing the number of colonies formed in replicates of doxorubicin treated cells (15nM over 24 hours) compared to non-treated controls. A total of 1.5 x 10^4 cells from each cell line were treated with doxorubicin. Error bars represent one standard error of the mean.
All derived cell lines are less sensitive to doxorubicin than parental A2780

Cell line	Mean surviving fraction (± 1 SEM)	Mean plating efficiency
A2780	0.14 ± 0.02	65
A1	1.02 ± 0.09	25
A2	0.99 ± 0.02	46
A3	0.99 ± 0.03	64
A4	1.07 ± 0.06	41
A5	1.04 ± 0.09	56
A6	0.83 ± 0.03	75
A7	0.9 ± 0.06	66
A8	0.98 ± 0.014	58
A9	0.89 ± 0.01	67
A10	0.87 ± 0.02	49

Cell line	Mean surviving fraction (± 1 SEM)	Mean plating efficiency
MCF7	0.04 ± 0.002	61
M1	0.8 ± 0.06	42.6
M2	0.93 ± 0.16	31.6
M3	1.02 ± 0.07	65.9
M4	0.61 ± 0.01	33.6
M5	1.0 ± 0.01	48
M6	0.92 ± 0.04	52.5
M7	1.06 ± 0.09	41.3
M8	0.74 ± 0.06	45.2
M9	0.98 ± 0.1	34.9
M10	0.74 ± 0.09	68.1

Table 7. Relative doxorubicin resistance of derived cell lines compared to parental cell lines.

Colony forming assays were used to determine the cell lines sensitivities to doxorubicin. 10^3 cells were plated out for each assay and exposed to 50nM doxorubicin (MCF7) and 15nM doxorubicin (A2780) for 24 hours and then grown in doxorubicin free supplemented RPMI medium. Results displayed are the mean of at least 15 repeat assays i.e. a total of at least 15,000 cells plated out for each cell line and exposed to doxorubicin, over 3 separate experiments. The colonies were counted after 10-14 days for both (no drug) control and drug exposed cells. The mean survival fraction was calculated by dividing the mean number of colonies formed by the drug exposed cells by the mean number of cells formed by the controls. The plating efficiency was calculated by dividing the number of colonies formed by the controls by the number of cells plated out.

5.4.2. Clonogenic Assay: Dose/response curves

The results presented above indicate that the derived cell lines are more resistant to doxorubicin than the parental cell lines. This data, however, represents only 1 dose point per cell line. To further compare differences in doxorubicin sensitivity two of the derived cell lines from MCF7 and A2780 were chosen at random and exposed to a wider range of doxorubicin concentrations. At least 10 plates of 10^3 cells were exposed to doxorubicin at each dose point. Mean survival fractions were calculated for each dose point and the results are shown in figures 11 and 12.

A6 and A9 were significantly more resistant than A2780 ($p < 0.001$), thus confirming that the derived lines had indeed acquired a resistant phenotype, see figure 12. M3 and M6 were significantly more resistant than the parental MCF7 ($p < 0.001$) shown in figure 13.

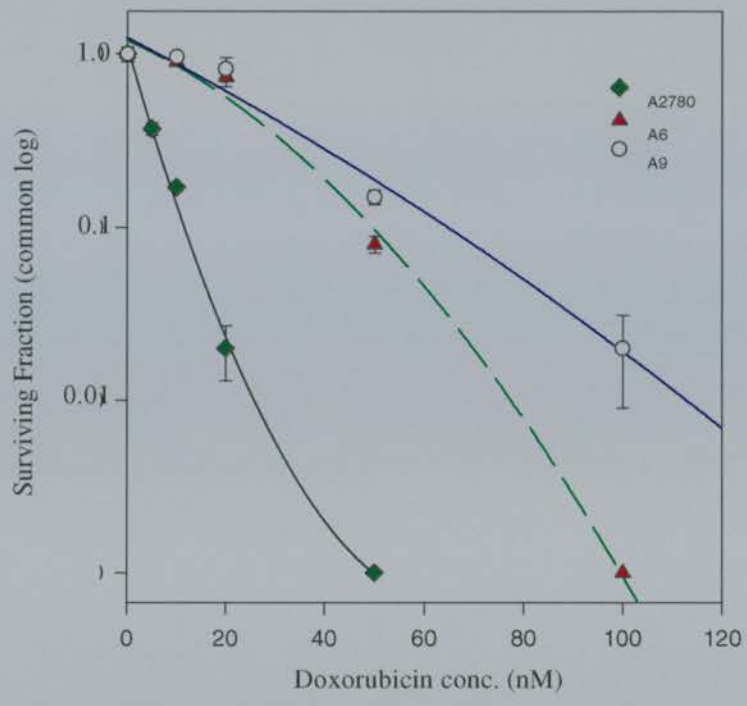


Figure 11. Doxorubicin dose/response curves for A2780 and its derived cell lines A6 and A9.

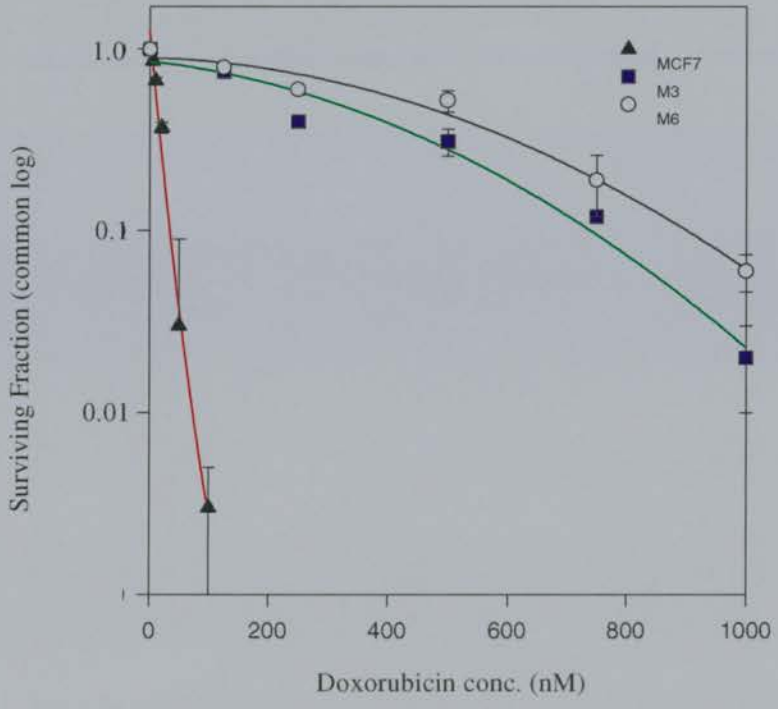


Figure 12. Doxorubicin dose/response curves for MCF7 and its derived cell lines M3 and M6.

Doxorubicin dose response curves derived using colony forming assay. At least 10 repeat colony forming assays (at least 1 x10^4 cells)were plated out and exposed to doxorubicin at each dose point. Mean survival fraction calculated by dividing mean number of surviving colonies on each plate by the mean number of colonies formed on the no drug control plates for each dose point. Error bars represent +/-one standard error of the mean.

The derived cell lines are less sensitive than parental A2780 and MCF7 to doxorubicin.

5.5. Characterisation of mismatch repair protein expression in the doxorubicin resistant derivatives of A2780 and MCF7

5.5.1. Western Immunoblot

Initially, the doxorubicin resistant cell lines were examined for expression of the mismatch repair proteins MLH1, PMS2 and MSH2. These were chosen as complete loss of expression of MLH1 and PMS2 had been demonstrated in 9 out of 10 independent cisplatin resistant derivatives of A2780 (Brown et al. 1997). Furthermore, human colon, endometrial and ovarian cancer cell lines deficient in MLH1 or MSH2 expression were less sensitive to cisplatin than sublines in which the MMR deficiency was complemented by chromosome transfer (Aebi et al. 1997; Durant et al. 1999). Western immunoblot of cell line extracts was performed using commercially available specific monoclonal antibodies to hMLH1, hPMS2 and hMSH2 (as detailed in **Material and Methods**). Protein extract from the cell lines A2780 (positive for MLH1, PMS2 and MSH2), A2780/CP70 (negative for MLH1 and PMS2) and LoVo colon cancer cell line (negative for MSH2) were used as controls (Mackean et al. 1998). Equal amounts of protein were loaded on to the gels.

The results of the hMLH1, hPMS2 and MSH2 immunoblots are shown in figures 13 to 15.

None of the cell lines appeared to have completely lost mismatch repair protein expression although given the quality of the western immunoblots it is impossible to say

if there is any evidence for a difference in the level of protein expression between the cell lines.

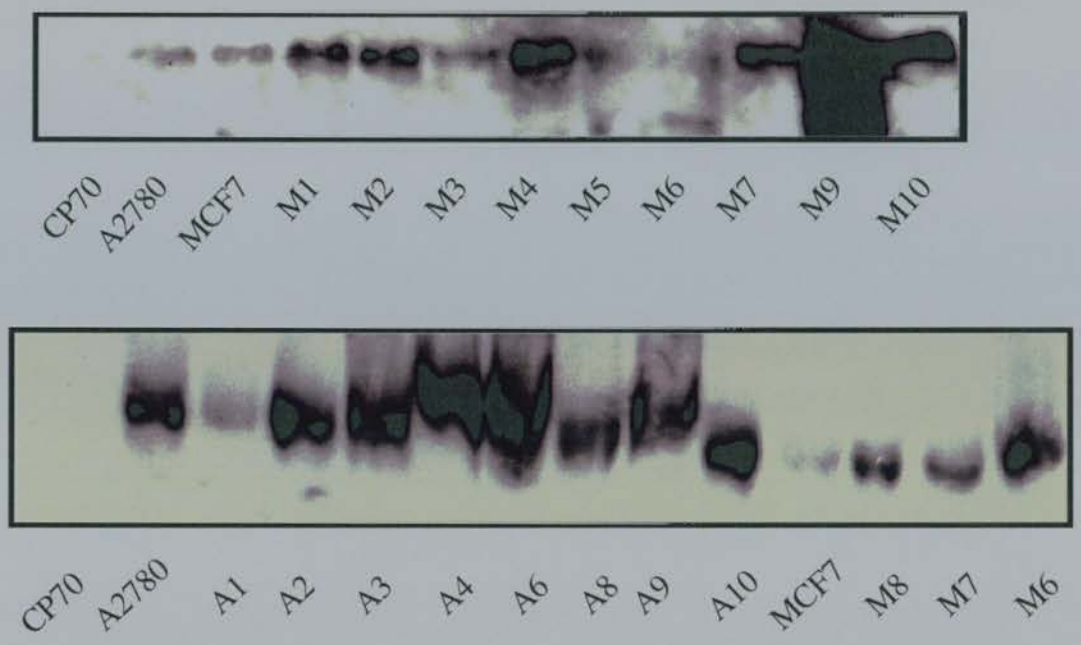


Figure 13. Basal levels of MLH1 protein in A2780, MCF7 and derived cell lines selected for resistance to doxorubicin.

Western immunoblot representing basal levels of MLH1 (Molecular Wt 80 kDa) in A2780, MCF7 and their doxorubicin resistant derivatives.
Equal amounts of protein were analysed per cell line.
No evidence for complete loss of MLH1 expression in derived cell lines.

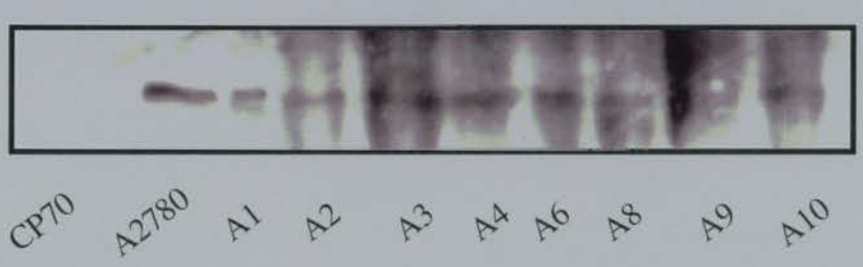


Figure 14. Basal levels of PMS2 protein in A2780 and derived cell lines selected for resistance to doxorubicin.

Western immunoblot representing basal levels of PMS2 (Molecular Wt. 95 kDa) in A2780 and its doxorubicin resistant derivatives. Equal amounts of protein were analysed per cell line.
No evidence for complete loss of PMS2 expression in derived cell lines.
MCF7 data not shown (no loss of PMS 2 expression)

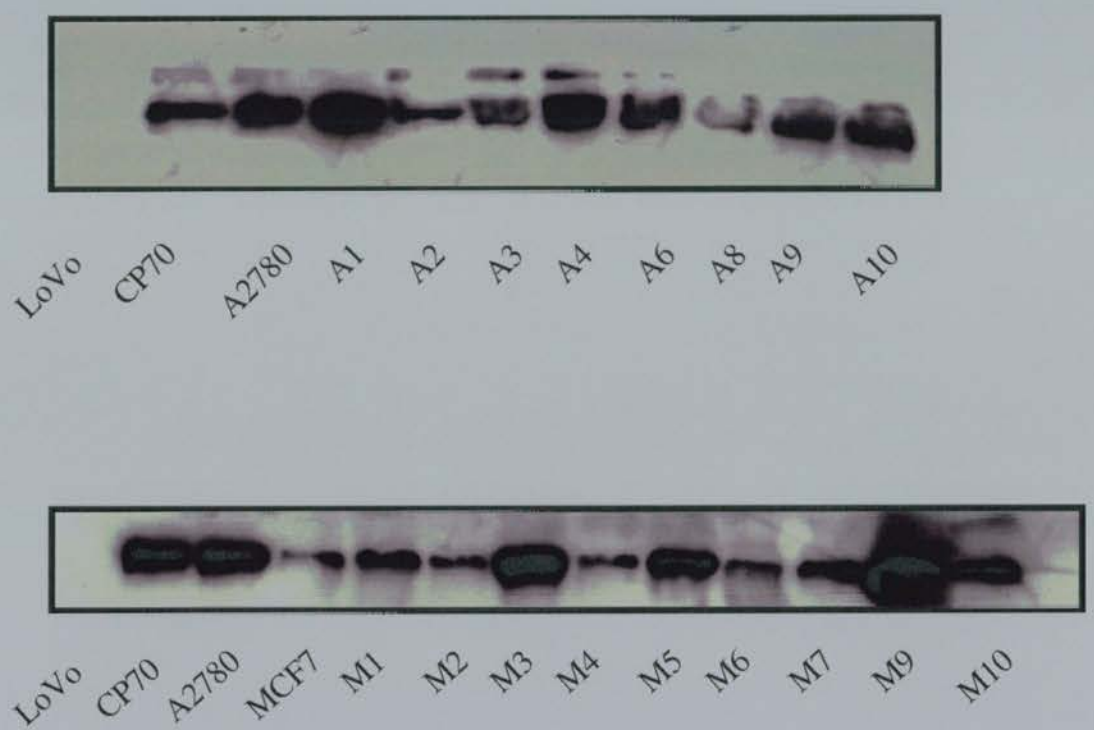


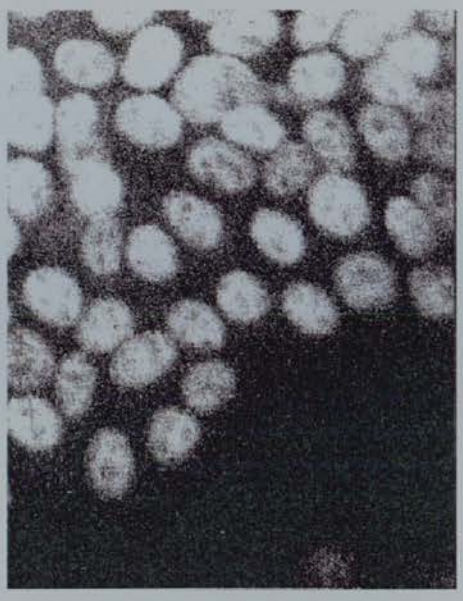
Figure 15. Basal levels of hMSH2 protein in A2780, MCF7 and derived cell lines selected for resistance to doxorubicin.

Western immunoblot representing basal levels of MSH2 (Molecular Wt. 100 kDa) in A2780, MCF7 and their doxorubicin resistant derivatives.
Equal amounts of protein were analysed per cell line.
No evidence for complete loss of MSH2 expression in derived cell lines.

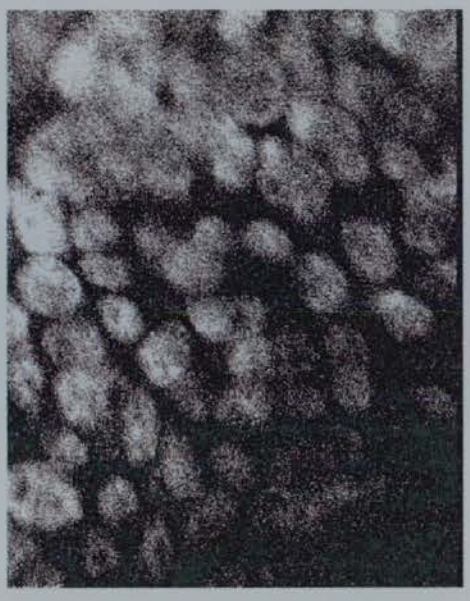
5.5.2. Immunohistochemical detection of hMLH1 protein in doxorubicin resistant derivatives of A2780 and MCF7

Western immunoblotting can demonstrate the presence and relative quantity of protein antigens within a cell. The sensitivity of this technique can be affected by such variables as the method of protein extraction or by protein denaturation. Furthermore, immunoblotting does not tell us about the subcellular location of the protein. Immunohistochemical detection of proteins in whole cells not only provides another method by which the presence or absence of a protein can be identified, but also gives information as to the subcellular localisation of the protein of interest.

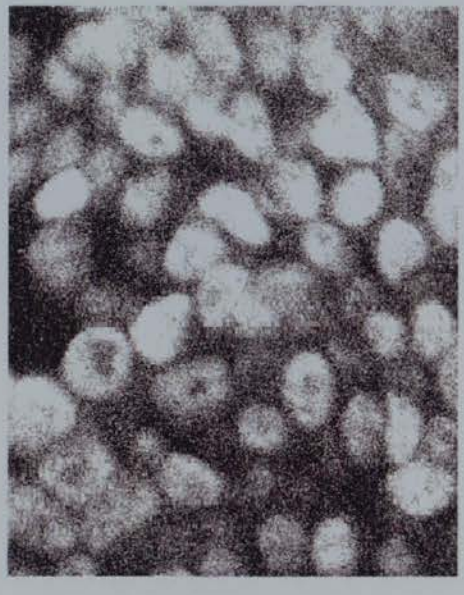
4×10^4 cells from each cell line were prepared by the method detailed in **Materials and Methods.** Analysis of the cells treated with hMLH1 antibody was performed using confocal microscopy. Propidium iodide was used as a counterstain enabling identification of the nucleus within the cell. The cell lines were examined for MLH1 expression only, as this was thought to be the most likely MMR protein to be lost based on the experience with cisplatin derived cell lines (Brown et al. 1997). Examples of the results are shown in figure 16. The signal from hMLH1 is localised to the nucleus and is present in all cells. There did not appear to be any evidence of loss of hMLH1 expression or of altered subcellular location in any of the cell lines examined.



