DNA damage repair proteins as determinants of sensitivity to platinum chemotherapy



Submitted by

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ABBREVIATIONS

5-FU	5-fluorouracil
A	adenine
ATM	ataxia telangiectasia-mutated kinase
ATR	ataxia telangiectasia mutated-Rad3-related kinase
BER	base excision repair
CI	confidence interval
CMR	complete metabolic response
CSA	Cockayne syndrome protein A
CSB	Cockayne syndrome protein B
CT-PET	positron emission tomography – computed tomography
DDR	DNA damage repair
DFS	disease free survival
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DSB	double-strand break
EME1	essential meiotic structure-specific endonuclease 1
ERCC1	excision repair cross-complementation group 1
FA	Fanconi anaemia
G	guanine
GG-NER	global genomic nucleotide excision repair
HR	Hazard Ratio
HR	homologous recombination
ICL	interstrand cross-link
IHC	immunohistochemistry

IQR	inter-quartile range
MMR	Mismatch repair
mRNA	messenger ribonucleic acid
MUS81	crossover junction endonuclease MUS81
NER	nucleotide excision repair
NHEJ	non-homologous end joining
OS	overall survival
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PFS	progression free survival
PMR	partial metabolic response
Pol	Polymerase
RNA	ribonucleic acid
RPA	replication protein A
siRNA	small interfering ribonucleic acid
SSB	single strand break
ssDNA	single strand deoxyribonucleic acid
TC-NER	transcription coupled nucleotide excision repair
TLS	translesion synthesis
XPA-G	xeroderma pigmentation complementation groups A - G

ABSTRACT

DNA damage repair proteins are determinants of sensitivity to platinum chemotherapy in preclinical models and in patients with cancer. The XPF-ERCC1 heterodimer incises DNA strands adjacent to platinum-DNA adducts and is essential for repair of platinum-DNA interstrand cross-links (ICLs). High expression of ERCC1 has been correlated with lack of response to platinum chemotherapy, but less is known about the value of XPF in predicting response to platinum-based chemotherapy. MUS81-EME1 is also involved in replication-coupled ICL repair. The role of MUS81 as a biomarker for response to platinum-based chemotherapy has not been examined.

This project tested the hypothesis that high tumour levels of the DNA repair proteins XPF and MUS81 are associated with lack of response to platinum chemotherapy.

In the first part of this thesis, microarray analysis of gene expression in samples from patients with oesophageal adenocarcinomas treated with neoadjuvant oxaliplatin-fluorouracil demonstrated an association between high levels of genes encoding ICL repair proteins (including *ERCC1*, *MUS81* and *EME1*) and poorer clinical outcomes. In addition, functional pathway analysis suggested a link between down regulation of cell cycle and anti-apoptotic pathways and response to treatment.

In the second part, high levels of XPF and MUS81 proteins in pre-treatment biopsies were correlated with worse outcomes following neoadjuvant oxaliplatin-fluorouracil chemotherapy for oesophageal adenocarcinoma. It was found that, in keeping with the microarray results presented in the first part, high pre-treatment levels of the G2/M-phase marker Cyclin B1 were associated with worse clinical outcomes. The relationship between XPF and MUS81 levels and response to DNA cross-linking agents in colorectal and anal cancers was also studied.

In the third part, it was demonstrated *in vitro* that reducing levels of XPF and MUS81 proteins increased sensitivity of colorectal and oesophageal cancer cells to oxaliplatin treatment.

These data suggest that XPF and MUS81 have the potential to be developed as biomarkers for clinical response to oxaliplatin-based chemotherapy.

1. INTRODUCTION

1.1 PLATINUM CHEMOTHERAPY

1.1.1 Platinum chemotherapeutic agents

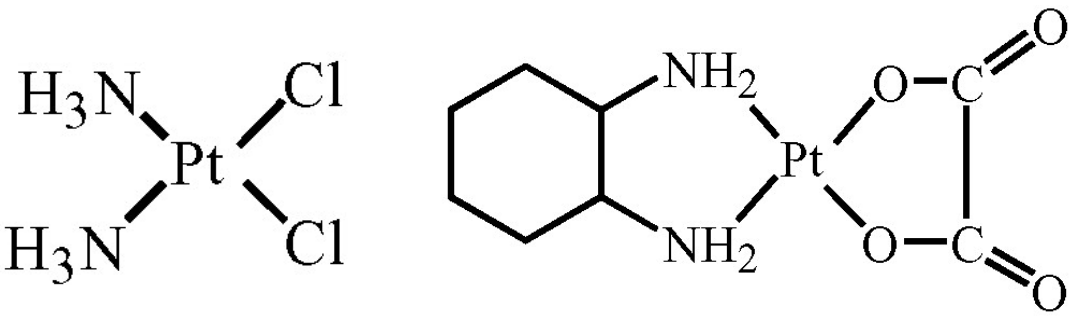
Cell division inhibition by platinum compounds was first observed in 1965 by Rosenberg and colleagues, who were working with *Escherichia coli*. By serendipity, they discovered that passage of current via platinum electrodes into a medium growing *E. coli* caused arrest of division (Rosenberg et al., 1965); they subsequently demonstrated that it was the product of the electrolysis of the media, specifically the platinum salts generated from the electrode, rather than the current itself, that produced the effect (Rosenberg et al., 1969).

The first platinum compound to enter clinical use was cis-diamminedichloroplatinum (II) (cisplatin). It was approved for use as a chemotherapeutic agent in Canada in 1978 (Lebwohl and Canetta, 1998). Cisplatin is noted for a number of toxic side effects, notably to the kidney and cochlea cells; however, the resultant nephrotoxicity can, to some extent, be prevented by concurrent high-volume fluid administration and forced diuresis (Hayes et al., 1977).

Since the discovery of cisplatin, other platinum-based compounds have been engineered and developed with improved therapeutic indices, less toxicity, and better pharmacological characteristics (Lebwohl and Canetta, 1998). The two

alternative platinum compounds that have entered widespread clinical use are carboplatin and oxaliplatin.

Oxaliplatin [l-OHP, oxalate(*trans-l*-1,2-diaminocyclohexane)platinum(II)] is a third generation platinum compound in which the amine groups present in cisplatin are replaced by a diaminohexane (DACH) ligand (**FIGURE 1.1**). It was first synthesized in Japan, and was noted to be less nephrotoxic than cisplatin in mouse models (Mathe et al., 1985); in humans it has subsequently been shown to be less nephrotoxic and myelosuppressive than cisplatin, but it has a higher incidence of neuropathy (Misset et al., 2001). Following work where a lack of cross-resistance to oxaliplatin in a cisplatin-resistant colorectal cancer cell line was demonstrated (Pendyala and Creaven, 1993), successful trials were undertaken in metastatic colorectal cancer, a condition for which this drug is now widely indicated in combination with the anti-metabolite, 5-fluorouracil (de Gramont et al., 2000). Latterly, the REAL 2 study, in patients with advanced oesophagogastric cancer, suggested that oxaliplatin may be superior to cisplatin in this patient group, with improved overall survival (OS) and decreased rates of renal toxicity, neutropenia, alopecia and thromboembolism. These findings have subsequently been supported by a German phase III trial (Cunningham et al., 2008) (Al-Batran et al., 2008).