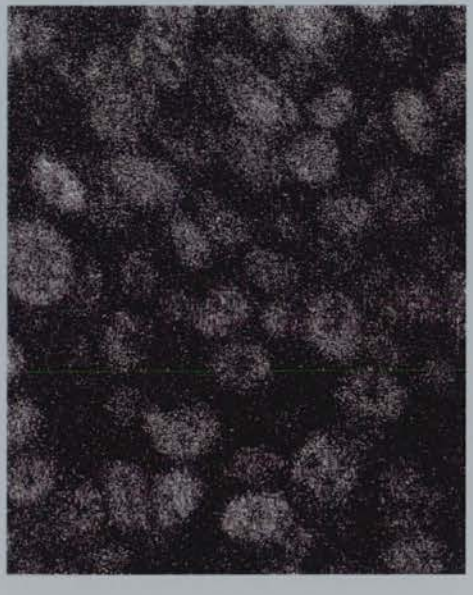
M3



M6



M7



MCF7

Figure 16. Immunohistochemical detection of basal MLH1 levels in MCF7 and its doxorubicin resistant derivative cell lines.

Confocal micrographs representing the basal expression of MLH1 protein in fixed MCF7, M3, M5 and M6 cells. No evidence of loss of MLH1. All MLH1 localised to the nucleus.

5.6. Characterisation of p53 expression in the doxorubicin resistant derivatives of A2780 and MCF7

5.6.1. Introduction

Increasingly the evidence suggests that mismatch repair is necessary to engage apoptosis in response to certain types of DNA damage. How mismatch repair is coupled to apoptosis is not known; the theories are discussed in the **Chapter 1**. At the time the data in this thesis was obtained papers linking MMR to p73 had not been published. Studies had shown that platinum resistant derivatives of A2780 have not only lost MMR activity but had lost functional p53 and ability to undergo p53-dependent apoptosis (Drummond et al. 1996; Anthoney et al. 1996). This suggested an association between loss of MMR and p53 function. However, restoration of MMR activity, although it restored drug sensitivity, does not restore p53 function (Brown et al. 1998). These data were obtained in cell-lines treated with cisplatin; there is no published data for doxorubicin. In addition, mutant *p53* has been linked with drug resistance both *in vivo* and *in vitro* to a range of chemotherapeutic agents including doxorubicin (Aas et al. 1996; Lowe et al. 1993; Lowe et al. 1994). Given the importance of p53 the doxorubicin resistant derived cell lines were examined for p53 expression.

In normal, unstressed cells, p53 protein is present in extremely low levels. This is largely due to the very short half-life (6 to 20 minutes) caused by rapid degradation of the protein via the ubiquitin system (Levine, 1992). Mutations affecting the p53 gene result in a stabilised protein with a much longer half life of up to 6 hours (Bartek et al. 1990; Finlay et al. 1988). A range of antibodies

have been raised against different epitopes of the p53 protein. These can be used to detect the presence of the mutant protein by immunohistochemistry, acting as a surrogate marker for the presence of mutant *p53*. Basal levels of wild type p53 are usually undetectable by this method. This technique does have its limitations and caution must be used in interpreting results. Firstly, several studies have shown that elevated intracellular p53 can arise from an apparently wild-type gene. This has been clearly demonstrated in the cisplatin resistant derivative of A2780, A2780/CP70 which exhibits no mutational differences in *p53* compared to the parental line (Brown et al. 1993). Secondly, immunohistochemical detection provides no information regarding the functional ability of the p53 protein. Direct sequencing of *p53* is more accurate but interpretation of results are still problematic: Mutations may or may not lead to loss of the wild type allele (Levine, 1997). P53's apoptotic activity is highly sensitive to *p53* dosage (Weinstein et al. 1997). Various mechanisms can compromise p53 function , including extragenetic mutations in the p53 pathway or interactions with other proteins, such as Mdm2 (Levine 1997). The doxorubicin resistant derived cell lines were examined for p53 expression by western immunoblot as this was the most rapid and convenient of the accepted techniques.

5.6.2. Western Immunoblot

To determine whether there was a gross difference in the levels of p53 between the doxorubicin resistant derived cell lines and the parental lines western immunoblot was performed. A commercially available specific monoclonal

antibody to p53 DO-1 was used as detailed in **Materials and Methods.** Both A2780 and MCF7 which are known to express wild-type p53, usually undetectable by this method, were used as negative controls. A2780/CP70 was used as a positive control. Equal volumes of protein were loaded and immunodetection of vinculin was used to control for equivalence of total protein on the western blot.

All of the doxorubicin resistant cell lines appear to exhibit elevated levels of p53 protein in comparison to the parental cell lines (figure 17).

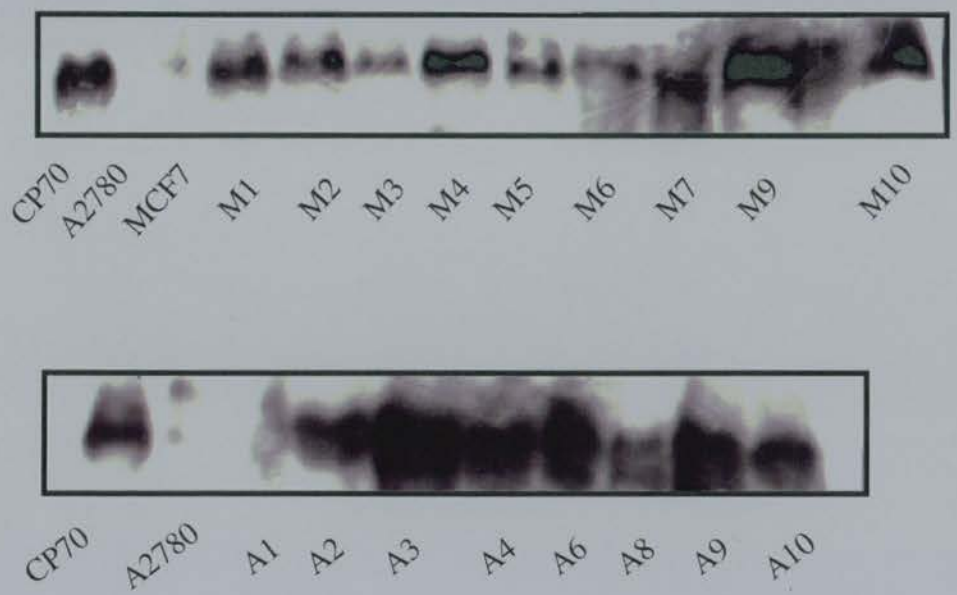


Figure 17. Basal levels of p53 protein in A2780, MCF7 and derived cell lines selected for resistance to doxorubicin.

Western immunoblot representing basal levels of p53 in A2780, MCF7 and their doxorubicin resistant derivatives. Equal amounts of protein were analysed per cell line. P53 is detectable in all the derived cell lines.

5.7. Determination of the frequency of microsatellite instability (MSI) in doxorubicin resistant derivatives of A2780 and MCF7

5.7.1. Introduction

Loss of functional DNA mismatch repair results in destabilization of the genome. This leads to high mutation rates particularly in microsatellite sequences in both non-coding and coding portions of the genome, so-called microsatellite instability (MSI). To date the detection of MSI has been used as the hallmark for loss of MMR activity. Studies have demonstrated MSI both in inherited and sporadic tumours (Aaltonen et al. 1993; Wooster et al. 1994) and in cell lines as a result of drug exposure (Anthoney et al. 1996). Having failed to demonstrate loss of MMR protein expression, the doxorubicin resistant cell lines were examined for MSI in order to determine if they had lost functional MMR activity.

MSI is defined as a change of any length due to either insertion or deletion of repeating units in a microsatellite within a tumour when compared to normal tissue (Boland et al. 1998). In this case comparison was made between the doxorubicin resistant derivatives and the parental cell lines. The analysis of microsatellite repeats was performed using the polymerase chain reaction (PCR). Certain parameters influence the success and accuracy of amplification of microsatellite DNA and are discussed below. In each cycle of amplification the DNA template and primer sequences go through steps of denaturation, annealing and elongation. The temperature at which the primers anneal to the template

strand is critical and is a function of the length and sequence context of the primers themselves. If the temperature of the annealing step is above the optimal annealing temperature for the specific primers then there will be a much reduced efficiency of amplification. If it is below optimal then the primers become more likely to anneal not only to the specific template sequences but also to non-specific sequences leading to an increase in non-specific amplified products. In the analysis of microsatellite repeat sequences reported here a technique of Touchdown PCR was used to improve the efficiency of DNA amplification (Don et al. 1991) This technique is further discussed in **Materials and Methods**. Investigating MSI is problematic:

- How many loci should be studied? And how many of these need to show MSI before a tumour can be defined as having a particular phenotype? MSI was initially described in inherited colonic carcinomas (HNPCC) in 1993 (Thibodeau et al. 1993; Peltomaki et al. 1993; and Aaltonen et al. 1993). International criteria for the investigation of MSI were not established until November 1998 (Boland et al. 1998) after the cell lines described in this thesis had been investigated. Prior to 1998 different approaches and criteria were used depending on which group had published the paper. Some considered 1 locus in 12 to be sufficient (Toyama et al. 1996), whilst others considered over 30% of loci examined to be appropriate (Thibodeau et al. 1996). This lead to the situation whereby the mutation rate in normal tissues such as peripheral T-lymphocytes, which may be as high as 3×10^{-3} per allele, was similar to that observed in some sporadic tumours labelled as displaying MSI (Hackman et al. 1995; Hearne et al. 1992).

- High or low frequency MSI ? In colonic tumours there appears to be two levels of MSI depending on the number of markers displaying MSI and on the degree of increase or decrease in the fragment size. High frequency MSI (MSI-H) is characterised by widespread instability with a majority of markers exhibiting instability. Low frequency MSI (MSI-L) is characterised by a minority of markers exhibiting MSI (Lothe et al. 1993; Dietmaier et al. 1997; Thibodeau et al. 1998). MSI-H has been shown to be associated with mutations in *MSH2*, *MLH1* and *PMS2* (Borrensen et al. 1995; Liu et al. 1995,1996; Wu et al. 1997). Less clear are the mechanisms underlying MSI-L . Based on observations in yeast MSH3 and MSH6 have been implicated in the phenomenon (Marsischky et al. 1996). MSH6 mutant human tumours and cell lines show MSI at lower levels than tumour cells with mutation in MSH2 and MLH1 (Papadopoulous et al. 1995; Percesepe et al. 1998). The presence of and molecular basis for a non-colonic MSI-L and MSI-H group of tumours remains to be established. Given that the cohort of human genes responsible for MSI is not fully known and that the doxorubicin derived cell lines do not appear to have lost expression of MLH1, MSH2 and PMS2 it is important to identify cell lines displaying MSI-L in order to investigate the underlying MMR pathway. Several issues, however, are not clear:
- Are the results obtained from colonic and other tumours applicable to the development of drug resistance in cell lines? Only one paper has been published examining MSI in the context of drug resistance (Anthoney et al. 1996).
- Which loci? The chances of discovering MSI are highly dependent on which loci are studied (Boland et al. 1998).

5.7.2 Results

On the basis of the available literature, 5 loci were examined in the doxorubicin resistant cell lines and compared to their respective parental cell line. If MSI was found at 2 or more loci the cell line was judged to exhibit a MIN+ phenotype (Sometimes referred to as a replication error, RER+, phenotype). If MSI was observed at less than 2 loci the number of loci examined would be expanded to 10. Each cell line was examined for alterations in the length of CA microsatellite repeats on chromosomes 2, 3 and 17. The choice of PCR primers was made on the basis of work carried out by G Hirst (personal communication) and A Anthoney (Anthoney et al. 1996) on doxorubicin and cisplatin resistant cell lines. Primers were chosen which were reliable and had demonstrated MSI in A2780AD, A2780CP70 or platinum resistant derivatives of A2780 as summarised in Table 8.

When analysis of microsatellite repeats at 10 loci was performed only one cell line MCF7/M6, at one locus D2S391 displayed MSI, figure 18. All the other cell lines did not exhibit any evidence of microsatellite mutation at the loci studied. Examples of the resulting autographs can be seen figures 18 and 19. The presence of fainter, so-called stutter, bands represent artefacts that arise during PCR by several different mechanisms (Tautz, 1990). The results are summarised in table 9. These results indicate that the observed doxorubicin resistance in these 20 cell lines is unlikely to be associated with the loss of DNA mismatch repair activity. Loss of MMR following doxorubicin exposure must occur at a very low frequency given that A2780AD remains the only cell line which has lost MLH1 expression.

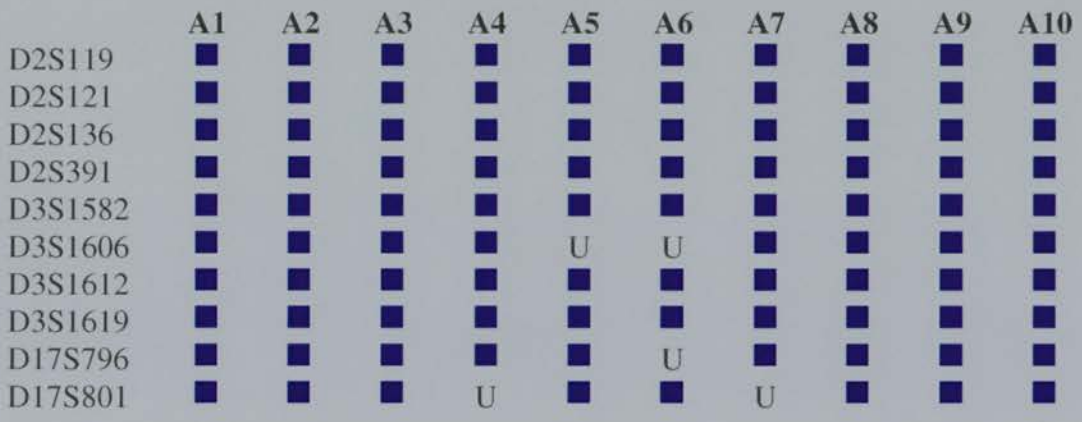
LOCUS	A2780	A2780AD	A2780/CP70
D2S119	■	■	■
D2S121	■	NA	■
D2S136	■	■	■
D2S391	■	■	■
D3S1582	■	■	■
D3S1606	■	■	■
D3S1612	■	■	■
D3S1619	■	■	■
D17S796	■	■	■
D17S801	■	■	■

■ No MI or LOH

■ MI no LOH

■ LOH

Table 8. Incidence of microsatellite mutation using primers located on chromosome 2, 3 and 17 in doxorubicin and platinum resistant derivatives of A2780. (Results obtained by G Hirst and A Anthoney.)



Microsatellite instability

No change

U Unknown

Table 9. Incidence of microsatellite mutations in multiple step selected doxorubicin resistant MCF7 and A2780 cell lines.

DNA from doxorubicin resistant A2780 and MCF7 cell lines, derived by selection with increasing concentrations of doxorubicin, was amplified by PCR using microsatellite specific primers. Mutation was characterised by a shift in the size of microsatellite DNA compared to that from parental MCF7 and A2780.

Only 1 cell line demonstrates MSI at 1 locus. None of the doxorubicin selected cell lines displays evidence of loss of functional mismatch repair.

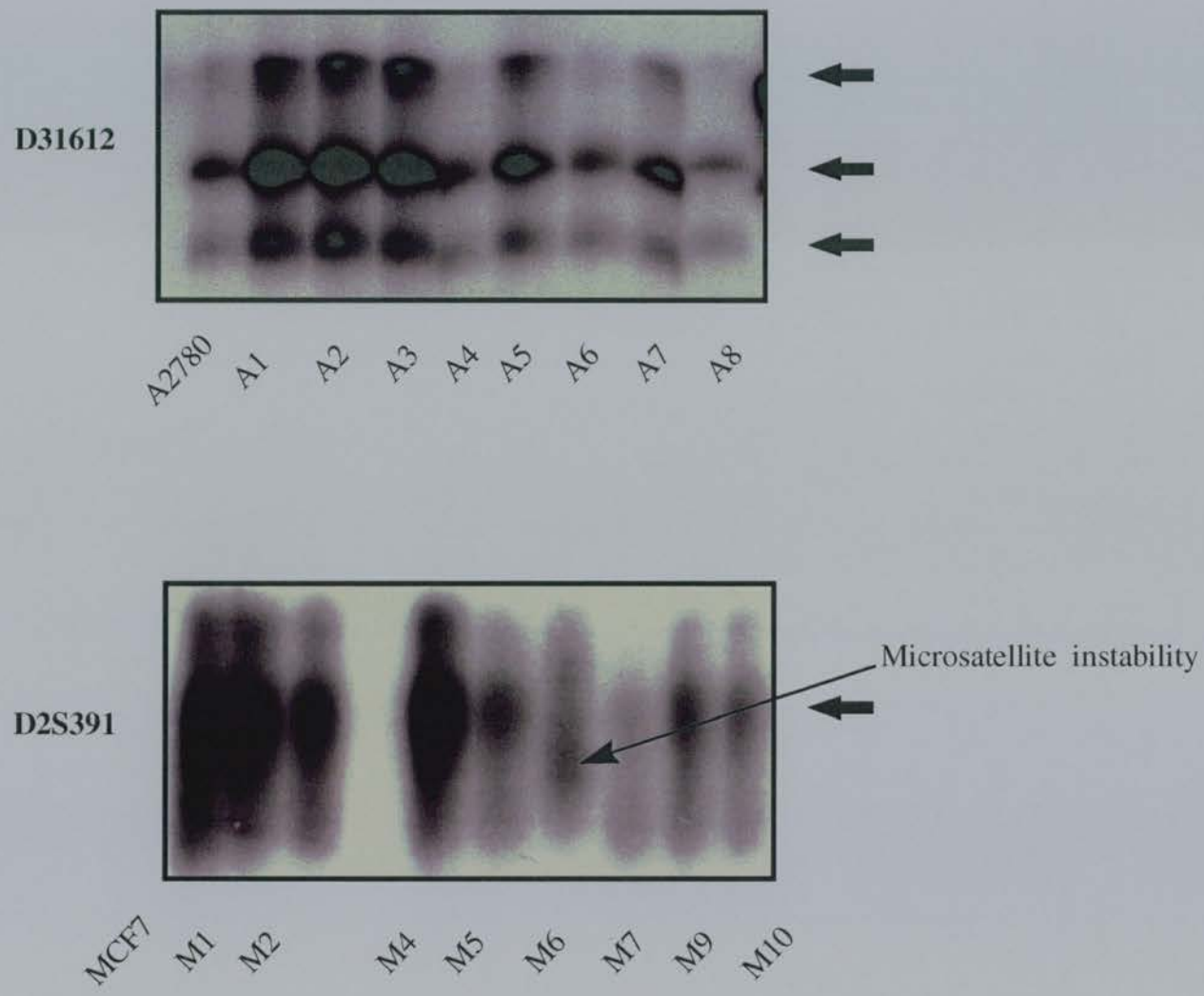


Figure 18. PCR amplification of microsatellite loci in A2780, MCF7 and their derived cell lines selected for resistance to increasing concentrations of doxorubicin.