Cisplatin Oxaliplatin

Figure 1.1. The chemical structures of cisplatin and oxaliplatin. Note that in oxaliplatin, the amine groups are replaced by a diaminohexane (DACH) ligand.

1.1.2 Mechanism of action of platinum chemotherapy

Platinum compounds exert their effect on cells by binding the purine residues on DNA strands. This can result in three different types of adduct: monofunctional adducts, were a single nucleobase residue is bound; intrastrand cross-links, where nucleobases on a single DNA strand are bound together by a molecule of the platinum agent; and interstrand cross-links, where nucleobases on adjacent strands of DNA are linked by the agent, thus bridging between the two strands (**FIGURE 1.2**) (Woynarowski et al., 2000). Although they are structurally different, cisplatin and oxaliplatin form the same types of DNA cross-links at the same sites, and in the same relative distributions. Approximately 60-65% of these adducts are intrastrand 1,2-GG, 25-30% are intrastrand 1,2-AG, 5-10% are intrastrand 1,3-GNG, and the remaining 1-3% are interstrand GG adducts (Chaney et al., 2005; Eastman, 1987).

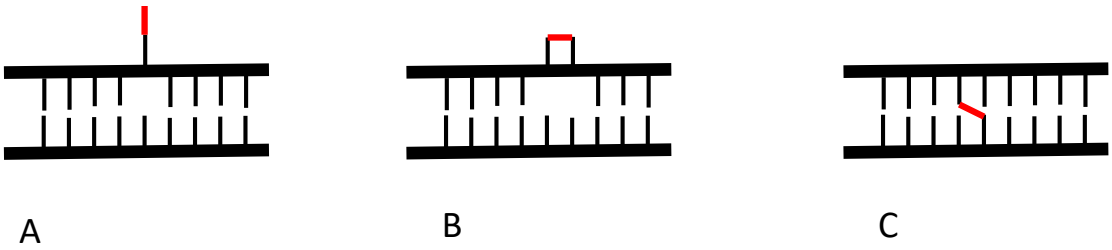


Figure 1.2. DNA adducts formed by platinum compounds. Platinum compounds may form either monofunctional adducts (A), intrastrand cross-links (B) or interstrand cross-links (C) with DNA.

There are, however, some important differences between the interaction of cisplatin with DNA and oxaliplatin with DNA. The lesions formed by oxaliplatin create different distortion of DNA strands due to the presence of the DACH ligand, and this may account for the fact that lower concentrations of oxaliplatin are required for equivalent toxicity to cisplatin (Rixe et al., 1996), despite oxaliplatin inducing fewer lesions in both naked and cellular DNA than equimolar cisplatin (Saris et al., 1996). For example, 1,2-GG intrastrand cross-links due to oxaliplatin bend the DNA by 31° in the direction of the major groove, compared with those with cisplatin which bend the DNA by 60-80°, the result of this is that the minor groove with the oxaliplatin cross-link is relatively narrow when compared with that of cisplatin (Wu et al., 2004). It is known that the DNA damage repair proteins hMSH2 (Fink et al., 1996) and MutS (Zdraveski et al., 2002) bind primarily to the minor groove of DNA, and their preferential binding to cisplatin-GG adducts over oxaliplatin ones is likely due to the narrowing of the minor groove by oxaliplatin excluding their binding (Chaney et al., 2005; Fink et al., 1996; Zdraveski et al., 2002). These differences in DNA deformation between cisplatin and oxaliplatin may go some way towards explaining the different sensitivities exhibited clinically to the two drugs in different tumour types.

These DNA adducts have a number of deleterious effects on cells including DNA strand distortion; prevention of transcription; prevention of DNA replication; stalling and collapse of replication forks; and induction of DNA damage signalling cascades leading to cell cycle arrest or death by apoptosis or necrosis. It is controversial as to which adducts are the most toxic for cells (Ngan et al., 2008; Xu et al., 2009), although there is some evidence that interstrand cross-links are the most toxic lesion (Palom et al., 2002). This is probably due to the complexity of dealing with the situation in which a replication fork has stalled at an interstrand cross-link.

Sensitivity to platinum chemotherapy may be influenced by a number of processes, including intracellular transport; extracellular detoxification by glutathiones (GSH), metallothioneins and antioxidants; removal of platinum-DNA adducts; altered expression of oncogenes; and activation of anti-apoptotic pathways (Rabik and Dolan, 2007).

1.1.3 Recognition and repair of DNA damage caused by cisplatin and oxaliplatin

In order to avoid disruption of cellular function and potential cell death due to platinum-DNA adducts, cells employ a variety of DNA damage repair (DDR) pathways in an attempt to remove the adducts and resume normal cellular function. The majority (65-75%) of these adducts form intrastrand cross-links. Here, there is the advantage that the opposite DNA strand is available as a

template for resynthesis. Pathways that have been implicated in repair of intrastrand crosslinks include nucleotide excision repair (NER), mismatch repair (MMR) and translesion synthesis (TLS). Repair of interstrand cross-links is a more complex process, which is thought to involve elements of NER, TLS and homologous recombination (HR).

1.1.4 Nucleotide excision repair

NER can occur by one of two mechanisms – global genome NER (GG-NER) or transcription-coupled NER (TC-NER). In GG-NER, the whole genome undergoes surveillance for DNA distortions and is active throughout the cell cycle, whereas TC-NER is initiated in response to the stalling of RNA polymerase II (RNA Pol II) by a template strand lesion during transcription. These two types of NER will be discussed in turn.

Global genome nucleotide excision repair

The key protein for detection of damage in GG-NER is XPC, which is assisted in this damage detection by RAD23B and CETN2 (Masutani et al., 1994; Nishi et al., 2005). After XPC binding has taken place, verification of DNA damage occurs with the binding of the TFIIH (transcription initiation factor IIH) complex (Riedl et al., 2003). This complex includes the DNA helicases XPB (Schaeffer et al., 1993) and XPD (Sung et al., 1993). These have opposite polarities, allowing extension of the open DNA configuration around the lesion – it is hypothesised that this permits the presence of the lesion to be verified (Marteijn et al., 2014). Damage verification itself is undertaken by XPA (Camenisch et al., 2006) and XPD (Sugasawa et al., 2009).

The next step of NER is irreversible – strand incision. The incision 5' to the lesion is undertaken by a heterodimeric endonuclease complex composed of xeroderma pigmentosum group F (XPF) protein (encoded by *ERCC4*) and excision repair cross-complementation group 1 (ERCC1) protein (Matsunaga et al., 1995), whilst the incision 3' to the lesion is made by XPG (Habraken et al., 1994). The stabilisation and regulation of these two nucleases is undertaken by replication protein A (RPA) (Matsunaga et al., 1996) and XPA (Riedl et al., 2003).

The final stage of the process is gap-filling synthesis and ligation. This is carried out by proliferating cell nuclear antigen (PCNA) and replication factor C (RFC), acting in concert with DNA polymerases and ligases (Ogi et al., 2010). The polymerases and ligases used depend on the proliferative status of the cells. In replicating cells, DNA Pol ε-dependent repair and ligation by DNA ligase 1 occurs (Ogi et al., 2010). In non-replicating cells, DNA polymerases δ and κ carry out repair (Ogi et al., 2010), with XRCC1-DNA ligase 3 completing the process (Moser et al., 2007).

Transcription coupled nucleotide excision repair

TC-NER is the main route by which platinum adducts are thought to be removed (Furuta et al., 2002; May et al., 1993; Moggs et al., 1997). Stalling of RNA Pol II at a lesion initiates TC-NER (Hanawalt and Mellon, 1993). This leads to recruitment of the Cockayne syndrome proteins CSA and CSB, which in turn allow construction of the NER machinery (Fousteri et al., 2006). This machinery is the same as is constructed for GG-NER, except that a few GG-NER-specific proteins including XPC are excluded (van Hoffen et al., 1995), and there are several TC-NER-specific proteins such as XPA-binding protein II (XAB2) (Fousteri et al.,

2006; Nakatsu et al., 2000) and high mobility group nucleosome-binding domain-containing protein 1 (HMGN1) (Birger et al., 2003; Fousteri et al., 2006). From this point onwards, the incision, gap filling and ligation stages proceed as per GG-NER.

1.1.5 Mismatch repair and futile repair cycles

MMR has two major roles: firstly it acts as a post-replicative repair system, correcting DNA polymerase errors that have escaped proof-reading, thus safeguarding the fidelity of the genome (Umar and Kunkel, 1996). Secondly, it activates cell-cycle regulatory proteins including p73 and c-ABL that induce cell cycle arrest and apoptosis (Gong et al., 1999; Nehme et al., 1999). It is also thought that activation of the MMR pathway causes futile repair cycles, where a futile cycle of synthesis past the DNA lesion is followed by the MMR system recognising and removing the newly synthesised strand, eventually resulting in gaps or strand breaks (Vaisman et al., 1998). This in turn causes activation of ATM/ATR leading to cell cycle arrest or apoptosis (Pabla et al., 2011).

Interestingly, loss of MMR due to a lack of hMSH2 or MutS activity causes resistance to cisplatin, but does not cause resistance to oxaliplatin (Fink et al., 1996; Vaisman et al., 1998; Zdraveski et al., 2002). It is hypothesised that this resistance is due to the fact that hMSH2 (Fink et al., 1996) and MutS (Zdraveski et al., 2002) recognise and bind to the strand deformation caused by cisplatin intrastrand cross-links, stimulating pro-apoptotic pathways, thus loss of function of these proteins promotes resistance to cisplatin. It is further proposed that as the DNA deformation due to oxaliplatin is different, with a relatively narrow minor groove and protrusion of the non-polar DACH ligand into the minor

groove (Wu et al., 2004), the potential binding sites of hMSH2 and MutS are blocked (Zdraveski et al., 2002), therefore rendering this pathway irrelevant in cellular response to oxaliplatin intrastrand cross-links.

1.1.6 Translesion synthesis

Stalling of DNA replication by DNA adducts results in ubiquitination of PCNA by RAD6 and RAD18 (Hoege et al., 2002) and substitution of normal DNA polymerases with specialised TLS polymerases such as pol η (Masutani et al., 1999; McDonald et al., 1999). When the lesion has been bypassed, the replicative polymerases can be switched back in if the TLS has been error-free (Masutani et al., 2000). If there has been a mismatch, then pol ζ is required to extend the mismatch before normal replication can resume (Yuan et al., 2000). Translesion synthesis does not in itself repair damage from platinum-based chemotherapy, but instead confers tolerance to platinum adducts induced in S phase and permits their subsequent removal by post replication repair mechanisms.

Pol η bypasses oxaliplatin-DNA adducts more readily than cisplatin-DNA adducts (Vaisman et al., 2000). This may help to explain why oxaliplatin has a lower mutagenic potential than cisplatin (Chaney et al., 2005). Another TLS polymerase, pol κ, has also been associated with the bypass of platinum-DNA lesions. It forms a complex with pol δ to perform resynthesis of the excised nucleotide fragment in the last stage of NER (Ogi et al., 2010).

1.1.7 Interstrand cross-link repair

Interstrand cross-links (ICLs) are links between two paired DNA strands and cause stalling of replication forks. Although ICLs form <5% of lesions created by cisplatin and oxaliplatin, it is thought that they fundamentally determine

cytotoxicity in cancer cells (Palom et al., 2002). ICLs cause damage to tumour cells by a number of different mechanisms: they can block the progression of replication forks by inhibiting progression of the replisome, they can prevent transcription, and they can distort the chromatin structure, preventing access for DNA-interacting proteins. ICLs can cause tumour cell death by induction of p53 and FAS-ligand dependent apoptosis (programmed cell death) or p53-independent mitotic catastrophe (cell death in the absence of apoptosis due to loss of vital DNA integrity). Multiple pathways are involved in the repair of ICLs (**FIGURE 1.3**); a unique feature of ICL repair is that both DNA strands must be incised to remove the lesion. The repair of ICLs can be either replication-dependent or replication-independent.