PCR amplification of microsatellite loci D2S391 and D3S1612 in A2780, MCF7 and derived cell lines. The microsatellite alleles are indicated by arrows. The presence of fainter, so-called stutter, bands occurs as a result of artefacts which arise during PCR. Microsatellite instability is demonstrated at D2S391 in MCF7/M6.

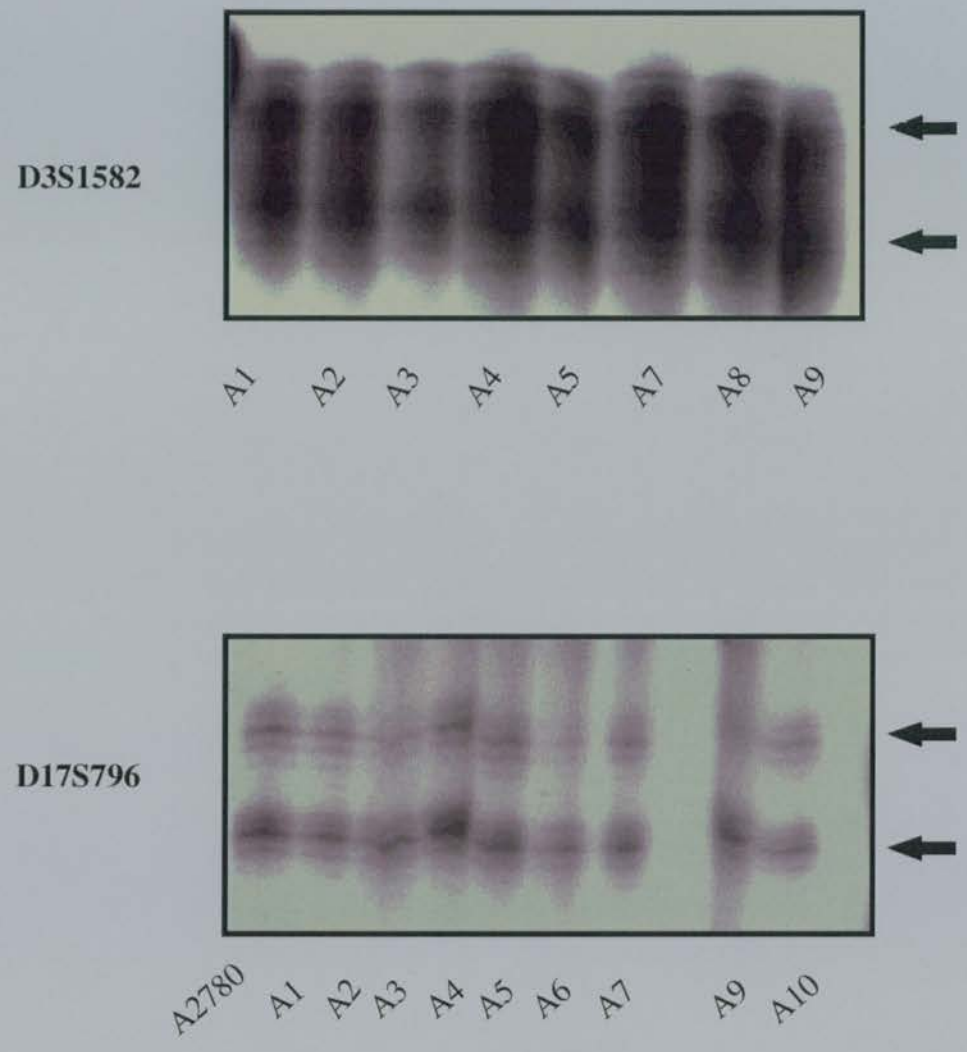


Figure 19. PCR amplification of microsatellite loci in A2780, MCF7 and their derived cell lines selected for resistance to increasing concentrations of doxorubicin.

PCR amplification of microsatellite loci D3S1582 and D17S796 in A2780, MCF7 and derived cell lines. The microsatellite alleles are indicated by arrows. The presence of fainter, so-called stutter, bands occurs as a result of artefacts which arise during PCR. No microsatellite instability is observed.

Following publication in November 1998 (Boland et al. 1998) of criteria for determining MSI in colorectal carcinoma it was reassuring to note that we had inadvertently followed the main guidelines, summarised below:

1. Five microsatellites should be examined, MSI-H is characterised by finding microsatellite instability at 2 out of the 5 loci. If MSI is observed at 1 locus the number of markers should be expanded to distinguish MSI-L from microsatellite stable tumours.
2. A panel of markers was identified which should be used in identifying MSI in colonic tumours.
3. Panels of markers for other tumour types were not established due to limited experimental information. The recommendations, however, for the examination of breast and ovarian tumours were to, initially, consider 5 loci and that these should consist of dinucleotide repeat sequences of DNA (breast tumours).
4. It was stated that markers, where possible, should have been previously validated.

5.8. Conclusions

- Twenty doxorubicin resistant cell lines were derived independently from clonal populations of the ovarian and breast carcinoma cell lines A2780 and MCF7 following serial exposure to doxorubicin.

- None of the doxorubicin resistant cell lines had completely lost expression of the mismatch repair proteins MLH1, PMS2 and MSH2. A reduction in the level of expressed proteins, however, cannot be excluded on the basis of the data presented here.

- No evidence of loss of mismatch repair activity, as detected by the presence of microsatellite instability, could be found in these cell lines.

- Loss of MMR following doxorubicin exposure must occur at a very low frequency given that A2780AD remains the only independently derived doxorubicin resistant cell line which has lost MLH1 expression.

- Loss of MMR does not appear to be a factor in the development of resistance to doxorubicin in these cell lines. Although a reduction in MMR protein expression which does not result in MSI has not been excluded.

- All of the resistant cell lines appeared to have elevated levels of p53 protein in comparison to parental A2780 or MCF7 which may be implicated in the

development of resistance. The focus of this thesis is on mismatch repair. There is an extensive literature characterising mutant *p53* in doxorubicin resistant cell lines. Given this, further investigation at a molecular or functional level of p53 or of other mechanisms of resistance was felt to be outside the scope of this thesis.

CHAPTER 6

MLH1 AND P53 EXPRESSION IN BREAST TUMOURS PRIOR TO AND FOLLOWING CHEMOTHERAPY

6.1. Introduction

The data presented so far suggests that the loss of expression of mismatch repair proteins results in resistance to doxorubicin. Furthermore, re-expression of MLH1 in A2780/CP70, together with other re-expression studies, leads to sensitisation to doxorubicin (and a variety of other drugs) supporting a direct role for MMR in engaging in cell death (de Alas et al. 1997; Durant et al. 1999) . It would appear, *in vitro*, that loss of MMR and in particular MLH1 represents a mechanism by which cells can acquire resistance to a variety of DNA damaging agents. Unlike cisplatin, however, acquisition of doxorubicin resistance as a result of exposure to the drug does not seem to result in loss of MMR (Anthoney et al. 1996; Drummond et al. 1996). The most important question remains, is loss of MMR a clinically relevant mechanism of drug resistance?

6.1.1. MMR protein expression and drug resistance in vivo

An increase has been reported in the proportion of ovarian tumours negative for MLH1 observed in samples taken at second look laparotomy after cisplatin chemotherapy (4/11) compared to untreated tumours (4/39) (Brown et al. 1997). In addition, a 66% reduction in MLH1 expression following chemotherapy has been reported following carboplatin or cisplatin in a study of 38 ovarian cancer patients (Fink et al. 1998b). A recent study has shown that reduced expression of MLH1 prior to platinum based chemotherapy predicts for poor overall survival (Mackean et al, 1999). What are the implications in other tumour types?

6.1.2. Breast cancer

Why study MMR in breast cancer?

- Breast cancer accounts for 1 in 5 of all deaths in women aged between 35 and 54. Despite radical surgery, more than 50% of surgically treated patients eventually relapse. Metastatic disease remains incurable with patients becoming progressively less sensitive to systemic therapy.
- Anthracyclines and cisplatin are agents which are commonly used in the treatment of breast cancer. Resistance to these drugs is a considerable clinical problem.
- Chemotherapy before surgery (so-called primary or neoadjuvant chemotherapy) is increasingly being used in the treatment of primary breast cancer. Its effectiveness in inducing tumour regression and thus allowing more conservative surgery (particularly avoidance of mastectomy) is well established (Smith and Al Moundhri,1998; Bonadonna et al. 1998; Smith et al. 1995). During therapy the tumour response can be directly measured and thus can be used as a surrogate marker of drug sensitivity. Furthermore, treatment occurs between initial biopsy and definitive surgery allowing a system to study, *in vivo*, the effect of chemotherapy on tumour biology (Daidone et al. 1995; Linn et al. 1997; Frassoldati et al. 1997). This is a similar approach to that used to investigate MMR protein expression in ovarian cancer.

In order to study mismatch repair in breast cancer should we be using MSI analysis or detection of MMR protein expression by immunohistochemistry?

MSI analysis in tumours is dependent on a number of factors:

1. The availability of suitable biopsy material. That is, tissue from which DNA can be extracted with low amounts of contamination from normal cells.
2. The availability of a normal control. In order to determine if MSI is present DNA from normal tissue is required to compare with the tumour DNA.
3. The assumption that drug resistance correlates with MSI. The pathways from MMR to apoptosis remain unknown. It is possible that changes in MMR protein expression could influence apoptosis without leading to MSI.
4. Which loci? As discussed in the preceding chapter there are inherent problems in examining for MSI.

Detection of MMR protein expression by immunohistochemistry allows archival paraffin embedded tissue to be studied. Furthermore, tumour cells within the sample can be directly identified using light microscopy. This technique was first used by Wilson in 1995 (Wilson et al. 1995). In 1996 Thibodeau et al showed that, in paraffin embedded colorectal tumour samples, immunohistochemical staining for MLH1 and MSH2 corresponded very closely with loss of function of these genes as detected by MSI. All 14 tumours which had lost immunohistochemical staining were found to exhibit MSI, giving a low false positive rate and 100% specificity. 5 out of the 14 tumours with normal histochemistry had MSI, giving a sensitivity of 64% (Thibodeau et al. 1996). As discussed earlier loss of MLH1 and MSH2 are not the only causes of MSI. Subsequent studies have confirmed the validity of this technique in other tumour types (Mackean et al. 1999; Parc, 1999; Hartman et al. 1999, Friedrich et al, 1999). It is not known what level of expression of MMR proteins is required to affect immunostaining or to produce MSI.

6.1.3. P53

Mutation of the tumour suppressor gene *p53* is a frequent genetic change in breast cancer (Elledge and Allred, 1994). Although the results of studies vary, mutant *p53* is generally viewed as an indicator of poor prognosis in breast cancer (Isola et al. 1992; Thor et al. 1992). As discussed in the preceding chapters it has been postulated that mutation of *p53* may play a role in the development of resistance to chemotherapy. An *in vitro* study examining chemosensitivity in human breast cancer specimens reported a correlation between mutant p53 protein expression and enhanced chemoresistance (Koechli et al. 1994). These findings have not been supported in clinical studies, with mutant p53 failing to act as an indicator of response to chemotherapy (Makris et al. 1997; Rozan et al. 1998; Makris et al. 1995). Loss of functional p53 has been observed in association with loss of the MMR protein MLH1 in experimental models (Drummond et al. 1996; Anthoney et al. 1996).

6.2. Aims of Chapter:

- To investigate, using immunohistochemistry, MLH1 and p53 expression in sporadic breast cancer.
- Using paired samples obtained prior to and following neoadjuvant chemotherapy examine the effect of chemotherapy on MLH1 and p53 expression.
- Assess the correlation of these markers with clinical outcome and with other clinical parameters.

6.3. The patients

Archival paraffin embedded material was obtained from 36 patients treated with neoadjuvant chemotherapy for primary breast cancer between 1993 and 1997. Samples were obtained from either the Beatson Oncology Centre, Glasgow or the Western General Hospital Edinburgh. Samples consisted of a tru-cut biopsy taken at the time of diagnosis and tumour taken at mastectomy following chemotherapy. Of the 36 patients paired samples were available for 29; for seven patients, paired biological determinants were not available because of the few or no tumour cells present in their samples. Median age of the patients was 47 years (range, 26 to 66 years) The median maximum tumour diameter at diagnosis was 6cm (range 2.9-15cm). Other patient characteristics are as listed in table 10.

Eleven patients received continuous-infusion 5-fluorouracil 200mg/m^2 /day, doxorubicin 50mg/m^2, and cyclophosphamide 600 mg/m^2 every 3 weeks (FAC); 16 patients received doxorubicin 20-30 mg/m^2 / week and continuous-infusion 5-fluorouracil 200mg/m^2 /day (AcF); and nine patients received epirubicin 50 mg/m^2 and cisplatin 60 mg/m^2 three times weekly and continuous-infusion 5-fluorouracil 200mg/m^2 /day (ECF). All patients completed at least 9 weeks of treatment before the repeat biopsy at surgery. Radiotherapy was administered according to local practice after both chemotherapy and definitive surgery. Those who were oestrogen receptor-positive were placed on tamoxifen. Response was assessed by bidimensional tumour measurement obtained retrospectively from patients case records, according to International Union Against Cancer Criteria (UICC), and maintained over at least 4 weeks (Hayward et al, 1977) UICC. Oestrogen receptor

Patient characteristics	Number of patients	%
Menopausal status		
Pre	18	50
Peri	3	8
Post	15	42
Stage		
T3	3	8
T4	29	81
T4d	4	11
N0	7	19
N1	24	67
N2	4	11
Histological grade		
I	1	3
II	10	28
III	19	53
UK[a]	6	17
ER status[b]		
Positive	14	39
Negative	16	44
UK[a]	6	17
Response[c]		
CR	12	34
PR	18	51
SD	2	6
PD	3	9

a. Unknown

b. ER, Oestrogen Receptor

c. Response: CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

Table 10 Patient characteristics.

(ER) status was obtained from patients pathology records. Median follow-up was 41 months (range 30 to 66 months).

6.4. Immunohistochemistry

Immunohistochemistry was performed on histology sections taken from tru-cut biopsy and mastectomy specimens as detailed in **Materials and Methods**. Samples were examined, where ever possible for MLH1 and p53 expression.

6.4.1 Storage of samples.

Several studies have demonstrated that loss of antigenicity occurs following prolonged storage of paraffin-embedded sections on glass slides (Prioleau and Schnitt, 1995; Jacobs et al. 1996). This has been demonstrated in a variety of different tissue types (including breast) and for a variety of different antigens (including p53) (Bertheau, 1998). Antigen loss has been shown to be significantly greater over time when slides are stored at room temperature as compared to 4°C (Jacobs et al. 1996). Slides in this study were therefore stored at 4°C and used within a short period of time after being cut.

6.4.2. Scoring of slides

Slides were scored for intensity and percentage cells staining as detailed in **Materials and Methods** by Dr M. Rawhilly (Consultant Pathologist). The reporting of immunohistochemistry has been a subject of heated debate for many years. Most of the studies focus on Oestrogen receptor (ER) analysis in breast

cancer. The most commonly reported combined intensity and percentage score is the *H-score* (McLelland, 1990) calculated by:

H-score = (% of cells stained at intensity category 1x 1) + (% of cells stained at intensity category 2 x 2) + (% of cells stained at intensity category 3 x 3)

This has been shown to give a closer correlation with the gold standard, biochemical, assay for detecting ER status than either intensity or percentage staining as assessed by immunohistochemistry (Remmle, 1986). In another study, Detre et al. (Detre et al. 1995) compared 3 methods of scoring immunostaining for ER. They showed that there was no advantage to the *H-score* when compared with 2 *quickscores*, one additive (I + %) and the second multiplicative (I x %). Both *quickscores* gave as good a correlation as the, more complicated, H-score when assessed against the gold standard enzyme immunoassay. Furthermore, they were less time consuming. No advantage was found between either method of assessing the *quickscore*. The additive method was chosen for this thesis i.e.

I + % = IHC score.

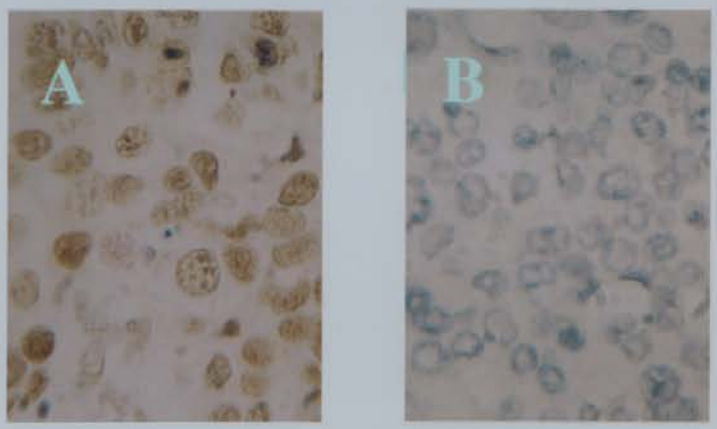
6.5. Changes in MLH1 and p53 expression in response to chemotherapy

Paired breast tumour samples from the same patient before and after neoadjuvant chemotherapy were evaluated for MLH1 immunohistochemical staining. An example of MLH1 immunohistochemical analysis of paired samples before and after chemotherapy is shown figure 20.

There was a significant reduction in MLH1 expression as assessed by the percentage staining ($p=0.01$, $n=28$) and combined IHC score ($p=0.01$, $n=28$) following chemotherapy, shown in Table 11. Furthermore, there was a reduction in the intensity of staining within cells seen after chemotherapy. Sixty-one percent of patients had immunostain intensity scores of 2 or greater before treatment compared with 36% after chemotherapy. This reduction held true for subgroup analysis by chemotherapy regimen although it did not reach significance shown in Table 12. Matched patient IHC, percentage and intensity staining scores prior to and following chemotherapy are shown in figure 21.

There was no significant reduction in p53 expression following chemotherapy shown in Table 11. Subgroup analysis of p53 IHC scores by chemotherapy regimen also failed to show any difference pre and post chemotherapy shown in figure 22.

There was no statistically significant correlation of percent, intensity or IHC MLH1 and p53 scores before or after chemotherapy shown in Table 13.



Samples taken prior to and following FAC chemotherapy (DFS=5months).

A Pre chemo biopsy immunostained for MLH1 (brown). Counterstain haemotoxylin. IHC=5

B Post chemo sample stained for MLH1. Counterstain haemotoxylin. IHC=0

Magnification x200

Figure 20. MLH1 immunohistochemistry (A) Tru-cut biopsy before chemotherapy; positive for MLH1 staining (brown). (B) Mastectomy specimen with low MLH1 staining (blue).

	Pre-chemo (mean)	Post-chemo (mean)	Pre vs post p-value
MLH1 staining:			
Intensity	1.7	1.3	**0.06**
Percentage	78	58	**0.01**
Intensity + Percentage	4	3	**0.0099**
			n=28
P53 staining:			
Intensity	1.1	1.0	0.16
Percentage	36.7	30.4	0.35
Intensity + Percentage	1.6	1.4	0.36
			n=29

Table 11. Comparison of MLH1 and P53 immunostaining prior to and following chemotherapy.