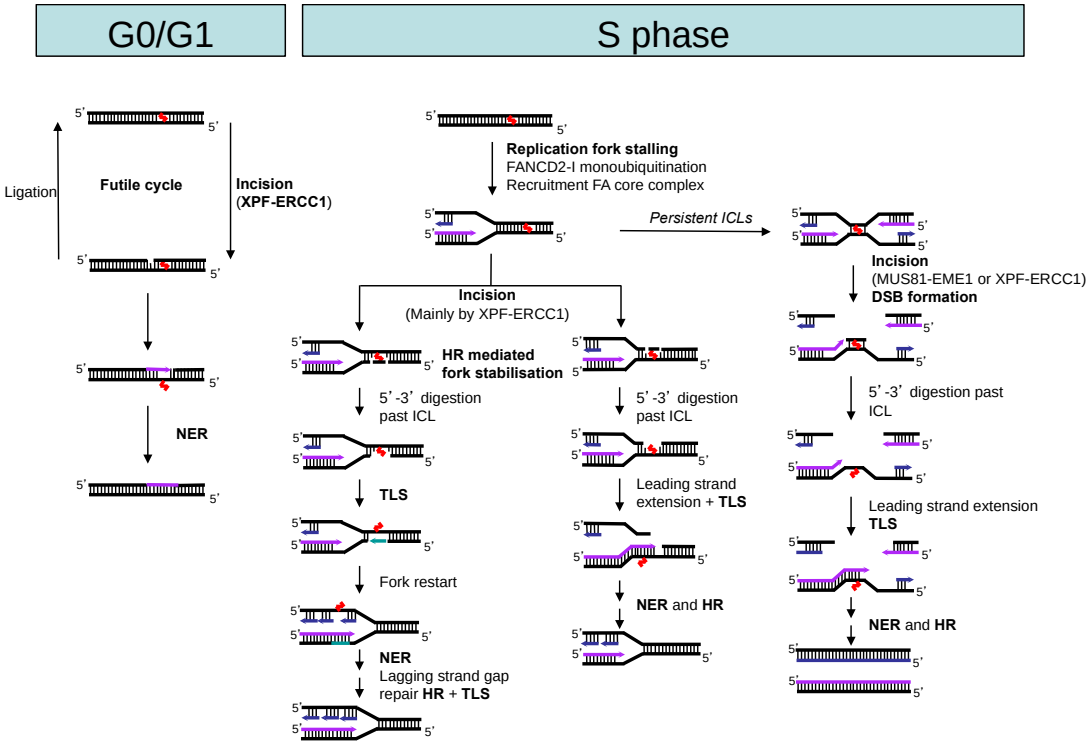


Figure 1.3. Mechanisms of interstrand cross-link repair at different stages of the cell cycle. In G0/G1 phase, nucleotide excision repair (NER) predominates, with the XPF-ERCC1 heterodimer performing the initial incision. Some lesions cannot be repaired by this pathway, and a futile cycle of excision and ligation occurs. In S phase, an ICL will lead to stalling of replication forks. Recruitment of the Fanconi Anaemia (FA) core complex stabilises the stalled replication fork, preventing collapse and the formation of double strand breaks (DSBs). In addition, monoubiquitination of FANCD1-I occurs. This is thought to regulate initial incision by XPF-ERCC1. In ICLs persisting into late S phase, two replication forks may converge on the ICL. In this situation, the MUS81-EME1 heterodimer is required for the generation of DSB ends for HR.

Replication-independent interstrand cross-link repair

There is increasing evidence for the existence of a replication-independent ICL repair pathway, but it is still ill-defined (Guainazzi and Scharer, 2010; Muniandy et al., 2010). This pathway is believed to use the NER XPC-RAD23B complex to recognise the ICL (Zhao et al., 2009). The NER proteins XPB and XPD then work with RPA to unwind the DNA strands before the ICL is "unhooked" by incisions

made by XPF-ERCC1 on the 5' side of the lesion and XPG on the 3' side (Rahn et al., 2010; Staresincic et al., 2009). The TLS polymerases REV1 and pol ζ are then used to bypass the unhooked ICL intermediate (Enoiu et al., 2012; Hlavin et al., 2010; Sarkar et al., 2006; Shen et al., 2006; Wang et al., 2001; Zheng et al., 2003). This leaves a template to repair the remaining strand by NER (Ho and Scharer, 2010). It has been demonstrated that this pathway depends on the TC-NER protein CSB, and the general NER factors XPF and XPG, but not on the GG-NER factor XPC, supporting a hypothesis that replication-independent ICL repair is associated with transcription (Enoiu et al., 2012). It is also possible that ICLs could, in the non-replicative context, stimulate a "futile cycle" of repair, where NER and TLS enzymes mediate nucleolytic cleavage of ICLs, but are unable to complete removal of the covalently liked bases (Mu et al., 2000).

Replication-dependent interstrand cross-link repair

ICLs show their greatest toxicity during S phase, when they cause stalling of the advancing replication fork. It is thought that ICLs persisting into early/mid S phase result in replication fork stalling. If they persist further into S phase, then two replication forks may converge on each side of the ICL (**FIGURE 1.3**).

The first stage of ICL repair is recognition of a stalled replication fork by FANCM (Niedernhofer, 2007). It then recruits the Fanconi anaemia (FA) core complex (Deans and West, 2009), which in turn performs monoubiquitination of FANCD2 (Matsushita et al., 2005) and FANCI (Smogorzewska et al., 2007). The monoubiquitination of FANCD2-FANCI causes it to be retained on the chromatin and recruit the nucleases and polymerases required for the repair process (Matsushita et al., 2005).

Excision of an ICL requires two incisions to be made on the lagging DNA strand – one 5' and one 3' to the lesion. This does not remove the ICL fully from the DNA, as it is still attached to the leading strand, but allows DNA synthesis past the lesion (Ho and Scharer, 2010). A number of nucleases have been implicated in this critical stage of the ICL repair process. These include the structure-specific endonucleases XPF-ERCC1, MUS81-EME1 and SLX1-SLX4, and the endo/exonucleases FAN1, SNM1A and SNM1B. The precise function of each individual nuclease in the ICL repair pathway has not been fully elucidated, and this may reflect the heterogeneity of DNA structures that are encountered during ICL repair. The nucleases may also be involved in downstream events such as the resolution of recombination intermediates. It is known that SLX1-SLX4, MUS81-EME1 and XPF-ERCC1 form a multiprotein complex that can be co-immunoprecipitated from cells (Svendsen et al., 2009). The potential roles of these nucleases are discussed in more detail below.

FANCM also recruits the Bloom's syndrome complex (BTR) (Deans and West, 2009), which activates cell cycle checkpoints via the replication protein A (RPA)-ataxia telangiectasia and Rad3-related (ATR)-CHK1 signalling cascade. Stabilisation of the stalled replication fork occurs via the BRCA1 and BRCA2 complexes, which initiate and regulate HR, with BRCA2 loading RAD51 onto the forks(Long et al., 2011). RAD51 then promotes strand invasion of the sister chromatid by the broken end to allow repair of the DSB by HR. BRCA1 also plays a role in non-homologous end-joining (NHEJ) of DSBs and may aid repair of ICL-associated DSBs by HR-independent mechanisms (Bunting et al., 2012).

Resolution of the DSB allows restoration of the stalled fork and replication to resume.

Following unhooking of the ICL by the action of the nucleases and stabilisation of the fork by HR, the nascent DNA strand must be extended past the lesion. Normal replicative DNA polymerases are unable to function when the replisome is blocked by a cross-link and lesion bypass therefore requires the use of TLS polymerases. A large number of TLS polymerases have been indicated as having potential roles in ICL repair. As in replication-independent ICL repair, REV1 and Pol ζ have been shown to be essential (Mirchandani et al., 2008; Moldovan et al., 2010; Raschle et al., 2008).

1.2 ENDONUCLEASES INVOLVED IN INTERSTRAND CROSS-LINK REPAIR

1.2.1 Role of XPF-ERCC1 in interstrand cross-link repair

As has been discussed above, XPF-ERCC1 is a key nuclease of the NER pathway, where it makes incisions 5' to the lesion. It has been shown that ERCC1 and XPF provide reciprocal stability with XPF containing the nuclease domain (Biggerstaff et al., 1993). Mutations in ERCC1 that disrupt its interaction with XPA, a key NER factor, stop NER but not ICL repair. This supports a hypothesis that the XPF-ERCC1 heterodimer plays a role in ICL repair separate to its function in NER (Orelli et al., 2010). Cells deficient in XPF-ERCC1 are hypersensitive to cross-linking agents (Andersson et al., 1996; De Silva et al., 2000).

Cells defective for XPF and ERCC1 accumulate DSBs following induction of ICLs (Niedernhofer et al., 2004). It is thought that these DSBs are MUS81-induced and represent an aberrant alternative pathway for the critical initial incision when there is a failure of XPF-ERCC1 (Hanada et al., 2006; Wang et al., 2011). This supports a hypothesis that XPF-ERCC1 is involved in the critical initial incision (Kuraoka et al., 2000; Niedernhofer et al., 2004; Wang et al., 2011).

1.2.2 Role of MUS81-EME1 in interstrand cross-link repair

MUS81-EME1 belongs to the XPF-ERCC1 nuclease family, with MUS81 containing the nuclease ERCC4 domain (Ciccia et al., 2008). MUS81-Mms4 is capable of cleaving branched DNA structures in yeast (Boddy et al., 2001). MUS81-EME1 is the human orthologue of these proteins and has the ability to cleave replication forks, 3' DNA flaps and Holliday Junctions (Ciccia et al., 2003; Ogrunc and Sancar, 2003). EME1 null embryonic stem cells are hypersensitive to the DNA cross-linking agents cisplatin and mitomycin C (Abraham et al., 2003).

As has been discussed above, depletion of XPF-ERCC1 leads to an accumulation of DSBs following induction of ICLs. This is suppressed by depletion of MUS81 (Wang et al., 2011). Interestingly, the formation of these DSBs is a relatively late response, occurring in late S/G2 phase, supporting a hypothesis that MUS81-EME1 represents an alternative pathway if XPF-ERCC1 has failed (Sengerova et al., 2011; Wang et al., 2011). In these circumstances, forks may converge more frequently on ICLs, leading to more complex structures, better suited to the endonuclease function of MUS81-EME1.

1.2.3 Other nucleases thought to be involved in interstrand cross-link repair

A number of other nucleases have been implicated in the resolution of ICLs. These include SNM1A, SLX-1-SLX4 and FAN1.

SNM1A is an exonuclease that has 5' to 3' activity and no apparent endonuclease function (Hejna et al., 2007). It is therefore not able to make the critical incisions required in ICL repair; however, cells depleted for SNM1A are sensitive to ICLs (Wang et al., 2011). It has been demonstrated in yeast models that SNM1A is capable of digesting past ICLs from the 5' side and that this can start from a single DNA nick. In the same study it was demonstrated that single and co-depletion of SNM1A and ERCC1 produced the same effect on ICL response, supporting a hypothesis that they participate in the same pathway for ICL processing (Wang et al., 2011). It has been postulated that XPF-ERCC1 provides an initial 5' incision with SNM1A then "trimming" past the ICL to allow unhooking of the ICL.

SLX1-SLX4 is another structure specific endonuclease. SLX1 contains the active nuclease domain (Aravind and Koonin, 2000). It cleaves a variety of structures including 5' flaps, 3' flaps, splayed arms and Holliday junctions (Fricke and Brill, 2003; Wyatt et al., 2013). There is growing evidence, however, that of the two proteins, SLX4 is far more important in the repair of ICLs. Deletion of SLX4 causes far greater sensitivity to ICLs than deletion of SLX1 (Castor et al., 2013). It has been shown that SLX4 co-precipitates with XPF-ERCC1, SLX1 and MUS81-

EME1 (Fekairi et al., 2009). SLX4 has domains for interacting with XPF-ERCC1, MUS81-EME1 and SLX1 (Fekairi et al., 2009; Munoz et al., 2009; Svendsen et al., 2009). It is thought that SLX4 is probably critical for the action of XPF-ERCC1 and SLX1 and works by recruiting them to the sites of ICLs, working as a scaffold protein (Castor et al., 2013). It has been shown that SLX4 potentiates the nuclease activity of XPF-ERCC1 (Hodskinson et al., 2014).

A nuclease has been found to interact specifically with monoubiquitinated FANCD2 – Fanconi-associated nuclease 1 (FAN1) (Kratz et al., 2010; MacKay et al., 2010; Moldovan et al., 2010). FAN1 is able to degrade single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA) exposed ends and also has 5' endonuclease activity, allowing it to cut DNA on the 5' side of an ssDNA-dsDNA junction. This ability makes it possible that FAN1 can incise DNA adjacent to a stalled replication fork causing "unhooking" and so converting the stalled replication fork into a DSB. FAN1 has therefore been proposed as an alternative candidate to XPF-ERCC1 for performing the critical initial incision of ICL repair (Deans and West, 2011; Raschle et al., 2008).

1.3 NEOADJUVANT CHEMOTHERAPY FOR OESOPHAGEAL CANCER

The use of platinum-based neoadjuvant chemotherapy has become standard practice in the UK for the management of operable oesophagogastric cancer (Allum et al., 2009; Cunningham et al., 2006). The OEO2 and MAGIC trials demonstrated improved disease-free survival in those receiving cisplatin-based neoadjuvant therapy with a hazard ratio for recurrence of 0.82 (95% confidence

interval 0.71-0.95, p= 0.03) in the OE02 trial and 0.66 (95% confidence interval 0.53-0.81, p<0.002) in the MAGIC trial (Allum et al., 2009; Cunningham et al., 2006). However, in the OE02 trial, 5% more patients in the neoadjuvant chemotherapy arm were found to have inoperable cancer at surgery than in the group treated with surgery alone (Medical Research Council Oesophageal Cancer Working Group, 2002). This discrepancy in the different trial populations highlights a key concern about neoadjuvant chemotherapy – it potentially delays curative surgery in patients whose tumours do not respond to the chemotherapy used; as a result some patients may progress to inoperable disease.

Despite the potential improvement in outcome offered by neoadjuvant chemotherapy, the outlook for these patients is often poor. For example, neoadjuvant chemotherapy results only in an improvement in 5-year survival from 17.1% to 23% (Allum et al., 2009). It should also be noted that response rates to neoadjuvant chemotherapy remain low; in a recently published trial of two pre-operative cycles of oxaliplatin and 5-fluorouracil, only 52% of patients exhibited a pathological response (defined as Mandard Tumour regression grades 1-3 (Fareed et al., 2010; Reynolds et al., 2007)), meaning that nearly half of these patients were subjected to the risks of multi-agent chemotherapy with no objective evidence of benefit (Gillies et al., 2012). There is therefore a need to develop biomarkers for prediction of response to platinum chemotherapy. This might allow personalised neoadjuvant treatment, whereby drugs to use for chemotherapy are selected on the basis of whether an individual patient's tumour is likely to regress in response those agents. Those that are identified as likely to respond could potentially receive more intensive regimens, and those

predicted to be less likely to respond could be offered alternative chemotherapy (e.g. a taxane (Bader et al., 2008)) or chemoradiotherapy (Stahl et al., 2009).