The within-patient comparison of MLH1 and p53 scores before and after chemotherapy was assessed using the Wilcoxon signed rank sum test.
MLH1 expression is reduced as a result of chemotherapy.

Chemotherapy Regimen	Pre-chemo (mean)	Post-chemo (mean)	N	P
FAC	4	2.5	11	0.07
AcF	3.8	3.2	12	0.07
ECF	4.3	3.2	6	0.06

Table 12 Comparison of MLH1 combined IHC scores prior to and following FAC, AcF and ECF chemotherapy regimens

The within-patient comparison of MLH1 IHC scores before and after FAC, AcF, and ECF chemotherapy regimens was assessed using the Wilcoxon signed rank sum test.

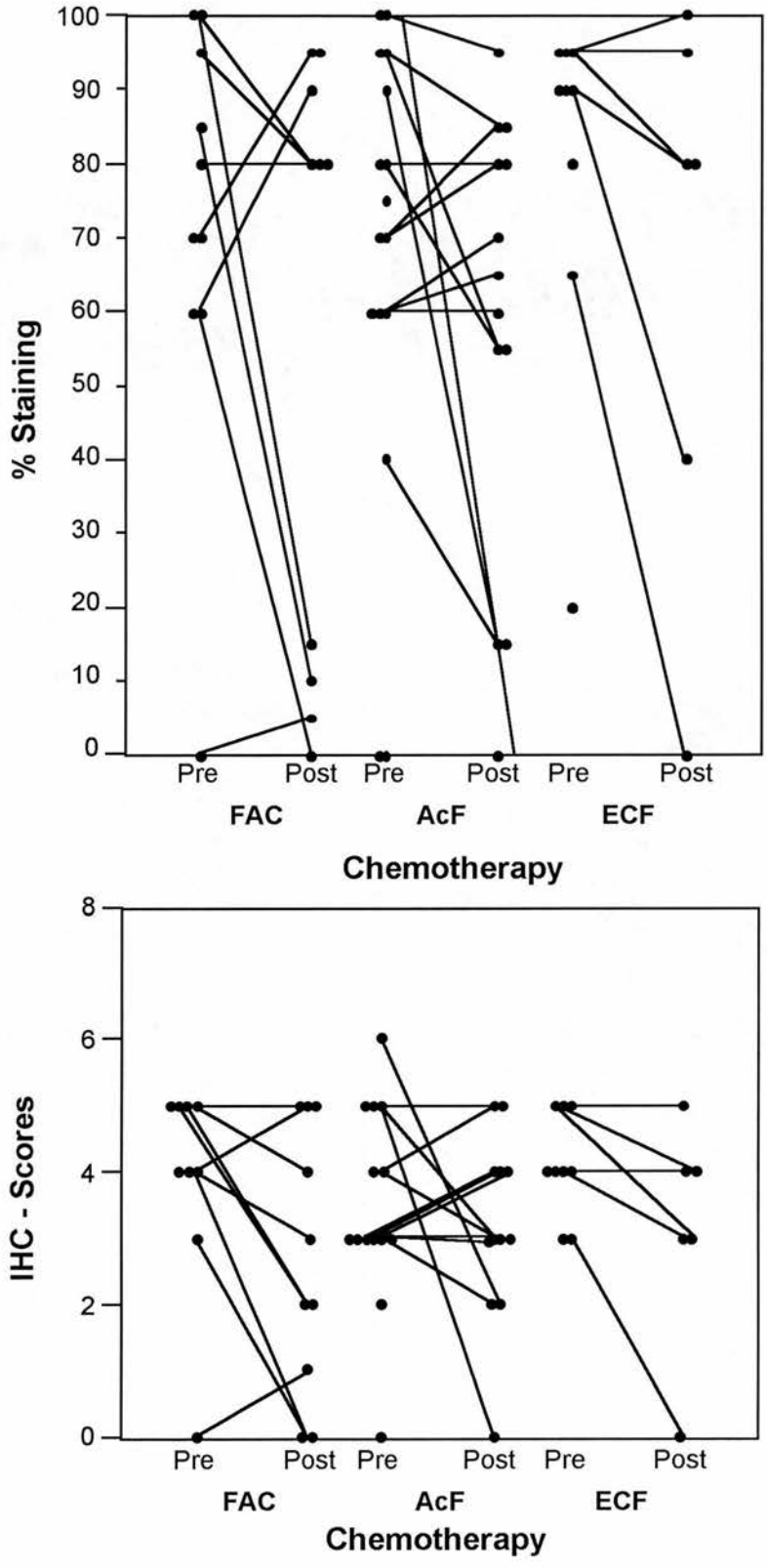


Figure 21. Paired MLH1 scores and percentage cells staining in breast cancer samples before and after chemotherapy

Percent and IHC (% staining + intensity) score are shown for each biopsy. Scores joined by a line are from the same patient before and after chemotherapy

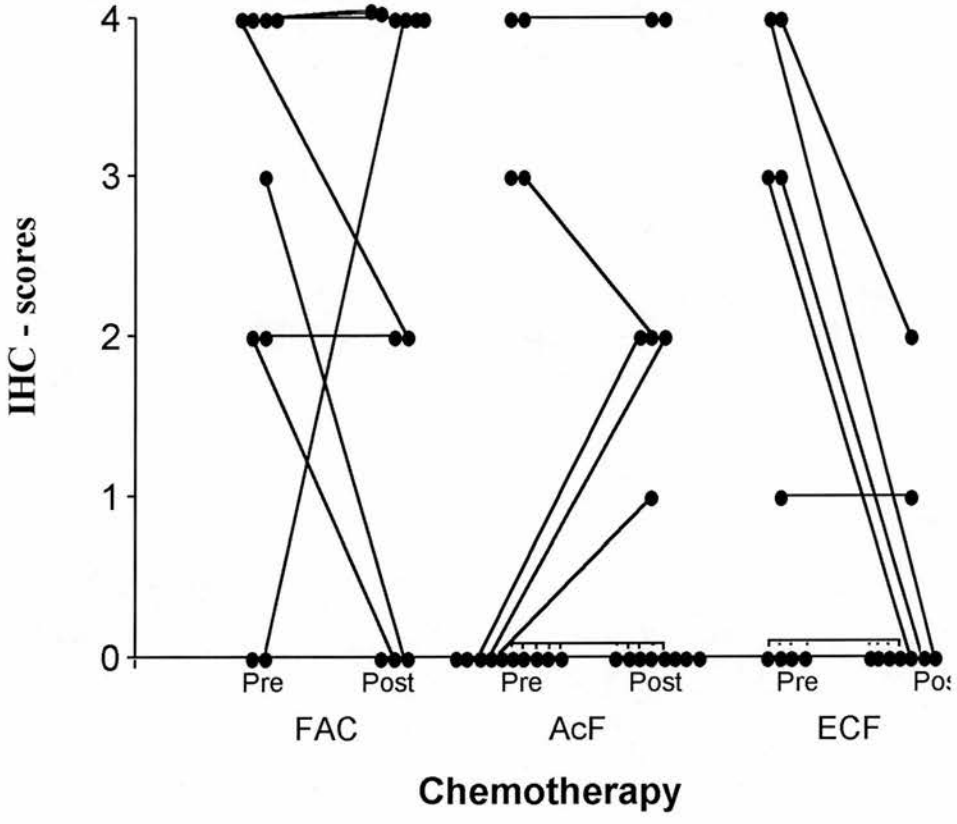


Figure 22. Paired p53 IHC scores in breast cancer samples before and after chemotherapy

IHC (% staining + intensity) score is shown for each biopsy. Scores joined by a line are from the same patient before and after chemotherapy.

6.6. Univariate and multivariate analysis

There was a highly significant correlation between poor disease free survival and a low level of MLH1 expression as expressed by percentage staining (p= 0.0031, n=28), intensity of staining within cells (p=0.016, n=28) and the overall IHC score (p=0.0005, n=28) obtained in the post-chemotherapy samples using Cox regression analysis. In addition, the difference between the pre and post-chemotherapy scores was found to correlate with a poor disease free survival (p=0.0007, n=27). Shown in Table 15, figures 23 and 24.

Pre-chemotherapy MLH1 expression did not predict for response to primary chemotherapy shown in Table 14. Furthermore, neither pre-chemotherapy intensity, percentage staining or IHC score for MLH1 predicted for disease-free survival. Shown in Table 15.

Figures 23 and 24 shows Kaplan Meir survival curves for MLH1 expression prior to and following chemotherapy.

Pre-chemotherapy p53 expression failed to predict tumour response to chemotherapy (Table 14) and disease free survival (Table 15). Figure 25 shows Kaplan-Meier survival curves for p53 IHC scores before and after chemotherapy. The post-chemotherapy p53 score did not predict DFS (p=0.12, n=29) shown in Table 15 and figure 25.

Analysis of individual patients with very low MLH1 expression (pre or post chemotherapy) has shown they have a very short disease free survival. Three patients had completely lost expression of hMLH1 following chemotherapy and they relapsed at 4, 8 and 15 months respectively. Two patients had very low expression of hMLH1 prior

to chemotherapy (0 and 10% of cells positive for immunostaining) these patients progressed at 12 months and 6 weeks respectively. Of the 7 patients for whom paired samples were unavailable, one had achieved a complete response (pre-chemotherapy sample 80% MLH1 positive) and remains in remission at 49 months. Of the remaining six patients, no clear pattern emerged and the distribution of pre-chemotherapy MLH1 scores was indistinguishable from the others. The failure to produce a post-chemotherapy hMLH1 score was because of poor sample quality.

A univariate analysis of clinical characteristics is shown in table16. There was an association between young age, pre-menopausal status and poor response to chemotherapy and poor disease free survival. A forward stepwise multivariate analysis was conducted using the statistically significant clinical features along with MLH1 intensity, percentage staining, combined IHC score after chemotherapy and change in IHC score (before and after). This identified percentage staining ($P = 0.019$), age ($P < 0.001$), response to chemotherapy ($P = 0.001$), and menopausal status ($P= 0.041$) as independent prognostic factors ($n = 27$).

MLH1vs P53	N	p-value
Pre-chemo staining:	32	
Intensity		0.07
Percentage		0.46
Intensity + Percentage		0.15
Post-chemo staining:	25	
Intensity		0.35
Percentage		0.46
Intensity + Percentage		0.41

Table 13 Correlation of MLH1 and P53 immunostaining.

There is no association between MLH1 and p53 expression as assessed by Spearman's rank correlation coefficient

	N	Tumour Response p-value
Pre-chemo MLH1 staining:	35	
Intensity		0.14
Percentage		0.9
Intensity + Percentage		0.39
Pre-chemo P53 staining:	32	
Intensity		0.4
Percentage		0.15
Intensity + Percentage		0.95
Age	36	0.57
Histological grade	30	0.31
Oestrogen receptor status	30	0.79
Menopausal status	36	0.39
Lymph node status	35	0.39

Table 14. Tumour response to chemotherapy and prognostic factors

The univariate examination of the association between tumour response and p53, MLH1 and clinical factors was made using the Cox Regression Analysis.

Prognostic Factor	N	DFS p-value
Pre-chemo MLH1 staining:	35	
Intensity		0.31
Percentage		0.33
Intensity + Percentage		0.39
Post-chemo MLH1 staining:	29	
Intensity		**0.016**
Percentage		**0.0026**
Intensity + Percentage		**0.0005**
Pre-Post MLH1 score	29	**0.0007**
Pre-chemo P53 staining:	32	
Intensity		0.24
Percentage		0.34
Intensity + Percentage		0.31
Post-chemo P53 staining:	29	
Intensity		0.11
Percentage		0.26
Intensity + Percentage		0.12

Table 15. Disease free survival, p53 and MLH1 expression

The univariate examination of the association between disease free survival and p53 and MLH1 expression was made using the Cox Regression Analysis.

Prognostic factor	N	DFS p-value
Age	36	**0.001**
Menopausal status	36	**0.03**
Progressive disease during chemotherapy	35	**0.022**
Lymph node status (pre-chemotherapy)	35	0.66
Lymph node status (post-chemotherapy)	28	0.99
Histological grade	30	0.28
Oestrogen receptor status	30	0.47

Table 16. Disease free survival and clinico-pathological factors

The univariate examination of the association between disease free survival and clinico-pathological factors was made using the Cox Regression Analysis.

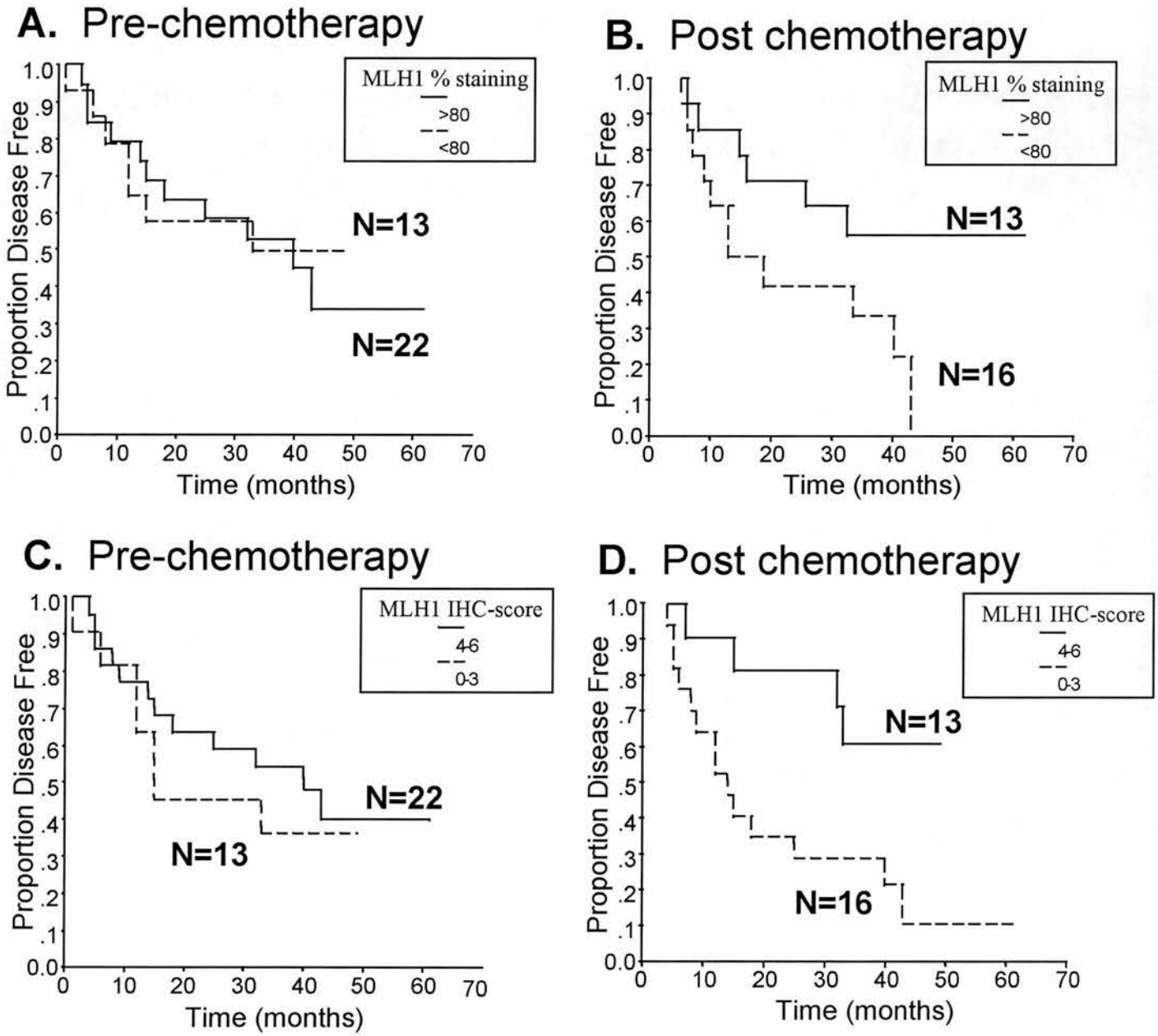


Figure 23. Kaplan Meier survival curves for MLH1 IHC scores and percentage cells staining prior to and following chemotherapy.

Kaplan Meier survival curves for MLH1 IHC scores pre-chemotherapy (A) and post-chemotherapy (B). The proportion of patients disease free is shown against time (months). MLH1 scores of 3 and less are shown with a dashed line and scores greater than 3 with a solid line.

The number of patients (N) in each group is shown.

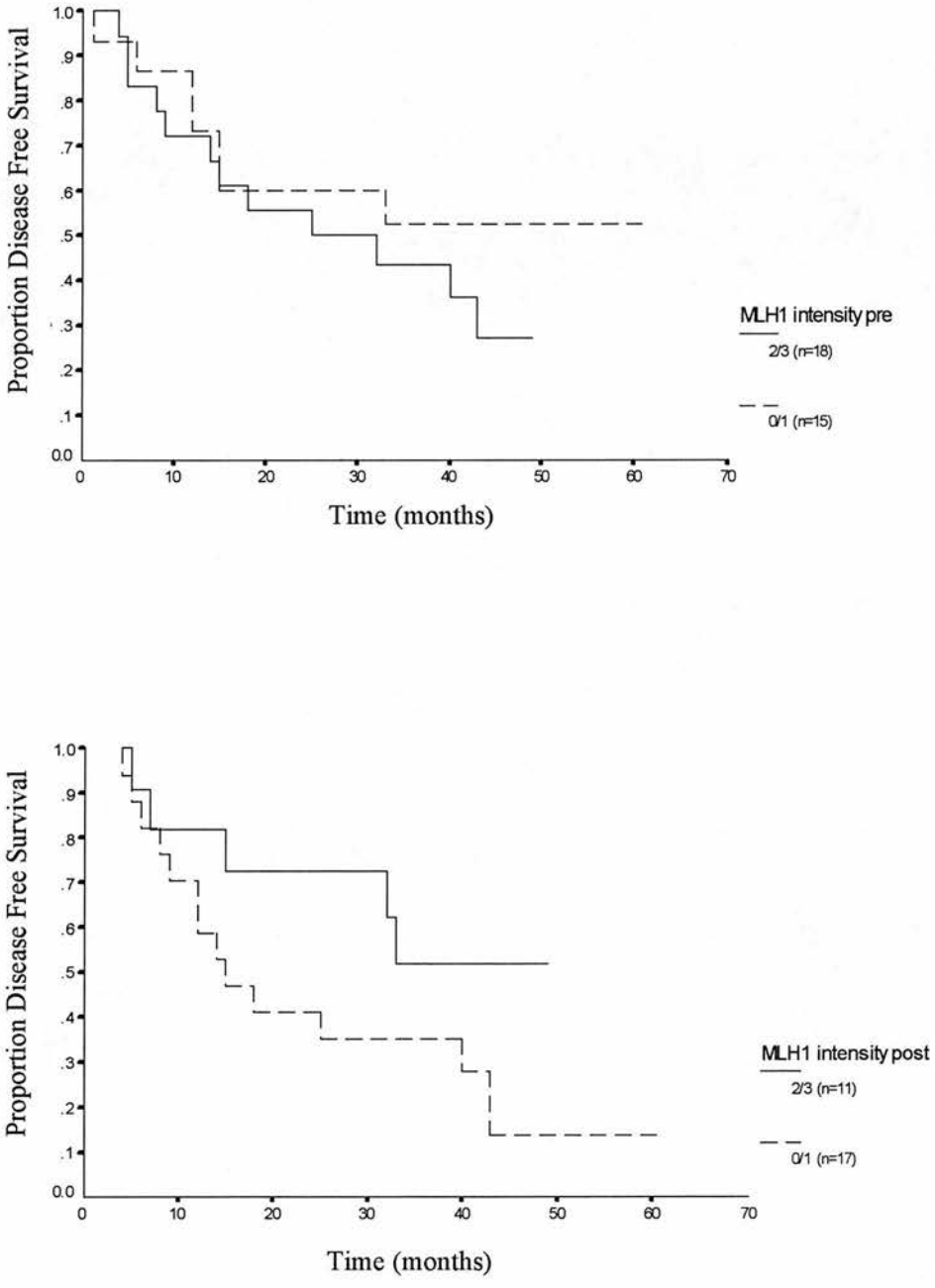


Figure 24. Kaplan Meier survival curves for MLH1 intensity prior to and following chemotherapy.