1.4 DNA DAMAGE REPAIR PROTEIN EXPRESSION AS A BIOMARKER FOR RESPONSE TO PLATINUM CHEMOTHERAPY

Biomarkers have been defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (2001). Broadly, biomarkers can be divided into prognostic and predictive biomarkers. Prognostic biomarkers help tell us the likely course of the disease in an untreated individual. For example, Ki-67 may be used as a prognostic biomarker in breast cancer. Predictive biomarkers identify subpopulations in a group of people with a disease who may benefit from a particular treatment. The use of HER2 status to predict response to Herceptin in breast cancer is an example of a predictive biomarker. Given the role of DNA damage repair pathways in cellular response to platinum chemotherapy, measurement of the DNA damage repair capacity of tumours has the potential to be developed as a predictive biomarker for response to platinum-based chemotherapy.

1.4.1 Evidence for XPF-ERCC1 as a biomarker for response to platinum chemotherapy

The role of XPF-ERCC1 in the repair of platinum-induced ICLs has been extensively studied *in vitro*. High levels of expression of XPF and ERCC1 have been shown to correlate with resistance to oxaliplatin in a variety of cell lines

(Hatch et al., 2014). Levels of *ERCC1* expression are increased in gastric cancer cell lines with acquired resistance to cisplatin, although, interestingly, these cells do not exhibit cross-resistance to oxaliplatin (Tozawa et al., 2008). High pre-treatment tumour levels of ERCC1 mRNA and protein correlate with worse outcomes following platinum-based therapy for patients with gastric, colorectal and lung cancer (Cobo et al., 2007; Metzger et al., 1998; Shirota et al., 2001; Wei et al., 2014).

The results for studies using immunohistochemistry to quantify ERCC1 levels reported in the literature must, however, be interpreted with caution due to ongoing controversy in the scientific community regarding the specificity of the commercially available antibodies (Bhagwat et al., 2009; Friboulet et al., 2013a).

It has been shown that there are four different isoforms of ERCC1, which are generated by alternative splicing of the *ERCC1* gene (designated 201, 202, 203, and 204). With regard to the aims of the research project described here, only one of these isoforms (202) has been shown to be active in the removal of cisplatin-induced DNA adducts (Friboulet et al., 2013a). It has been further demonstrated that ERCC1-202 is the only isoform capable of interacting with XPF to form the functional XPF-ERCC1 heterodimer (Friboulet et al., 2013b). In order to detect ERCC1, sixteen commercially available antibodies were tested by Friboulet *et al*. (including the frequently used 8F1 clone) and each was found to bind at least three isoforms of ERCC1 (Friboulet et al., 2013a), one of which was the ERCC1-202 isoform. The most commonly used antibody in the literature, the 8F1 antibody, has been shown to bind choline phosphate cytidylyltransferase-α

(CCTα), a phospholipid synthesis enzyme regulated by RAS, in addition to ERCC1. To confound the problem of this lack of specificity, CCTα expression itself may be associated with response to platinum chemotherapy (Vaezi et al., 2014). This lack of specificity has resulted in investigators advocating complete avoidance of this antibody in projects aiming to detect ERCC1 (Bhagwat et al., 2009; Friboulet et al., 2013a; Vaezi et al., 2014). A specific antibody to detect the 202 isoform of ERCC1 does not currently exist. There is, therefore, a clinical need to develop alternative biomarkers that provide the same prognostic information as immunoexpression of ERCC1, but more reliably.

XPF forms a functional heterodimer with ERCC1 and represents a potential alternative biomarker for platinum sensitivity. It has been shown that ERCC1 and XPF provide reciprocal stability; assay of one of these proteins is therefore likely to be representative of the functional expression of both (Biggerstaff et al., 1993; Hatch et al., 2014). Furthermore, it has been demonstrated that ERCC1-202 is the only ERCC1 isoform capable of interacting with XPF, and that expression of ERCC1-202 is required for immunodetection of XPF (Friboulet et al., 2013b). In the absence of a specific antibody for the DNA repair active ERCC1-202 isoform, XPF immunodetection therefore provides an attractive alternative for detection of the XPF-ERCC1 heterodimer. The SPM228 mouse monoclonal antibody against XPF has previously been demonstrated by Hatch et al., via knockdown experiments, to be specific for XPF (Hatch et al., 2014).

In oesophageal cancer, low levels of XPF protein have been shown to be associated with *shorter* time to recurrence following neoadjuvant

chemoradiotherapy (Alexander et al., 2012). It should be noted that this study did not specify what chemotherapy was used, it examined a heterogeneous mixture of squamous cell carcinomas and adenocarcinomas, and the results contradict published literature on ERCC1 and *in vitro* data on XPF. In contrast, a study in head and neck cancer patients treated with radiotherapy or platinum-based chemoradiotherapy showed that high XPF expression was associated with shorter time to progression on both univariate and multivariate analysis (Vaezi et al., 2011). These findings were supported by a recent important study in which Leichman et al. demonstrated an inverse relationship between intra-tumoural *ERCC1* gene expression (measured by real time polymerase chain reaction) and progression-free and overall survival in patients with operable esophageal cancer receiving neoadjuvant oxaliplatin, fluorouracil and external-beam radiation therapy (Leichman et al., 2011).

A meta-analysis in patients with non-small cell lung cancer showed that ERCC1 expression is not a prognostic biomarker for patients treated with surgery alone (Hubner et al., 2011). In patients with breast cancer or bladder cancer, it has been shown that *high* levels of ERCC1 and XPF may be associated with *improved* outcomes for patients managed with surgery alone, i.e. the converse of the evidence from the majority of studies that high levels of XPF-ERCC1 are associated with poor response to platinum-based chemotherapy (Alexander et al., 2010; Sun et al., 2012). Taken together, these studies suggest that, if low XPF expression predicts response to platinum-based chemotherapy, it may have clinical utility as a predictive biomarker.

1.4.2 Evidence for MUS81-EME1 as a biomarker for response to platinum chemotherapy

Mouse embryonic stem cells that have knockout of *EME1* are hypersensitive to cross-linking agents (Abraham et al., 2003). High levels of both EME1 and MUS81 have been shown to correlate with resistance to cisplatin in a variety of human cancer cell lines and knock-out of MUS81 or haploinsufficiency of EME1 render colorectal cancer cells sensitive to cisplatin and mitomycin C (Hiyama et al., 2006; Tomoda et al., 2009).

Three studies have examined MUS81 as a potential prognostic biomarker in cancer patients. In 2008 Wu et al. showed that decreased expression of MUS81, both mRNA and protein, was associated with worse overall and disease free survival in patients undergoing resection of hepatocellular carcinomas (Wu et al., 2008), they have further shown that low *MUS81* mRNA was also a marker of poor prognosis in colorectal (Wu et al., 2011) and gastric (Wu et al., 2010) carcinomas. Published studies have only looked at patients treated with surgery alone. No study has yet examined MUS81-EME1 as a predictive biomarker for response to platinum chemotherapy.