Kaplan Meier survival curves for MLH1 intensity pre-chemotherapy (A) and post-chemotherapy (B). The proportion of patients disease free is shown against time (months). MLH1 intensity of 1 and less are shown with a dashed line and scores greater than 2 with a solid line.

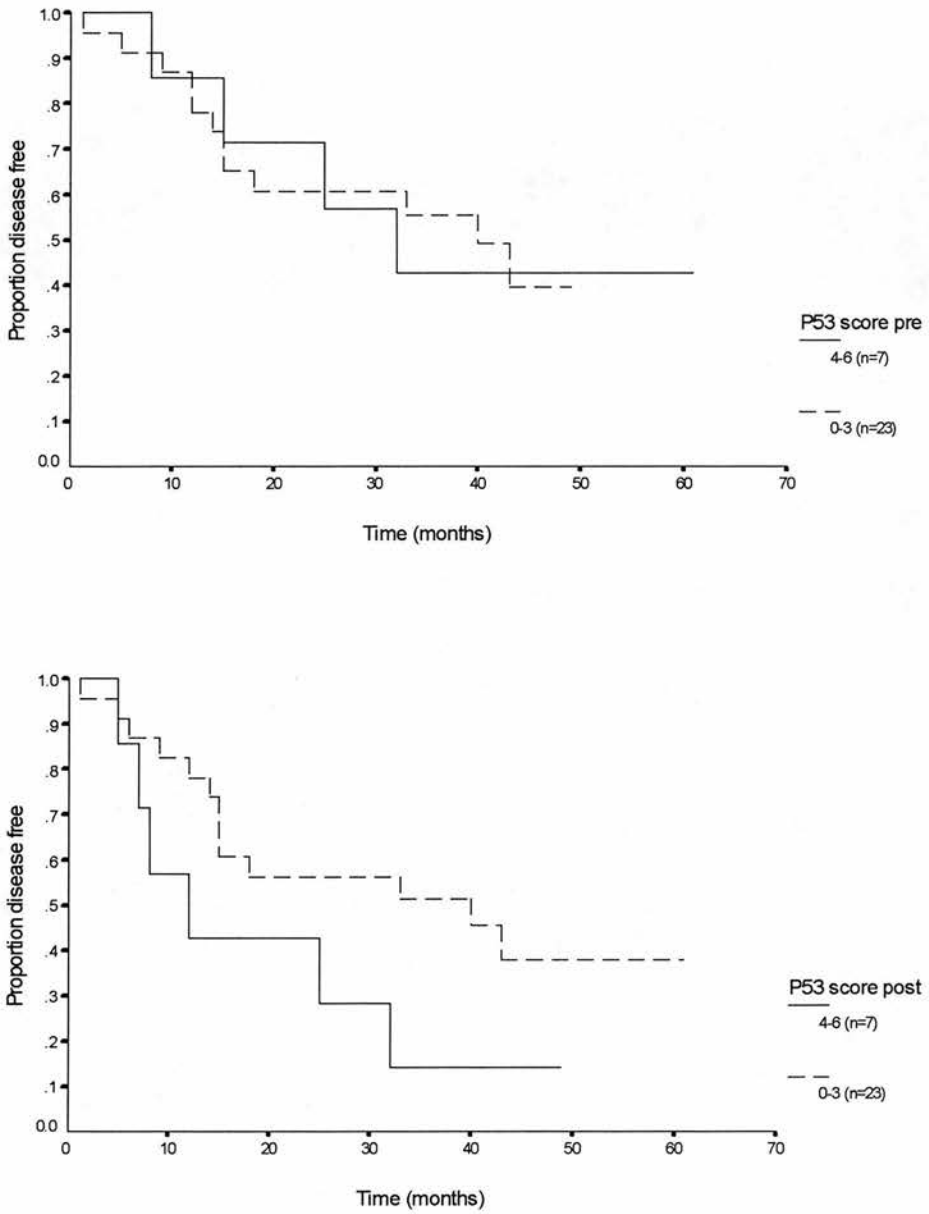


Figure 25. Kaplan Meier survival curves for p53 IHC scores prior to and following chemotherapy.

Kaplan Meier survival curves for p53 IHC scores pre-chemotherapy (A) and post-chemotherapy (B). The proportion of patients disease free is shown against time (months). p53 scores of 3 and less are shown with a dashed line and scores greater than 3 with a solid line.

6.9. Conclusion

- Loss of MLH1 expression occurs as a result of chemotherapy *in vivo*.

- Low levels of MLH1 in breast tumours following chemotherapy independently predicts poor disease free survival.

- There is no association between p53 and MLH1 expression *in vivo*.

- Loss of p53 expression does not occur as a result of chemotherapy.

- Pre-chemotherapy MLH1 and p53 levels do not predict tumour response to chemotherapy or disease free survival.

- Post-chemotherapy p53 expression does not predict disease free survival.

7. Summary

- For the first time, combined chemotherapy has been shown to result in loss of expression of the MMR protein hMLH1 in human breast cancer.

- Loss of expression of hMLH1 following chemotherapy is a poor prognostic indicator in patients receiving neoadjuvant chemotherapy in locally advanced breast cancer. It acts independently of other known prognostic factors and is a good predictor of poor disease-free survival.

- Genetic inactivation of *MLH1*, *MSH2*, *MSH3*, and *MSH6* leads to decreased sensitivity to doxorubicin in *S cerevisiae*. Furthermore, re-expression of MLH1, as a result of gene transfer, results in increased sensitivity. This supports a direct role for loss of MMR in resistance to doxorubicin.

- Re-expression of hMLH1 as a result of chromosome transfer into the cisplatin resistant ovarian tumour cell line A2780/CP70 increases sensitivity to doxorubicin. This supports a direct role for loss of MLH1 in resistance to doxorubicin in a cell line derived by exposure to another chemotherapeutic agent.

- Complete loss of MMR does not occur as a result of exposure to doxorubicin in the human breast and ovarian carcinoma cell lines examined, although a reduction in the level of expression of mismatch repair proteins has not been excluded.

CHAPTER 8

DISCUSSION

8.1 Mismatch repair and drug resistance.

Increasingly the data suggests a direct role for MMR in the development of resistance to a range of DNA damaging agents. Inactivation of the MMR genes in yeast not only decreases sensitivity to doxorubicin but to cisplatin and carboplatin as well. Furthermore, transformation of the yeast *MLH1* gene back into *mlh1* mutant strain increases sensitivity to cisplatin in addition to doxorubicin (Durant et al. 1999). Restoration of MMR activity by chromosome transfer into MMR-defective human colorectal (MLH1) and endometrial (MSH2) tumour cell lines results in increased sensitivity to a number of drugs (Aebi et al. 1997; Umar et al. 1997). Here, more importantly with regard to drug resistance, sensitivity has been examined in a cell line (A2780/CP70) which has lost MLH1 expression as a result of exposure to cisplatin. Re-expression of MLH1 in this cell line increases sensitivity to both doxorubicin and cisplatin (Durant et al. 1999). Furthermore, introduction of *MLH1* into A2780/CP70 sensitises cells to cisplatin, temozolomide and epirubicin when grown as a xenograft (Plumb et al. 2000). This data suggests, accepting the limitations of yeast and cell line studies, that MMR is directly involved in drug sensitivity, rather than the alternative hypothesis that loss of MMR leads to higher mutation rates at drug resistance genes. Furthermore, it implicates loss of MLH1 as a mechanism by which resistance to multiple drugs can occur. The chromosome transfer studies emphasise the potential for drug sensitisation by re-expression of MLH1 in drug resistant cells. How loss of MMR leads to drug resistance is not understood and potential models were discussed in **Chapter 1.**

Why is MLH1 lost as a result of cisplatin exposure? The promoter of *hMLH1* has been shown to undergo hypermethylation in tumours and cisplatin-resistant ovarian cancer cell lines, which correlates with loss of MLH1 expression (Strathdee et al. 1999; Herman et al. 1998; Leung et al. 1999). This may be the basis for the high frequency of MLH1 loss, rather than other MMR proteins observed in tumours and drug resistant models. Furthermore, hypermethylation of the hMLH1 promoter and the resultant loss of protein expression was seen at higher frequencies in tumours that had been exposed to chemotherapy (50% samples) than untreated samples (9%). In sporadic colon (Herman et al. 1998), gastric (Leung et al. 1999) and endometrial (Gurin et al. 1999) cancer in which the overwhelming majority of sporadic tumours with loss of MMR are MLH1 deficient similar results are seen, with the *hMLH1* promoter exhibiting hypermethylation.

Why was loss of MLH1 observed in the doxorubicin resistant derivative of A2780, A2780AD (Drummond et al. 1996) when the cell lines derived in this thesis had no disruption of MMR activity? Doxorubicin and cisplatin both produce DNA hypermethylation *in vitro*. Cisplatin, however, induces hypermethylation at doses which are 100 fold lower than those required by doxorubicin (Nyce, 1989). Hence, it may simply reflect a dose effect; if the cell lines discussed here had been exposed to higher doxorubicin concentrations loss of MLH1 may have been induced. Alternatively, loss of MLH1 in response to doxorubicin exposure may be a low frequency event. The most likely explanation, however, is that loss of MLH1 expression was induced by exposure to another agent. A2780AD was originally isolated after treatment of the A2780 cell population with the alkylating agent EMS to induce drug resistant mutations.

8.2. Mismatch repair and breast cancer.

Is loss of MMR a clinically relevant mechanism of drug resistance? Three clinical studies have reported an increase in the proportion of ovarian tumours with low expression of MLH1 as determined immunologically in samples taken at "second look" laporotomy after cisplatin chemotherapy compared to untreated tumours (Mackean et al. 1999, Brown et al. 1997, Samimi et al, 2000). These support the data presented in this thesis showing loss of expression of MLH1 following neoadjuvant chemotherapy in locally advanced breast cancer. The results presented here are open to criticism as the patients had received 3 different chemotherapy regimens. They did, however, all receive an anthracycline and in some cases cisplatin containing chemotherapy. Together with the ovarian studies this suggests that loss of MLH1 could be an important factor contributing to the frequent development of clinical resistance in these tumour types. Why is the level of MLH1 expression reduced following chemotherapy? There is evidence in ovarian cancer that cisplatin induces loss of expression of MLH1 as a result of hypermethylation of the MLH1 promoter (Strathdee et al. 1999). There is no evidence, however, that this is the case with the anthracyclines or in breast cancer. Furthermore, the data presented in this thesis would suggest that doxorubicin is not inducing loss of MLH1 expression, although this clearly requires further investigation. The alternate hypothesis is that the chemotherapy administered to these patients selects out a pre-existing sub-population of breast cancer cells which have low levels of MLH1 expression. Further research is ongoing to identify how MLH1 expression is reduced following chemotherapy in breast cancer patients.

Loss of MLH1 expression in breast cancer following chemotherapy is a predictor of poor disease free survival and may, therefore, define a subgroup of patients with drug-resistant breast cancer. Why does MLH1 expression prior to chemotherapy fail to act as a prognostic indicator or to predict for tumour response to chemotherapy? It is possible that expression analysis prior to chemotherapy may fail due to an inability to identify resistant sub-populations that are more readily identifiable after chemotherapy. However, notably, the two patients who had less than 10% of tumour cells expressing MLH1 prior to chemotherapy had rapidly progressive disease. It could be that loss of MLH1 is a marker for other changes which result in a more aggressive phenotype or that it represents tumour progression.

Tumours treated with neoadjuvant chemotherapy are unusual in that tissue samples are taken prior to and following chemotherapy. For most patients only pre-chemotherapy samples will be available and, in the case of recurrent disease, this may have been taken several years (and chemotherapy regimens) earlier. How useful, therefore, is a prognostic marker assessable only on post-chemotherapy samples? It has long been known that tumour cells release DNA into the circulation which may then be recovered from serum. Serum DNA measurements are elevated in approximately 50% of cancer patients (Leon et al. 1977). Studies in lung and head and neck cancer patients have demonstrated the feasibility of collecting serum DNA and testing this for microsatellite instability using PCR (Chen et al. 1996; Nawroz et al. 1996). MSI is a marker for MMR function (with the caveat discussed below). Potentially, MSI analysis of serum DNA could represent a novel way of assessing MMR function without the need for tumour biopsy. Alternatively, in ovarian tumours treated with cisplatin, loss of MLH1

occurs as a result of promoter hypermethylation (Strathdee et al. 1999). Methylation-specific PCR using bisulphite modification of DNA can be used to examine the methylation status of the *hMLH1* gene in tumour DNA (this could include tumour DNA within patient serum). Potentially, this technique provides a surrogate measure of functional MMR. Furthermore, a recent study using a mouse xenograft system and an HPLC based technique has demonstrated that changes in global DNA methylation within mouse peripheral lymphocytes can be correlated to expression of MLH1 following treatment with a demethylating agent (Plumb et al. 2000). Potentially this could be used to monitor MLH1 expression in patients throughout their treatment. It is not known in breast cancer if MLH1 expression is lost by promoter hypermethylation. Further work needs to be done in breast cancer to determine the mechanism of MLH1 loss and the potential use of acceptable surrogate markers.

In HNPCC tumours and the cell lines discussed in this thesis MLH1 expression is completely lost. Only 6 out of a total of 64 breast cancer samples examined had completely lost MLH1 expression. Are we observing down regulation of MLH1 expression in breast cancer rather than loss? What effect does this have on cellular phenotype? The answers are unknown. The relationship between levels of MLH1 expression as detected by immunohistochemistry and the MIN+ phenotype is not known. In addition, it is not clear what the cut-off points should be for determining prognostic risk. Further research is necessary to clarify these points.

In ovary cancer, loss of MLH1 prior to chemotherapy is an independent predictor for poor overall survival. Furthermore, it correlates with early stage disease, which is in itself a good prognostic factor (Mackean et al. 1999). This

suggests, in this tumour type at least, that loss of MLH1 does not represent a late stage of tumour progression. In gastric cancer microsatellite instability is associated with a poor prognosis (Choi et al. 2000) and in endometrioid cancers it is associated with improved survival (Maxwell et al. 2001). In both sporadic and HNPCC colonic carcinomas microsatellite instability correlates with an improved prognosis (Bubb et al. 1996, Elsaleh et al. 2000). Furthermore, patients who have MSI positive tumours appear to have a greater benefit from adjuvant chemotherapy(Elsaleh et al. 2000). These contrasts between the different prognoses associated with MSI and mismatch repair protein expression highlights the gap in our understanding of how defective mismatch repair ultimately effects genome stability. In addition it suggests that there may be a tissue specific response to the effects of MSI. It is possible that there are different underlying mutations or mechanisms of mismatch repair inactivation which may act to moderate or intensify tumour progression depending on the tumour type. Each tumour has a different genetic background and the expression and/or activation of the downstream effectors of mismatch repair may differ depending on the site of the tumour. It has even been suggested that the site of the tumour may be a factor in prognosis(Paulson et al. 1996). Thus, mismatch repair deficient tumours in the colon tend to form bulky tumours which grow rapidly into the lumen resulting in early detection (Kuismanen et al. 1999) thereby improving survival. The impact of adjuvant chemotherapy may be having an impact on prognosis. It is not really surprising that MSI positive colon tumours have an improved prognosis with chemotherapy as the drugs which are used are cytotoxic independent of the mismatch repair pathway. This is in contrast to both breast and ovarian cancer where loss of mismatch repair results in resistance to the anthracyclines and platinum based regimens which form the basis of adjuvant chemotherapy.

8.3. P53

Consistent with other studies, p53 showed no significant prognostic value before or after combination chemotherapy and no significant change in the levels of protein expression (Daidone et al, 1995). No association was demonstrated between the expression of p53 and MLH1. It should be remembered, however, that immunohistochemistry may not be a reliable measure of p53 function, and controversy exists as to its use in determining the value of p53 as a prognostic factor. In the light of recent publications it may be more appropriate to examine for p73 expression (discussed in the **Chapter 1**).

Further work is necessary to elucidate the apoptotic pathways following detection of doxorubicin induced DNA damage by the MMR system. The published data focuses on lesions produced by methylating agents and cisplatin, there are no studies examining the effects of doxorubicin or other topoisomerase inhibitors.

8.4. Circumvention of drug resistance

The clinical studies, together with the *in vitro* data, suggest that MLH1 may be a key regulator of chemosensitivity in breast, ovarian and possibly other types of cancer. This has implications for patient management:

Which drug?

Some drugs are not affected by MMR status *in vitro*, such as paclitaxel and oxaliplatin(Fink et al. 1998; Fink et al. 1996). Choosing these agents may be more

appropriate in patients whose tumours have lost expression of MLH1. Recently human MMR defective cells have been shown to be hypersensitive to CCNU(1-(2-chloroethyl)-3-cyclohexyl-nitrosurea) and to mitomycin C in a xenograft model (Fiumicino et al. 2000). These issues require further investigation.

Inhibition of replicative bypass.

As discussed in **Chapter 1** it has been suggested that loss of MMR leads to replicative or recombinational bypass of DNA lesions giving a drug-tolerant phenotype. Inhibition of this bypass could be used to sensitise cells. Aphidicolin, an inhibitor of DNA polymerases, sensitises MMR-deficient cell lines to CDDP (*cis* –diaminedichloroplatinum II) and MNU (a monofunctional methylating agent) to a greater extent than their MMR-proficient counterparts. Potentially, the use of polymerase inhibitors as modulators of drug resistance in MMR deficient cells could be utilised in the clinic (Moreland et al. 1999).

Restoring expression of MLH1

Inducing the expression of MLH1 in drug resistant cells would reverse the drug resistant phenotype. This has been demonstrated in the chromosome transfer studies. In ovary and colon cancer cell lines loss of MLH1 expression is due to methylation of the MLH1 promoter. Re-expression of MLH1 occurs *in vitro* and in a xenograft murine model as a result of treatment with the demethylating agent 2-deoxy-5-azacytidine (DAC) (Plumb et al. 2000). Re-expression of MLH1 corresponds to an increase in cisplatin sensitivity. Clinical trials of DAC in combination with cisplatin are imminent in patients with advanced ovarian cancer.

Gene Therapy

The identification of loss of MLH1 as a specific genetic defect makes it a potential target for gene therapy.

8.5. Conclusions

Loss of MMR, and in particular loss of expression of MLH1 appears to be a key factor in the development of resistance to a wide range of DNA damaging drugs. This thesis supports a direct role for loss of MLH1 in the development of resistance to doxorubicin. Which doxorubicin induced DNA lesion(s) MMR is detecting is not clear. Further work is necessary to identify both the lesions involved and the downstream events resulting in doxorubicin induced cell death. Loss of MLH1 occurs as a result of chemotherapy in locally advanced breast cancer and acts as a predictor of poor disease free survival. A large prospective study based on the results presented in this thesis is under way as part of the Anglo Celtic II Trial in breast cancer. If these results are confirmed in larger prospective studies there will be important implications for the future choice of drugs and treatment strategies employed in the treatment of breast cancer.

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Reduced MLH1 Expression in Breast Tumors After Primary Chemotherapy Predicts Disease-Free Survival

By H.J. Mackay, D. Cameron, M. Rahilly, M.J. Mackean, J. Paul, S.B. Kaye, and R. Brown

Purpose: Loss of function or expression of the mismatch repair protein MLH1 and the tumor suppressor protein p53 have been implicated in acquired resistance to anticancer drugs. We have compared the expression of MLH1 and p53 in tumors from women with clinically node-positive breast cancer before and after primary (neoadjuvant) chemotherapy. Further, we have assessed the value of these markers as predictors of response to therapy by correlation with disease-free survival.

Patients and Methods: Immunohistochemistry scores of MLH1 and p53 expression were made on 36 tru-cut prechemotherapy biopsies and 29 paired postchemotherapy tumor samples. The significance of the change in scores and their correlation with disease-free survival were evaluated by the Wilcoxon signed rank sum test and Cox proportional hazards regression analysis, respectively.

Results: Primary chemotherapy results in a significant reduction in the percent of cells expressing MLH1 ($P = .010$). This change in MLH1 expression after chemotherapy is strongly associated with poor disease-free survival ($P = .0025$). Expression of p53 was not significantly altered by chemotherapy. Neither MLH1 nor p53 expression before chemotherapy predicted disease-free survival or tumor response to chemotherapy. Low MLH1 expression after chemotherapy was an independent predictor of poor disease-free survival on multivariate Cox analysis when considered with other clinicopathologic prognostic factors.

Conclusion: Tumor cells that have reduced MLH1 expression seem to have a survival advantage during combined chemotherapy of locally advanced breast cancers, which supports the hypothesis that loss of MLH1 has a role in drug resistance. MLH1 expression after chemotherapy is an independent predictive factor for poor disease-free survival and may, therefore, define a group of patients with drug-resistant breast cancer.

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BREAST CANCER IS the most common malignancy among women in the Western world. The majority of women present with disease localized to the breast with or without axillary lymph node involvement.[1] Despite radical surgery, more than 50% of surgically treated patients eventually relapse. The introduction of adjuvant treatment (endocrine, chemo-, and radiotherapy) has resulted in a reduction in mortality, with a 25% survival improvement at 10-year follow-up.[2] Metastatic disease, however, remains incurable, with patients becoming progressively less sensitive to systemic therapy.

Primary (neoadjuvant) chemotherapy is increasingly being used in the treatment of primary breast cancer. Its effectiveness in inducing tumor regression and, thus, allowing more conservative surgery (particularly avoidance of mastectomy) is well established.[3-5] Disease-free and overall patient survival have been correlated with the clinical response to primary chemotherapy, although results have been conflicting.[6,7] Studies have failed to predict tumor response to primary chemotherapy using proliferation indices, expression levels of the tumor suppressor gene *p53* or the apoptosis inhibitor *BCL2*, and amplification of the oncogene c-*erbB*-2.[8-10] Data for the *MDR1* gene product P-glycoprotein (*P-gp*) are conflicting,[11] although coexistent p53 and *P-gp* expression has been reported to be an independent prognostic factor for short disease-free survival.[12] Overexpression of c-*erbB*-2 has also been reported as an independent prognostic factor for short disease-free and overall survival,[13] although again data are conflicting.[9]

Several studies have suggested a role for mismatch repair (MMR) deficiency in the development of drug resistance.[14-16] Loss of the MMR protein MLH1 occurs in tumor cell lines selected for resistance to methylating agents, cisplatin, and doxorubicin.[14,15] The promoter of the *hMLH1* gene has recently been shown to undergo hypermethylation in tumors and cisplatin-resistant cell lines, which correlates with loss of MLH1 expression.[17,18] This may be the basis for the high frequency of MLH1 loss, rather than other MMR proteins, observed in tumors and drug-resistant models.[14-17] MLH1-deficient tumor lines with MMR activity

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restored by chromosome transfer are more sensitive than the MMR–deficient parental line to several clinically important agents, including cisplatin, carboplatin, doxorubicin, etoposide, and thioguanine.[16,19] Exposure to such agents in vitro and in vivo results in enrichment of the MLH1-deficient population.[20,21] Loss of MMR leads to an increased rate of frameshift mutations in DNA, which is manifested by genetic instability at repeat sequences in DNA, giving increase to microsatellite instability (MSI).[22] Thibodeau et al[23] examined paraffin-embedded tissue from colorectal tumors for both MLH1 and MSH2 by immunohistochemistry (IHC) and compared the results with detection of MSI and MMR gene mutation analysis. They showed that loss of immunohistochemical staining for these MMR proteins corresponded closely with loss of function of these genes detected by MSI.

Mutation of the tumor suppressor gene *p53* is a frequent genetic change in breast cancer.[24] Although the results of studies vary, mutant *p53* is generally viewed as an indicator of poor prognosis in breast cancer.[25,26] In view of the central role of wild type *p53* in cell cycle control and induction of apoptosis,[27,28] mutation of *p53* may play a role in the development of resistance to chemotherapy. An in vitro study examining chemosensitivity in human breast cancer specimens reported a correlation between mutant *p53* protein expression and enhanced chemoresistance.[29] These findings, however, have not been supported in clinical studies, with mutant *p53* failing to act as an indicator of response to chemotherapy.[8,9,30]

The aim of the present study was to investigate MLH1 and p53 expression in sporadic breast cancer. The study focused on these two proteins because of the limited amount of biopsy material available for analysis, especially before treatment, and previous in vitro studies implicating their importance in drug resistance.[16,17,29] Using samples obtained before and after neoadjuvant chemotherapy, we have examined the effect of chemotherapy on MLH1 and p53 expression and have assessed the correlation of these markers with clinical outcome.

PATIENTS AND METHODS

Patients

Archival paraffin-embedded material was obtained from 36 patients treated with neoadjuvant chemotherapy for primary breast cancer between 1993 and 1997. Samples were obtained from either the Beatson Oncology Center (Glasgow, United Kingdom) or the Western General Hospital (Edinburgh, United Kingdom). Samples consisted of a tru-cut biopsy taken at the time of diagnosis and tumor taken at surgery after chemotherapy. Of the 36 patients, paired samples were available for 29; for seven patients, paired biologic determinants were not available because of the few or no tumor cells present in their samples. Median age of the patients was 47 years (range, 26 to 66 years). The median maximum tumor diameter at diagnosis was 6 cm (range, 2.9 to 15 cm). Other patient characteristics are as listed in Table 1. Eleven patients received continuous-infusion fluorouracil 200 mg/m^2/d, doxorubicin 50 mg/m^2, and cyclophosphamide 600 mg/m^2 every 3 weeks; 16 patients received doxorubicin 20 to 30 mg/m^2 /wk and continuous-infusion fluorouracil 200 mg/m^2/d; and nine patients received epirubicin 50 mg/m^2 and cisplatin 60 mg/m^2 three times weekly and continuous-infusion fluorouracil 200 mg/m^2/d. All patients completed at least 9 weeks of treatment before the repeat biopsy at surgery. Radiotherapy was administered according to local practice after both chemotherapy and definitive surgery. Those who were estrogen receptor-positive were placed on tamoxifen. Response was assessed by bidimensional tumor measurement, obtained retrospectively from patients' case records, evaluated according to International Union Against Cancer criteria, and maintained over at least 4 weeks.[31] Median follow-up was 41 months (range, 30 to 66 months).

Table 1. Patient Characteristics

Characteristics	Patients	
	No.	%
Menopausal status		
Pre	18	50
Peri	3	8
Post	15	42
Stage		
T3	3	8
T4	29	81
T4d	4	11
N0	7	19
N1	24	67
N2	4	11
Histologic grade		
I	1	3
II	10	28
III	19	53
UK	6	17
ER status		
Positive	14	39
Negative	16	44
UK	6	17
Response		
CR	12	34
PR	18	51
SD	2	6
PD	3	9

Abbreviations: UK, unknown; ER, estrogen receptor; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

Immunohistochemistry

IHC was performed on histology sections taken from the tru-cut biopsy and mastectomy specimens. All samples were formalin-fixed and paraffin-embedded. Mouse monoclonal antibodies G168-15 (1/100 dilution; PharMingen, Oxford, United Kingdom) and DO-1 (1/200 dilution; Oncogene Sciences, Cambridge, United Kingdom) were used to detect MLH1 and p53, respectively, using a peroxidase-labeled streptavidin-biotin technique.[32] Slides were deparaffinized in Histoclear (Fischer Scientific Ltd, Loughborough, United Kingdom) then rehydrated through graded alcohols and water. Removal of endogenous

peroxidase activity was achieved by incubation in 0.1% hydrogen peroxide for 20 minutes. Sections were immersed in 10 mmol/L sodium tricitrate buffer pH6, subjected to heat-induced antigen retrieval by microwaving for 15 minutes, and then cooled for 20 minutes. The commercially available Vectastain Elite ABC Kit (Vector Laboratories Burlingame, CA) was used to detect the antigen. Sections were lightly counterstained with hematoxylin. Internal controls were obtained for each slide by omitting the primary antibody. For MLH1, external controls included A2780 cells (positive control for MLH1 and negative control for p53) and A2780/CP70 cells (negative control for MLH1 and positive control for p53).[14,33] Two slides per patient sample were immunostained in separate runs. Slides were scored by a consultant pathologist using light microscopy. A score of 0 to 3 for stain intensity was assigned: no staining = 0; weakly positive = 1; moderately positive = 2; and strongly positive = 3. Percentage staining was assessed both as a percentage figure and according to the scoring system: 0 = 0% to 5%; 1 = 5% to 20%; 2 = 20% to 80%; and 3 = 80% to 100%. A combined immunohistochemical score (H-score) was achieved by multiplying the percent by the intensity score.[34] Both immunostaining and scoring were blinded to clinical outcome.

Statistical Methods

Variables taken into account for statistical analysis included estrogen receptor status, age, menopausal status, size, grade, nodal status, and response to chemotherapy. All information was obtained retrospectively from patient records. The within-patient comparison of MLH1 and p53 scores before and after chemotherapy was assessed using the Wilcoxon signed rank sum test. An association between the p53 and MLH1 scores was determined using Spearman rank correlation coefficient. The univariate examination of the association between disease-free survival and p53, MLH1, and clinical factors was made using the Cox regression analysis. The likelihood ratio P value is quoted. The multivariate analysis of factors associated with disease-free survival was also conducted using Cox (multiple) regression techniques and a forward selection procedure (P to enter = .05, P to remove = .10)

RESULTS

Changes in MLH1 and p53 Expression in Response to Chemotherapy

Paired breast tumor samples from the same patient before and after neoadjuvant chemotherapy were evaluated for MLH1 immunohistochemical (IHC) staining. An example of MLH1 immunohistochemical analysis of paired samples before and after chemotherapy is shown in Fig 1. There was a significant reduction in the percent of cells expressing MLH1 after neoadjuvant chemotherapy (P = .010, n = 28). Matched patient percent MLH1 H-scores before and after chemotherapy are shown in Fig 2. The median percent before and after chemotherapy was 83% and 75%, respectively. Furthermore, there was a reduction in the intensity of staining within cells seen after chemotherapy (P = .068, n = 28). Sixty-one percent of patients had immunostain intensity scores of 2 or greater before treatment compared with 36% after chemotherapy. The overall median MLH1 H-score (multiplying percentage and intensity) after chemotherapy was significantly lower than before chemotherapy (P = .036, n = 28). A subgroup analysis by chemotherapy regimen also showed a reduction in median MLH1 after chemotherapy.

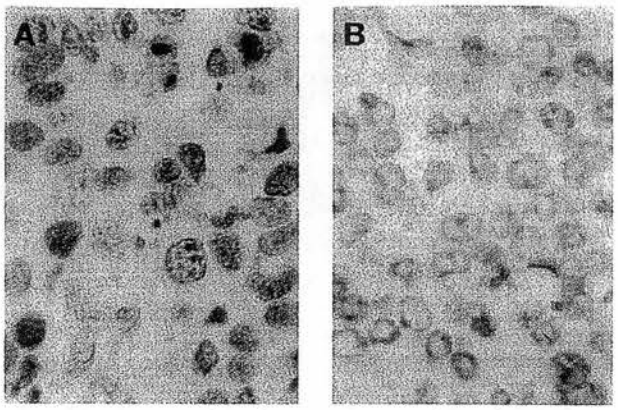

Fig 1. MLH1 immunohistochemistry. (A) Tru-Cut biopsy before chemotherapy; positive for MLH1 immunostaining (brown). (B) Mastectomy specimen with low MLH1 immunostaining. Both sections were counterstained with hematoxylin (blue). Samples are taken from same patient before and after fluorouracil, doxorubicin, and cyclophosphamide chemotherapy (disease-free survival, 5 months). Magnification, ×200.

There was no statistically significant correlation between percent or intensity of MLH1 and p53 staining before or after chemotherapy. There was no significant difference

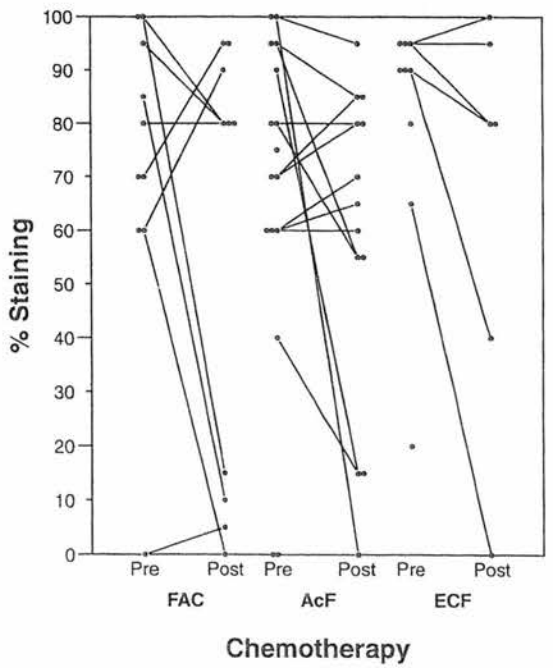

Fig 2. Paired MLH1 scores in breast cancer samples before and after chemotherapy. Percent H-scores are shown for each biopsy sample. Scores joined by a line are from the same patient before and after chemotherapy.

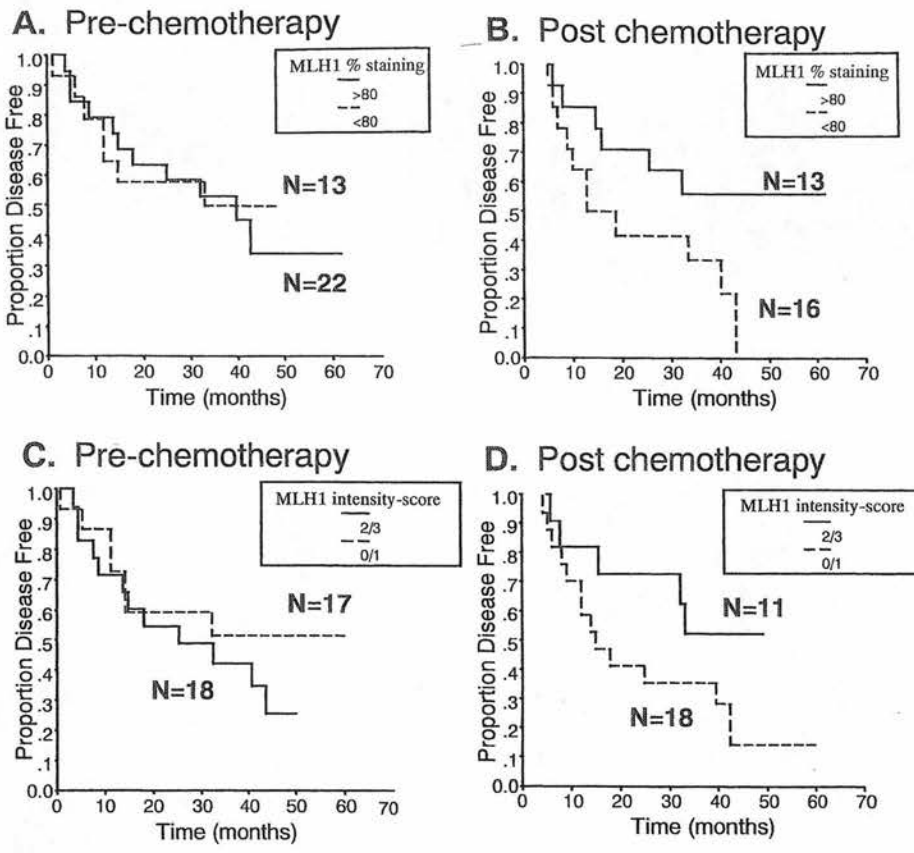

Fig 3. Kaplan-Meier survival curves. MLH1 H-scores before chemotherapy (A, C) and after chemotherapy (B, D). (A) and (B) show percent MLH1-positive; (——) > 80%, (- - - -) < 80%. (C) and (D) show MLH1 intensity; (——) scores of 2 to 3, (- - - -) scores of 0 to 1.

between the p53 H-scores after chemotherapy. Subgroup analysis of p53 H-scores by individual chemotherapy regimen also failed to show any difference before and after chemotherapy.

Univariate and Multivariate Analysis

There was a highly significant correlation between poor disease-free survival and a low level of MLH1 expression, as expressed by percentage staining ($P = .0022$, n = 28) or intensity of staining within cells ($P = .015$, n = 28) obtained in the postchemotherapy samples using Cox regression analysis. In addition, the difference between the pre- and postchemotherapy scores was found to correlate with a poor disease-free survival ($P = .0025$, n = 27). The prechemotherapy MLH1 and p53 H-scores did not predict for response to primary chemotherapy. Furthermore, neither the prechemotherapy MLH1 percent ($P = .32$, n = 33) nor intensity ($P = .38$, n = 33) predicted for disease-free survival. Likewise, prechemotherapy p53 percent ($P = .47$, n = 31) and intensity ($P = .34$, n = 31) did not predict for disease-free survival or postchemotherapy p53 score ($P = .12$, n = 29). Figure 3 shows Kaplan-Meier survival curves for MLH1 percent and intensity IHC scores before and after chemotherapy.