1.5 AIMS

1.5.1 Scope of the research project

The remit of this research project is to investigate the role of the DNA damage repair protein heterodimers XPF-ERCC1 and MUS81-EME1 in predicting tumour sensitivity to oxaliplatin-based chemotherapy.

It is known that platinum chemotherapy exerts its cytotoxic effects on tumour cells by a variety of mechanisms, but it is thought that interstrand cross-links represent the most toxic of the lesions induced by these drugs. Repair of these ICLs is a complex process, involving a number of DNA damage repair pathways, including NER, TLS and HR. A critical step in this process is the incision of the DNA strand to allow unhooking of the ICL. XPF-ERCC1 is implicated in this process in both replication-dependent and replication independent ICL repair. MUS81-EME1 is thought to be critical for incisions in the more complex structures found in replication-dependent ICL repair. The activity of these two protein heterodimers may therefore represent potential critical steps in the ICL repair pathway.

It is known that ERCC1 expression is a potential biomarker for response to both cisplatin and oxaliplatin. Measurement of ERCC1 protein expression has, to date, been hampered by the lack of a robust antibody for its immunodetection. The use of XPF protein as an alternative to ERCC1 is attractive as the two proteins provide mutual stabilisation, thus levels of one protein reflect the level of the other protein. Few studies have been performed using XPF immunodetection as an assay of XPF-ERCC1, and its role as a biomarker for response to platinum chemotherapy remains to be defined.

MUS81-EME1 has not been investigated as a biomarker for response to platinum chemotherapy. It is known that high levels of MUS81 and EME1 correlate with resistance to cisplatin in a number of cancer cell lines, but there is no *in vitro* evidence about the effect of MUS81-EME1 expression on cellular sensitivity to

oxaliplatin. MUS81-EME1 therefore represents a potential novel biomarker for response to platinum chemotherapy.

1.5.2 Aims of the research project

This research project aimed to test the hypothesis that high levels of tumour expression of the DNA repair proteins XPF and MUS81 before treatment is associated with lack of response to platinum chemotherapy.

The first aim of this project was to use microarray analysis of gene expression in pre-treatment tumour specimens to assess for a potential association between XPF-ERCC1 expression or MUS81-EME1 expression and clinical response to oxaliplatin chemotherapy. Functional pathway analysis was also carried out to identify other potential expression biomarkers for response to oxaliplatin.

The second aim was to validate immunohistochemical assays for expression of XPF and MUS81 protein levels and then test their potential use as biomarkers for response to ICL-generating chemotherapy in a variety of gastrointestinal tumour types.

The third aim was to test the association between cell cycle distribution and response to oxaliplatin-fluorouracil chemotherapy in oesophageal cancer.

The final aim was to test whether MUS81 expression in vitro affected sensitivity to oxaliplatin in oesophageal adenocarcinoma cell lines.

2. MATERIALS AND METHODS

2.1 GENERAL LABORATORY SUPPLIES

2.1.1 Chemical reagents

Analytical grade chemicals were used in these studies. They were obtained from Invitrogen (Paisley, UK), National Diagnostics (Atlanta, Georgia, USA), Sigma-Aldrich (St Louis, Missouri, USA) and ThermoFisher (Waltham, Massachusetts, USA).

2.1.2 Buffers and solutions

All buffers were made up in water, purified by reverse osmosis with the Millipore system (Merck, Darmstadt, Germany). Buffers prepared in the laboratory are listed below in **TABLE 2.1**. For Western blotting, the running (tris glycine SDS) and transfer (tris glycine + 10% methanol) buffers were obtained from Invitrogen. The blocking buffer used on the membranes post-transfer was from LI-COR (Cambridge, UK). Sterilisation of buffers for cell culture was performed by autoclaving them at a temperature of 121°C and a pressure of 15 psi for 15 minutes.

Table 2.1. Buffers and solutions.

Buffer/Solution	Constituents
Phosphate buffered saline (PBS)	1 PBS tablet in 100 ml purified water
Modified radioimmunoprecipitation assay (RIPA) buffer	50 mM Tris, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 0.1% SDS
SDS-PAGE loading buffer (5X)	25 mM Tris (pH6.8), 2.5% mercaptoethanol, 1% sodium dodecyl sulphate (SDS), 5% glycerol, 0.05 mg/ml bromophenol blue, 1mM EDTA
Blocking buffer	LiCor buffer 1:1 with PBS
Incubating buffer	LiCor buffer 1:1 with PBS, 0.1% Tween 20
Running buffer	5 ml NuPage running buffer stock solution per 100 ml purified water
Transfer buffer	5 ml NuPage transfer buffer stock solution per 100 ml purified water, 10% methanol
Washing buffer	PBS, 0.1% Tween 20
Stripping solution for Western blots	Purified water, 25 mM glycine-HCl, 1% (w/v) SDS
Citrate buffer	2.94g sodium citrate tribasic dehydrate per litre dH_20 corrected to pH 6.0

2.2 EQUIPMENT

Spectrophotometric assessment of assays was performed using the POLARstar Omega plate reader from BMG Labtech (Ortenberg, Germany), with the LI-COR Odyssey scanner (LI-COR, Cambridge, UK) used to visualise Western blots. Colonies from clonogenic survival experiments were counted using an automated colony counter (Oxford Optronics, Milton Park, UK).

Processing of formalin-fixed specimens was performed with a Leica ASP200 S tissue processor (Leica Microsystems, Wetzlar, Germany), paraffin wax embedding on the Histostar embedding workstation (ThermoFisher, Waltham, Massachusetts, USA) and automated immunohistochemistry on the BOND-MAX immunostainer (Leica Microsystems, Wetzlar, Germany).

2.3 HUMAN TISSUE

Oesophageal tissue from oxaliplatin treated patients was prospectively collected as part of a trial in Oxford (Ethics Committee reference number 06/Q1604/24) and stored within a Good Laboratory Practice (GLP) compliant laboratory. The characteristics of these patients and treatment received are outlined in more detail in Section 3.2. Pre-treatment biopsies were taken from the centre of the tumour and from macroscopically normal squamous mucosa 10 cm proximal to the tumour at endoscopy. Post-treatment tumour specimens were taken from the luminal aspect of the middle of the residual tumour on resection specimens. Biopsies of macroscopically normal mucosa were again taken 10cm proximal to the tumour in the resected specimens.

Tissue from oesophageal cancer patients treated with surgery alone was obtained from the Oxford Radcliffe Biobank (ORB). Long-term follow-up data for both groups of were obtained from the Oxford University Hospitals NHS Trust patient administration system, patient's case notes and multidisciplinary team (MDT) meeting records. Where any details remained to be clarified, the patient's general practitioner (GP) was contacted. Patients were considered to have had

disease recurrence on the basis of either histology or imaging alone, if considered conclusive by the MDT.

Samples from oesophageal cancer patient who received neoadjuvant cisplatin were obtained, with follow-up data, from Dr Richard Turkington and the Northern Irish Biobank, Queens University, Belfast. Colorectal cancer tissue samples and outcome data came from ORB and the FOCUS2 triallists. Anal tumour specimens and outcome data were received from Dr Duncan Gilbert, Sussex Cancer Centre, Brighton.