Analysis of individual patients with low MLH1 expression (before or after chemotherapy) has shown they have a short disease-free survival. Three patients had completely lost expression of MLH1 after chemotherapy, and they relapsed at 4, 8, and 15 months, respectively. Two patients had significantly low expression of MLH1 before chemotherapy (0% and 10% of cells positive for immunostaining); these patients progressed at 12 months and 6 weeks, respectively. Of the seven patients for whom paired samples were unavailable, one achieved a complete response (prechemotherapy sample 80% MLH1-positive) and remains in remission at 49 months. Of the remaining six patients, no clear pattern emerged and the distribution of prechemotherapy MLH1 scores was indistinguishable from the others. The failure to produce a postchemotherapy MLH1 score was because of poor sample quality.

A univariate analysis of clinical characteristics showed an association between poor disease-free survival and young age ($P = .001$, n = 34), premenopausal status ($P = .024$,

n = 34), and poor response to chemotherapy ($P = .005$, n = 33). The associations between disease-free survival and estrogen receptor status, nodal stage, tumor grade, and pathologic nodal status after chemotherapy were not statistically significant at the 20% level. A forward stepwise multivariate analysis was conducted using the statistically significant clinical features along with MLH1 intensity, percentage staining, combined IHC score after chemotherapy, and change in IHC score (before and after). This identified percentage staining ($P = .019$), age ($P < .001$), response to chemotherapy ($P = .001$), and menopausal status ($P = .041$) as independent prognostic factors (n = 27).

DISCUSSION

Neoadjuvant treatment provides a system to study in vivo the effect of chemotherapy on the proportion of cells expressing given genes in the initial biopsy compared with the tumor after chemotherapy from the same patient. This provides an ideal opportunity to study clinical drug resistance. A small number of studies have examined the impact of neoadjuvant chemotherapy on biologic marker expression, with changes observed in proliferation indices, proliferating cell nuclear antigen, expression of *P-gp*, and epidermal growth factor receptor.[35-37] However, a consistent picture correlating these markers with clinical drug resistance has not yet emerged. There have been numerous in vitro studies linking the loss of DNA MMR to drug resistance,[14-16] but little is known about its clinical significance. We have shown, for the first time, that combined chemotherapy results in loss of MLH1 expression, and that this is a poor prognostic indicator in patients receiving neoadjuvant treatment for locally advanced breast cancer. Loss of MLH1 expression seems to act independently of the other prognostic factors in this study and to be a good predictor of poor disease-free survival.

These observations are consistent with the hypothesis that tumor cells with reduced MLH1 have a survival advantage during chemotherapy and also support the contention that loss of MLH1 plays a role in drug resistance. MLH1 expression after chemotherapy, but not before chemotherapy, is an independent predictive factor for poor disease-free survival and may, therefore, define a group of patients with drug-resistant breast cancer. Expression analysis before chemotherapy may fail to predict disease-free survival because of an inability to identify resistant subpopulations that are more readily identifiable after chemotherapy. This holds the caveat that postchemotherapy biopsy represents residual and not necessarily clinically resistant disease.

It is conceivable that, rather than causing drug resistance, loss of MLH1 is a marker for other changes that result in a more aggressive tumor phenotype. However, in vitro studies show that restoration of MMR in deficient cells restores drug sensitivity,[16,19] strongly arguing that MMR can have a direct role in drug sensitivity and, therefore, potentially in response of patients to chemotherapy. Acquisition of microsatellite instability phenotype, a marker for loss of MMR, has previously been shown to correlate with reduced survival and poor disease prognosis in breast cancer.[38] Conversely, a microsatellite phenotype correlates with good prognosis in colon cancer.[39] These differences may reflect the different impact of a mutator phenotype on tumor progression (in the case of colon cancer) versus lack of MMR on drug sensitivity (in the case of breast cancer).

How MMR deficiency could lead to the development of drug resistance is not yet fully understood. The MMR protein MutSα (a heterodimer of MSH2 and MSH6) recognizes and binds to sites of DNA damage, such as O^6-methylguanine and 1,2-cisplatin intrastrand crosslinks, and is proposed to lead to recruitment of MutLα (a heterodimer of MLH1 and PMS2 O^6-methylguanine) into the complex.[40,41] This has been suggested to lead to either futile rounds of DNA repair[42] or replication stalling[14] and activation of an apoptotic pathway. For topoisomerase II inhibitors (such as doxorubicin), MMR proteins may serve as a detector of the cleavable complex produced by the binding of the drug to topoisomerase II.[43] Alternatively, doxorubicin is known to participate in redox cycling reactions that produce DNA damage including crosslinks[44] that may be recognized by MMR.

We chose to examine MLH1 expression specifically as this is the MMR protein that has been shown in vitro to be most frequently lost as a result of serial exposure to chemotherapy.[14] An increase in ovarian tumors immunologically negative for MLH1 is observed in samples taken after chemotherapy compared with untreated tumors.[14] The frequent loss of MLH1 may be because of the higher probability of inactivation by methylation of the promoter of this gene.[17,18] Patients in the present study received regimens containing either doxorubicin or cisplatin, drugs exhibiting resistance in vitro associated with loss of MLH1 expression.[14-17]

Consistent with other studies,[36] p53 showed no significant prognostic value before or after combination chemotherapy and no significant change in the levels of protein expression. It should be stated, however, that IHC may not be a reliable measure of p53 function, and controversy exists as to its use in determining the value of p53 as a prognostic factor. We could find no association between MLH1 and p53 expression as assessed by IHC.

Our results should be viewed with caution because of the relatively small patient numbers, different chemotherapy regimens, and the use of a selected patient population. However, this study does demonstrate, for the first time,

clear evidence for loss of MLH1 as a result of chemotherapy in matched pairs of breast tumor samples. Further, it suggests that loss of expression of MLH1 is an important prognostic factor in predicting disease-free survival in this group of patients. Large prospective studies are now necessary to further validate these observations. If loss of MLH1 is confirmed as being involved in clinical drug resistance in breast cancer, this opens up a number of possibilities for improving treatment results, including the development of agents that may be active in MMR-deficient cells or that may be capable of reversal of resistance.

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Dependence on *RAD52* and *RAD1* for anticancer drug resistance mediated by inactivation of mismatch repair genes

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Mismatch repair (MMR) proteins repair mispaired DNA bases and have an important role in maintaining the integrity of the genome [1]. Loss of MMR has been correlated with resistance to a variety of DNA-damaging agents, including many anticancer drugs [2]. How loss of MMR leads to resistance is not understood, but is proposed to be due to loss of futile MMR activity and/or replication stalling [3,4]. We report that inactivation of MMR genes (*MLH1*, *MLH2*, *MSH2*, *MSH3*, *MSH6*, but not *PMS1*) in isogenic strains of *Saccharomyces cerevisiae* led to increased resistance to the anticancer drugs cisplatin, carboplatin and doxorubicin, but had no effect on sensitivity to ultraviolet C (UVC) radiation. Sensitivity to cisplatin and doxorubicin was increased in *mlh1* mutant strains when the *MLH1* gene was reintroduced, demonstrating a direct involvement of MMR proteins in sensitivity to these DNA-damaging agents. Inactivation of *MLH1*, *MLH2* or *MSH2* had no significant effect, however, on drug sensitivities in the *rad52* or *rad1* mutant strains that are defective in mitotic recombination and removing unpaired DNA single strands. We propose a model whereby MMR proteins – in addition to their role in DNA-damage recognition – decrease adduct tolerance during DNA replication by modulating the levels of recombination-dependent bypass. This hypothesis is supported by the finding that, in human ovarian tumour cells, loss of *hMLH1* correlated with acquisition of cisplatin resistance and increased cisplatin-induced sister chromatid exchange, both of which were reversed by restoration of *hMLH1* expression.

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Results and discussion

We examined drug sensitivities of isogenic haploid strains of *S. cerevisiae* that had specific MMR genes disrupted (Table 1; for a review of the biochemistry and genetics of eukaryotic MMR, see [5]). Disruption of the MutS homologues *MSH2*, *MSH3* and *MSH6* and the MutL homologues *MLH1* and *MLH2* (but not *PMS1*) conferred a significant increase in resistance to cisplatin and carboplatin compared with the wild type (Table 1, Figure 1a). Genetic inactivation of *MLH1*, *MSH2*, *MSH3* and *MSH6* also led to increased resistance to doxorubicin. None of the MMR mutants, however, showed any significant increase in resistance or sensitivity to UVC radiation (Table 1). Transformation of the *MLH1* gene back into the *mlh1* mutant strain (*mlh1* + p*MLH1*) led to increased sensitivity to cisplatin and doxorubicin compared with vector-alone controls (*mlh1* + vector; Table 1). Together, these results demonstrate a direct role of MMR proteins in cisplatin, carboplatin and doxorubicin sensitivity.

Loss of MMR usually leads to increased gene mutation rates, leading to a mutator phenotype [6,7]. We examined the MMR mutant strains used for mutant frequency (Table 1) and mutation rate [8]. The wild-type strain had a mutation rate of 3.7×10^{-8} per viable cell, *mlh2* mutants a rate of 6.7×10^{-8} per viable cell and *pms1* mutants a rate of 8.7×10^{-6} per viable cell. The drug-resistance phenotype of *mlh2* mutants and absence of it in *pms1* mutants suggests that Mlh2p, but not Pms1p, has a role in processing of the type of damage induced by these agents. The mutator phenotype in *pms1* but not in *mlh2* mutants argues that loss of MMR activity *per se* (or at least MMR activity requiring Pms1p) need not lead to resistance, and that acquisition of drug resistance is not due to the mutator phenotype of these strains.

The *S. cerevisiae RAD52* gene is involved in meiotic and mitotic recombination [9]. *RAD52* inactivation led to increased sensitivity to cisplatin and UV radiation (Table 1). Inactivation of *MLH1*, *MLH2* or *MSH2* in a *rad52* mutant strain had no significant effect on sensitivity to cisplatin, carboplatin or UV radiation (Table 1, Figure 1b). Thus, inactivation of *RAD52* leads to loss of the resistance mediated by MMR gene inactivation and a sensitisation of the yeast to these agents. This suggests that drug resistance mediated by loss of MMR is dependent on Rad52p activity and implicates a recombination-dependent process in damage tolerance. Possible models for recombinational bypass of lesions during DNA replication, that would be Rad52p dependent, have been proposed previously [10].

Inactivation of *RAD1* also led to increased sensitivity to cisplatin, carboplatin and UV radiation (Table 1). The

Table 1

Sensitivities of MMR mutants to anti-cancer drugs and UV radiation.

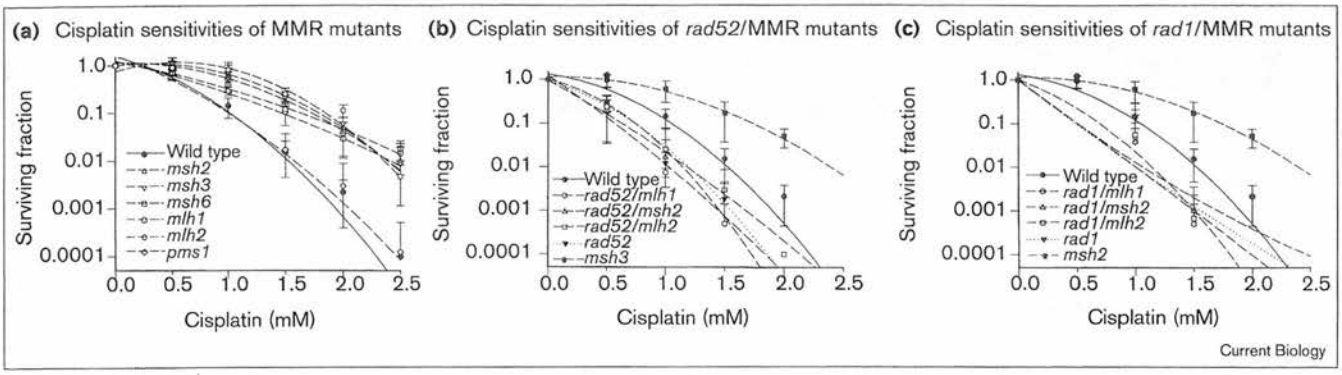
Genotype	Strain	IC90 Cisplatin (mM)	RF	IC90 Carboplatin (mM)	RF	IC90 Doxorubicin (mM)	RF	IC90 UV (J/m^2)	RF	Mutant frequency
Wild type	2096-1B	1	1	13	1	70	1	170	1	1.6×10^{-6}
msh2	RHB 2348	1.7	1.7*	20	1.5*	370	5.3*	210	1.2	2.5×10^{-5}
msh3	RHB 2347	1.8	1.7*	28	2.2*	310	4.4*	130	0.8	n.d.
msh6	NHT 173	1.7	1.6*	29	2.2*	420	6*	190	1.1	n.d.
mlh1	RBT 311	1.5	1.4*	36	2.8*	150	2.1*	150	0.9	4.7×10^{-5}
mlh2	RBT 324	2	2*	40	3.1*	n.d.	–	150	0.9	9.6×10^{-7}
pms1	RBT 269	1.1	1.1	10	0.7	n.d.	–	140	0.8	8.7×10^{-5}
mlh1 + vector	RBT311:v	1.6	1.6*	29	2.2*	120	1.7*	n.d.	–	n.d.
mlh1 + p*MLH1*	RBT311:mlh1	0.5	0.5*	10	0.8	30	0.4*	n.d.	–	n.d.
rad52	RHB 2692	0.7	0.7*	13	1	n.d.	–	70	0.4*	n.d.
rad52/msh2	RHB 2700	0.7	0.7	13	1	n.d.	–	70	0.4	n.d.
rad52/mlh1	RHB 2698	0.5	0.5	12	0.9	n.d.	–	80	0.5	n.d.
rad52/mlh2	RHB 2699	0.6	0.6	13	1	n.d.	–	100	0.6	n.d.
rad1	RBT 302	0.6	0.6*	7	0.5*	n.d.	–	10	0.06*	n.d.
rad1/msh2	RHB 2694	0.6	0.6	7.5	0.6	n.d.	–	13	0.08	n.d.
rad1/mlh1	RHB 2693	0.7	0.7	8	0.6	n.d.	–	11	0.06	n.d.
rad1/mlh2	RHB 2695	0.6	0.6	8	0.6	n.d.	–	11	0.06	n.d.

All strains are isogenic derivatives of a *Matα* wild-type strain. The construction of all of the mismatch-repair-deficient strains except *mlh2* has been described [22]. *MLH2* was deleted using an oligonucleotide-based KANMX disruption cassette. *RAD52* was disrupted with *LEU2* using plasmid pMS20 obtained from D. Schild [23]. *RAD1* was deleted using a *LEU2* disruption/deletion plasmid obtained from R. Keil [24]. IC90, concentration of drug inducing a 90% reduction in surviving fraction. RF, resistance factor relative to the wild-type strain. Those marked with an asterisk are significantly different in drug sensitivity from the wild-type strain or the corresponding single mutant, as assessed by a two-tailed Student's *t*-test at 1.5 mM cisplatin or 15 mM carboplatin. Mutant frequency, the number of L-canavanine-resistant colonies per 10^6 colony forming units; n.d., not determined.

RAD1 gene in *S. cerevisiae* is involved in nucleotide excision repair (NER), but has also been implicated in mitotic recombination [11]. Purified Rad1p interacts with DNA bubble structures, is required for the endonucleolytic cleavage that removes 3′ single-stranded DNA ends [11], and is also required for removal of non-homologous ends during recombination [12]. These are activities that could be required for recombination-dependent DNA-damage bypass by removing bases to allow initiation of replication after bypass [13]. Inactivation of *MLH1*, *MLH2* or *MSH2* had no significant effect on cisplatin or carboplatin sensitivities in a *rad1* mutant strain (Table 1, Figure 1c). These observations are consistent with Rad1p being necessary for increased damage bypass.

We propose a model whereby loss of MMR proteins can increase *RAD52/RAD1*-dependent recombinational bypass of adducts (see Figure 2). Treatment of cells with cisplatin and carboplatin induces predominantly 1,2 intrastrand DNA crosslinks which is believed to be the major cytotoxic lesion [14], although a role for the minor lesions cannot be excluded. The 1,2 intrastrand crosslink induced by cisplatin and carboplatin is poorly repaired, either due to not being recognised by NER [15] or by inhibition of repair, for instance by damage-recognition proteins [16]. Consistent with this, inactivation of *RAD1* in *S. cerevisiae* did not give the extreme hypersensitivity to cisplatin observed for UV radiation (Table 1). Persistent or non-repaired DNA lesions could lead to a cytotoxic signal being generated during DNA replication. The heterodimer of Msh2p and Msh6p — hMutSα —recognises 1,2 cisplatin crosslinks in a complementary duplex DNA and with greater affinity if the platinated guanine residues are opposite non-complementary bases [17]. Bypass of cisplatin DNA lesions during DNA replication has been shown in cisplatin-resistant human cells which correlates with defects in MMR [18]. The mechanisms leading to bypass are largely unknown, but possibilities include recombinational mechanisms, as well as trans-lesion DNA synthesis. It has been shown that MMR proteins can affect levels of homologous recombination in yeast [19,20]. Alternatively, MMR proteins recognising cisplatin adducts may lead to inefficient MMR, which competes with recombinational bypass of the lesion. If *RAD52/RAD1*-dependent recombinational bypass of DNA adducts occurs during replication, then inhibition of, or competition with, either initiation or progression of this process by MMR proteins will lead to sensitivity. Conversely, loss of MMR proteins will reduce the probability of lethal signals being generated during replication by increasing adduct bypass, leading to resistance. Consistent with DNA replication being necessary for MMR-dependent drug sensitivity, we observed a significantly increased resistance of cells exposed to cisplatin during the stationary phase of growth compared with exponentially growing cells — for an exponentially growing wild-type strain of *S. cerevisiae*, the concentration of cisplatin that results in 90%

Figure 1



Drug toxicity was measured by exposing exponentially growing cells in liquid culture for 24 h to cisplatin, plating out 400 cells onto YPD medium and allowing colony formation. Values shown are the means of multiple independent experiments representing at least 15 repeat values at each drug concentration and using independent clonal isolates. Curves through data points represent second order linear regression. Error bars represent 99% confidence limits.

inhibition of clonal growth (ID90) is 1.0 mM, whereas that of stationary cells is 1.8 mM. On the other hand, the cisplatin sensitivity of *msh2* mutants (ID90 = 1.7 mM) is not affected by growth phase.

To examine the potential relevance of the observations made in *S. cerevisiae* to how tumours may acquire resistance to these chemotherapeutic drugs, we examined cisplatin sensitivities in a matched set of human ovarian tumour cell lines. If recombinational bypass of DNA adducts during DNA replication occurred, this would be manifested by a sister chromatid exchange (SCE). A2780 is a human ovarian carcinoma cell line derived from an untreated patient, whereas A2780/cp70 is an *in-vitro*-derived cisplatin-resistant derivative that has lost MMR activity due to loss of *hMLH1* expression [4]. We introduced a human chromosome 3 containing a wild-type *hMLH1* gene into the A2780/cp70 line and showed restoration of Mlh1p expression, MMR activity and partial restoration of cisplatin sensitivity (Table 2). We also observed increased resistance of A2780/cp70 to doxorubicin and the methylating agent N-nitrosomethylurea (MNU) and restoration of sensitivity of the chromosome 3 transfers (Table 2). The restoration of drug sensitivities in the A2780/cp70/ch3 line supports a direct role for *MLH1* in cell death induced by these drugs in these ovarian cells. Furthermore, as shown in Table 2, A2780/cp70 cells, which

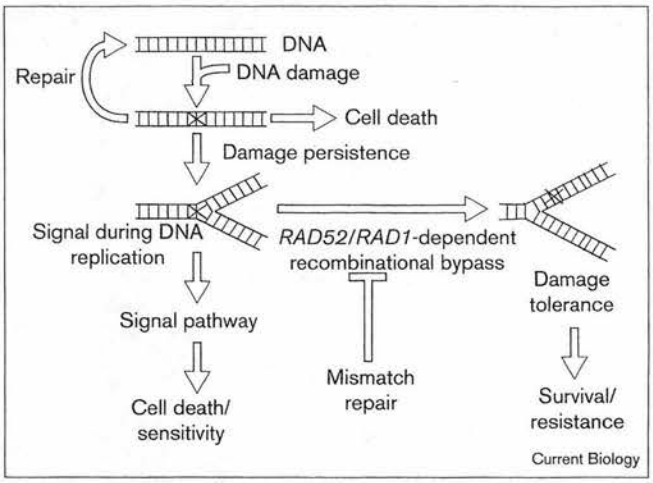
Table 2

Drug sensitivities and SCE induction in ovarian tumour cell lines.

Cell line	MMR status	IC50 MNU (mM)	IC50 Cisplatin (μM)	IC50 Doxorubicin (nM)	Untreated*		Cisplatin-treated*	
					Number of SCEs			
					per cell	per chromosome	per cell	per chromosome
A2780	+	0.2	10	15	1.2	0.026	6.3	0.14
A2780/cp70	–	0.79	65	25	1.5	0.034	16.2	0.37
A2780/cp70/ch2	–	0.65	56	25	2.0	0.044	18.2	0.4
A2780/cp70/ch3	+	0.21	12	16	1.0	0.022	9.6	0.21

A2780/cp70/ch3 and A2780/cp70/ch2 are derivatives of A2780/cp70 that have, respectively, chromosome 3 or 2 introduced by microcell-mediated chromosome transfer. MMR status was determined by *in vitro* assays using plasmid substrates with defined mismatches (P. Karran and O. Humphries, personal communication). Western analysis with hMlh1-specific antibodies (data not shown) indicated that MMR+ lines express hMlh1, whereas MMR– lines do not. IC50 values are the concentrations of MNU, cisplatin or doxorubicin necessary to reduce the surviving fraction of cells by 50%. Cells were treated with MNU after depletion of O^6-alkyltransferase are the mean of multiple independent clonogenic assays. A2780 and A2780/cp70/ch3 are significantly more sensitive to MNU, cisplatin and doxorubicin than A2780/cp70 and A2780/cp70/ch2 as determined by two-tailed Student's *t*-test ($p < 0.01$). *The number of SCEs in exponentially growing cells that were either untreated or treated with 10 μM cisplatin for 1 h. SCEs were quantified by Hoechst staining, followed by Giemsa staining of 5-bromo-2′-deoxyuridine (BrdU)-labelled metaphase spreads. At least 40 metaphases were scored. A2780 and A2780/cp70/ch3 have significantly less SCEs after cisplatin treatment than A2780/cp70 and A2780/cp70/ch2, as

Figure 2



Model of MMR modulation of recombination bypass affecting drug sensitivity. Certain types of DNA damage induced by chemotherapeutic drugs such as cisplatin are poorly repaired and may persist in the genome. We propose that signals are generated during DNA replication of this unrepaired damage that could lead to cell death, but have the potential to be bypassed in a *RAD52/RAD1*-dependent manner that will lead to damage tolerance and cell survival. This recombinational bypass can be inhibited by MMR expression. Thus, loss of MMR leads to increased drug resistance because of increased bypass.

have lost Mlh1p expression, have a higher level of SCEs induced by cisplatin than the MMR-proficient parental A2780 line. Restoration of Mlh1p expression in the A2780/cp70/ch3 line reduced the level of SCEs induced, whereas introduction of chromosome 2 (A2780/cp70/ch2), which does not restore MMR activity, had no effect. These observations are consistent with a chromatid exchange mechanism being modulated by MMR and with the hypothesis that recombination bypass of cisplatin adducts leads to damage tolerance in MMR-defective tumours.

The data presented in yeast and mammalian cells provide evidence for MMR proteins directly affecting cytotoxicity induced by cisplatin, carboplatin and doxorubicin. Although loss of MMR is associated with methylation tolerance in mammalian cells, increased tolerance to methylating agents has not been observed in yeast strains defective in MMR genes, except for *msh5* mutants [21]. This may imply that O^6-methyl guanine induces toxicity by a different mechanism or that this lesion is processed by other repair pathways in yeast masking any effects of MMR on sensitivity to methylating agents. The yeast strains described in the present study will provide a means to examine other anticancer agents and platinum analogues for MMR-dependent cytotoxicity.

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Original Article

A Pilot Study of Continuous Infusional 5-Fluorouracil, Doxorubicin and Cyclophosphamide in Breast Cancer

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Abstract. The purpose of this study was to evaluate the toxicity and activity of continuous infusional 5-fluorouracil (5-FU) given at three dose levels in combination with cyclophosphamide and doxorubicin (FAC) in women with breast cancer.

Thirty-nine patients with either primary tumours >3 cm prior to surgery ($n = 24$) or metastatic disease ($n = 15$) received cyclophosphamide 600 mg/m^2 and doxorubicin 50 mg/m^2 as an intravenous bolus every 3 weeks for six courses. Continuous infusional 5-FU was delivered via a central venous line for a maximum of 18 weeks at dose levels of 100 mg/m^2 per day ($n = 6$), 150 mg/m^2 per day ($n = 3$) and 200 mg/m^2 per day ($n = 30$).

At the 200 mg/m^2 per day dose level, 36% of patients required dose delays and 23% dose reductions; there was one death due to neutropenic sepsis. Hickman line complications occurred at all dose levels, particularly thrombosis (18%) and infection (33%). The response rate was 62% (95% confidence interval (CI) 32–84) for metastatic disease, including five complete responses (CRs). The response rate for primary tumours prior to surgery was 81% (95% CI 57–95) including six clinical CRs.

Infusional FAC is an active regimen and has an acceptable toxicity profile. It does not, however, appear to offer any significant advantage over other chemotherapy regimens. This study does not support the further evaluation of infusional 5-FU at these doses in combination with doxorubicin and cyclophosphamide.

Keywords: Breast neoplasms; Chemotherapy; Infusional 5-fluorouracil

Introduction

The combination of bolus 5-fluorouracil (5-FU), doxorubicin and cyclophosphamide is a standard regimen, with proven activity in the treatment of breast cancer. As primary therapy in women with large tumours (>3 cm), response rates of 70%–87% have been reported [1]; in metastatic disease, the reported response rate ranges between 30% and 70% [2]. Attempts have been made to optimize this regimen by dose escalation with colony stimulating factor support [2]. An alternative approach in view of the short half-life and S-phase specificity of 5-FU is to administer it as a continuous infusion [3].

Phase II studies in solid tumours have demonstrated activity for continuous infusional 5-FU both as a single agent and in combination with other drugs. In breast cancer, single-agent studies have shown response rates of between 12% and 50% using doses ranging from 150 mg/m^2 per day to 500 mg/m^2 per day [4]. In some cases, patients have remained sensitive to infusional 5-FU when they have become resistant to bolus therapy. Higher total doses of 5-FU can be given by continuous infusion with comparable levels of toxicity as for conventional bolus administration.

When this study was devised there had been no direct comparison between FAC (5-FU, doxorubicin, cyclophosphamide) regimens using bolus 5-FU and continuous infusion. The first aim of the study was to identify the dose of continuous infusional 5-FU that could be given with standard doses of adriamycin and cyclophosphamide. The second aim was to make a preliminary evaluation of the activity of infusional FAC. If these data were sufficiently encouraging, a randomized comparison of bolus and infusional FAC was planned. Based on previously published response data for bolus FAC, our criterion for proceeding to a larger trial was a minimum overall response rate for primary tumours and metastatic disease of 90% and 80% respectively.

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Patients and Methods

Patients

Between January 1994 and May 1996, a total of 39 patients were treated in this pilot study. Those who had histologically confirmed breast carcinoma with either large primary (>3 cm) tumours or metastatic disease were eligible if they had an ECOG performance status of 0 or 1 and were aged between 18 and 70 years. For patients with metastatic disease, previous adjuvant chemotherapy was allowed provided it was completed at least 12 months prior to study entry and did not include an anthracycline; prior hormonal therapy and radiotherapy were permitted. Patients had to be deemed able to manage a central venous line and an ambulatory intravenous infusion pump. Written informed consent was obtained in accordance with local ethics committee guidelines.

Initial staging investigations included clinical examination with bidimensional tumour measurement (if appropriate), full blood count, plasma biochemistry, serum liver function tests and chest radiography. Cardiac assessment was by electrocardiogram and echocardiogram. A bone scan and a liver ultrasound examination were performed in patients with metastatic disease or when clinically indicated. Primary tumours were assessed by clinical measurement and mammography was repeated at the end of treatment.

Treatment

All patients received bolus cyclophosphamide 600 mg/m^2 and doxorubicin 50 mg/m^2 intravenously every 3 weeks for a maximum of six courses. In addition, 5-FU was administered as a continuous infusion via a Hickman line using a Walkmed portable infusion pump (Medfusion Incorporated) for 18 weeks. The dose of 5-FU was 100 mg/m^2 per day in the first cohort of patients. Provided that dose-limiting toxicities (e.g. complicated or prolonged grade 4 myelosuppression, grade 3/4 stomatitis or diarrhoea) were present in <2/6 patients, it was planned that the 5-FU dose would be increased to 150 mg/m^2 per day and then to 200 mg/m^2 per day in successive cohorts.

A double-lumen Hickman line was inserted into the superior vena cava under radiographic screening with antibiotic cover (vancomycin 1 mg intravenously). At the start of the study, patients received prophylactic low dose warfarin (1 mg/day). Despite this, several developed Hickman line-associated thrombosis, so subsequent patients received full warfarin anticoagulation (target international normalized ratio (INR) 2 to 3). Patients with line-associated thromboses were managed by thrombolysis using heparin and streptokinase [5].

Patients were assessed clinically every 3 weeks and toxicity was evaluated according to standard WHO criteria. Responses were assessed according to UICC criteria [6,7] and are shown with 95% confidence intervals (CI). Patients receiving neoadjuvant therapy underwent surgery within 4 weeks of completion of chemotherapy.

Statistical Analysis

The time elapsing from the date of starting chemotherapy to first documented treatment failure was considered the disease-free survival and the time to progression in patients with primary tumours and metastatic disease respectively. Death from all causes was taken as the endpoint for overall survival, which was also measured from the date that chemotherapy started. KaplanMeier estimates were used to assess disease-free survival, time to progression and overall survival.

Results

The clinical characteristics of all 39 patients are shown in Tables 1 [8] and 2. Six women were treated at the first 5-FU dose level, three at the second and 30 at the third.

Toxicity

The pattern of toxicities is shown in Table 3. Treatment was generally well tolerated, with the most frequent problems being related to Hickman lines. Of the first 15 patients treated, who all received warfarin 1 mg daily, four (26%) developed line thromboses. Subsequent patients received full anticoagulation with the aim of achieving an INR of 2–3. In total, seven (18%) patients had Hickman line-related thrombosis, all occurring in those whose INR was below the target range. Fourteen (36%) women developed line-related infection; these were mostly minor exit site problems not resulting in disruption to treatment.

Table 1. Characteristics of 24 patients with large (>3 cm) primary tumours

	No. patients
SBR grade [8]	
I	0
II	6
III	11
Unknown	7
ER status	
Positive	6
Negative	8
Unknown	7
Menopausal status	
Premenopausal	12
Postmenopausal	12

SBR, Standardized Bloom Richardson; ER, oestrogen receptor.

Table 2. Characteristics of 24 patients with metastatic disease

	No. patients
Site of disease	
Local recurrence	4
Single visceral site	2
Bone	1
Liver/bone	1
Local/bone	1
Lung/local	3
Lung/liver/bone	2
Local/liver/bone	1
ER status	
Positive	4
Negative	6
Unknown	5
Premenopausal	7
Postmenopausal	8
Previous treatment	
Endocrine alone	1
Radio/chemo/endocrine	6
Radio/endocrine	6
No prior therapy	2

ER, oestrogen receptor.

Table 3. Toxicity of infusional FAC in 39 patients

	WHO grade					
	Dose level 1		Dose level 2		Dose level 3	
	1–2	3–4	1–2	3–4	1–2	3–4
Nausea and vomiting	4	1	1	2	18	2
Haematological	–	3	1	2	2	27
Cutaneous	–	–	1	–	7	–
Mucositis	5	–	3	3	21	6
Lethargy	1	–	2	–	10	–
Diarrhoea	–	–	1	7	–	
Conjunctivitis	–	–	–	–	5	–

Initially, three patients were treated at the first dose level, of whom two developed complications in relation to the Hickman lines (infection and thrombosis not requiring discontinuation of treatment, respectively). An additional three patients were treated at this dose level, one of whom had a line thrombosis but continued treatment. Four of 36 (11%) cycles were delayed owing to incomplete recovery of the neutrophil count by day 22.

As none of the patients at the first dose level had dose-limiting drug-related toxicity, the dose of 5-FU was escalated. Again there were no dose-limiting toxicities. However, of the three patients receiving 5-FU 150 mg/m^2 per day, two had central line infections not related to neutropenia and one had grade 3 vomiting despite prophylactic antiemetics. Only two of 18 cycles (11%) were delayed (owing to vomiting and line infection respectively).

Finally, the dose of 5-FU was increased to 200 mg/m^2 per day. As this was the final dose level, a total of 30 patients were treated in order to define more clearly the pattern of toxicities and antitumour activity. In the third cohort, 12 patients completed treatment without delay and 11 (37%) patients had dose delays. In total, 25 of 144 cycles (17%) given were delayed. The main toxicities were myelosuppression and mucositis, with seven (23%) patients requiring a reduction of 5-FU and one requiring an additional reduction of doxorubicin. There was one death secondary to neutropenic sepsis. Ten women (33%) failed to complete the planned treatment programme owing to: line complications (3), non-specific malaise (4) and progressive disease (3). Four patients developed Hickman line thrombosis; in each case the INR was below the target of 2 when the thrombosis developed. After the introduction of an anticoagulation policy, only three of 24 (13%) patients developed a line-related thrombosis (half the previous rate).

Antitumour Activity

As the number of patients treated was relatively small and clinical activity was a secondary endpoint, responses were considered together for all three dose levels. Response was evaluable only in women receiving at least six weeks of treatment.

Of the 24 patients with large primary tumours, 21 were evaluable, with three failing to complete 6 weeks of treatment. Seventeen (81% CI 57–95) responded, including six clinical complete responses (CRs) and four (19%) with stable disease. All subsequently underwent mastectomy and axillary clearance followed by radiotherapy and/or tamoxifen. There were no pathological CRs, but ten of the 17 responding patients were axillary lymph node negative. To date, seven patients have developed distant metastases but there have been no locoregional recurrences. Thirteen patients remain well. The median disease-free survival has not been reached but 67% (standard error (SE) 10%) are estimated to be still alive and disease free at 3 years. Furthermore, the median survival has not been reached but the estimated percentage still alive at 3 years is 63% (SE 12%).

Of the 15 women with metastatic disease, 13 were evaluable. Eight responded (62% CI 32–84) including five with a CR; a further two had stable disease and three progressed through treatment. Two patients remain in CR 28 and 31 months after treatment. The median time to progression was 6 months (95% CI 4–8). The median survival is 14 months (95% CI 7–21). Those patients with stable disease on completion of therapy progressed at 7 and 5 months.

Discussion

In this prospective pilot study, we have shown that the combination of infusional 5-FU, doxorubicin and

cyclophosphamide is both safe and effective in the treatment of primary breast cancer and metastatic disease.

Having demonstrated the safety and activity of infusional FAC, it is important to put this in the context of other regimens. The benefits of primary chemotherapy have previously been clearly demonstrated, with response rates for conventional chemotherapy combinations lying between 70% and 80%, with a median time to progression of around 20 months [1,9,10]. The combination of infusional 5-FU (200 mg/m^2), epirubicin and cisplatinum (ECF) has shown an improved response rate of 98% [11]. In women with metastatic disease, conventional anthracycline-based regimens show response rates between 30% and 70% and a median survival of 9–30 months [2]. Other continuous infusional 5-FU regimens in combination with doxorubicin [12], epirubicin and cisplatinum/carboplatin [13,14] give higher response rates (76% and 84% respectively) but median survival figures are similar. Although comparisons of different series are subject to criticism, it appears that this infusional FAC regimen offers no advantage over the use of existing drug combinations. The disparity between this combination and other infusional 5-FU regimens may be due to the inclusion of lower 5-FU doses, smaller patient numbers, patient selection and, in the case of those with metastatic disease, the level of pretreatment.

The toxicity profile of this study was dominated by Hickman line complications. The 18% level of line-related thromboses was significantly higher than that of approximately 10% in other studies [4,11–13]. This highlights the importance of dedicated specialist nursing backup and careful anticoagulation. Lokich et al. [3] established that 5-FU could be given safely over prolonged periods in doses up to 300 mg/m^2, with plantar–palmar syndrome and mucositis replacing myelosuppression as the dose-limiting toxicity. In this study, cutaneous toxicity was mild, as was mucositis. Side effects were generally less severe than with infusional ECF, with similar levels of myelosuppression necessitating dose reductions and delayed courses of treatment. This infusional FAC regimen does not appear to offer a significant improvement in side-effect profile over conventional regimens, inparticular over the combination of infusional ECarboF or 5-FU with weekly doxorubicin [12,14].

What is the future for infusional 5-FU? Although we could have escalated the drug dose further, single-agent studies have not proved a dose-related effect beyond 150 mg/m^2 [4]. Infusional 5-FU as part of ECF and ECarboF is undoubtedly very active, although it is not clear if the prolonged infusion contributes beyond bolus administration. Moreover, single-agent studies have shown tumour response in bolus 5-FU refractory tumours [4]. The future for prolonged fluoropyrimidine therapy probably lies in the new generation of oral drugs. Capecitabine is a fluoropyrimidine carbamate given orally that may be targeted to tumour cells by differential expression of the enzyme involved in its activation (personal communication M. Mackean [15]). UFT comprises Tegafur, another fluoropyrimidine, and the dihydropyrimidine dehydrogenase inhibitor Uracil [16]. Both these compounds are active and may replace infusional 5-FU because of better tolerability.

In conclusion, infusional FAC, although safe and effective, does not seem likely to offer any advantages over existing regimens. The response rates for primary and metastatic disease of 71% and 61% did not meet our minimum criteria for proceeding to a larger trial. Given the cost and intensity of labour required to maintain Hickman lines, we did not pursue this combination further.

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