2.4 MEDIA AND CELL CULTURE SUPPLIES

2.4.1 Media

Cells were cultured in either Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich) or Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen). Foetal calf serum, penicillin/streptomycin and trypsin EDTA were obtained from Invitrogen. Complete media contained the base medium with 10% foetal calf serum, 100 units of penicillin and 100 µg/mL streptomycin.

2.4.2 Cell culture supplies

Cell culture flasks (25 cm^2, 75 cm^2 and 175 cm^2), 10 cm dishes, 6- and 96-well plates and pipettes were bought from Corning (Brimingham, UK). Pipette tips and filtered pipette tips were from VWR (Lutterworth, UK).

2.5 CELLS

HCT116 Cells were obtained from Bert Vogelstein (John Hopkins University, Baltimore, USA). Oxaliplatin–resistant HCT116 cells were a gift from Patrick Johnston (Queens University, Belfast, UK). Oxaliplatin-resistant and parental HT29 cells were obtained from Vanessa Almendro (Institutd'Investigacions Biomèdiques, Bareclona, Spain). Oxaliplatin-resistant and sensitive AGS, MKN45 and ST16 cells were received from Srinavasa Madhusudan (University of Nottingham, UK). HCT116 cells haploinsufficient for EME1 (EME1+/-) and deficient for MUS81 (MUS81-/-) were gifted by Professor Miyagawa of Tokyo University, Japan. Characterisation of the cell lines used in these experiments is detailed in **Tables 2.2 – 2.4**.

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2.6 SIRNA

siRNA was bought from Dharmacon (Epsom, UK) (XPF and MUS81) and Qiagen (Crawley, UK) (non-targetting siRNA for negative control). The sequences used can be found in **Table 2.5**.

Table 2.5. siRNA sequences for knockdown experiments.

Target	Sequence
***MUS81* siRNA**	5'-CAGCCCTGGTGGATCGATA-3'
***MUS81* siRNA**	5'-GGGTATACCTGGTGGAAGA-3'
***MUS81* siRNA**	5'-CAGGAGCCATCAAGAATAA-3'
***MUS81* siRNA**	5'-GGGAGCACCTGAATCCTAA-3'
***XPF* siRNA**	5'-CTCGCCGTGTAACAAATGAAA-3'
***XPF* siRNA**	5'-CTCCTTGATGCACCACGTTAA-3'
***XPF* siRNA**	5'-CTGCTCGACACTGACGGGCTA-3'
Non-targetting siRNA (sense)	AllStar Negative Control

2.7 ANTIBODIES

Antibodies were purchased from Abcam (Cambridge, UK), Dako (Cambridge, UK), Leica Microsystems (Wetzlar, Germany), LI-COR Biosciences (Cambridge, UK) and Santa Cruz (Dallas, Texas, USA).

Table 2.6. Antibodies for Western Blotting.

Epitope	Clone	Concentration	Species	Manufacturer
β-actin	mAbcam8226	1:20,000	Mouse	Abcam
XPF	SPM228	1:1,000	Mouse	Abcam
MUS81	MTA302G10/3	1:1,000	Mouse	Abcam
Anti-mouse 680nm	Polyclonal	1:10,000	Goat	LI-COR Odyssey
Anti-mouse 800nm	Polyclonal	1:10,000	Goat	LI-COR Odyssey

Table 2.7. Antibodies for immunohistochemistry.

Epitope	Clonality	Clone/Ref. Number	Concentration	Species	Manufacturer
Cyclin A	Monoclonal	6E6	1:50	Mouse	Leica
Cyclin B1	Monoclonal	7A9	1:100	Mouse	Leica
Cyclin D1	Monoclonal	P2D11F11	1:100	Mouse	Leica
Cyclin E	Monoclonal	13A3	1:200	Mouse	Leica
Ki-67	Monoclonal	MIB-1	1:200	Mouse	Dako
XPF	Monoclonal	SPM228	1:200	Mouse	Abcam
MUS81	Monoclonal	MTA302G10/3	1:1,000	Mouse	Abcam
ERCC1	Polyclonal	FL297	Not Suitable	Rabbit	Santa Cruz

2.8 DRUGS

The oxaliplatin used in this work was sourced from Sigma-Aldrich. It was supplied in its powder form and made up to stock solutions with bimethyl sulphide to a stock concentration of 50 μM. All stock solutions were stored at -20°C and kept for a maximum of one month. Particular care was taken to avoid exposure of oxaliplatin to direct light.

2.9 *IN VITRO* EXPERIMENTAL PROCEDURES

Assistance with *in vitro* experiments was provided by Dr Rebecca Carter and Dr Adam Westbrook. Dr Westbrook performed the Western blotting in Section 6.2 and Dr Carter performed repeats of the experiments in Sections 6.5 and 6.6.

2.9.1 Cell culture

Growth of cells

Cells from frozen stock were thawed rapidly at 37°C and resuspended in complete medium. Following centrifugation, the medium was aspirated and the cell pellet resuspended again in complete medium to remove the residual DMSO. Cells were monitored for viability and confluence ever 24 hours. When 80% confluence was reached, cells were washed with PBS, trypsinised and then diluted in fresh medium. Incubation of cells was carried out at 37°C in 5% CO_2.

Growth of oxaliplatin-resistant cell lines

Following thawing, oxaliplatin-resistant AGS, MKN45 and ST16 cell lines were grown in complete RPMI 1640 medium. HCT116, HT29 and KM12-L4 cell lines were grown in complete DMEM medium. After the first passage, all of the cell lines except HCT116 were permanently cultured in the presence of oxaliplatin. AGS and ST16 cells were grown in media containing 5 μM oxaliplatin, MKN45 in 3.3 μM oxaliplatin, and HT29 and KM12-L4 in 2 μM oxaliplatin. Oxaliplatin-resistant HCT116 cells were grown in complete medium without oxaliplatin, and were spiked with 8 μM oxaliplatin once a month.

Cell storage

Cells for permanent storage were prepared from cultures grown to 70% confluence. These cells were then trypsinised and resuspended in freezing

medium (complete medium for the cell line plus 10% DMSO). After aliquoting into CryoTubes (Nalgene Nunc, Penfield, NY, USA), the suspension was frozen at -80°C for 24 hours and then permanently stored in liquid nitrogen.

2.9.2 siRNA transfection

Cells were transfected with 10 nM siRNA against Mus81 (Dharmacon Mus81 Smartpool) or XPF (Dharmacon XPF siRNA #5, #6 and #7) in serum free media containing a 1/500 dilution of RNAiMax Lipofectamine (Life Technologies, Paisley, UK). Incubation for siRNA depletion was for 48 hours after which cells were re-plated and the siRNA treatment was repeated. After a further 48 hours, cells were harvested for IC_{50} assay and protein quantification, or for use in making cell pellets for IHC. All experiments included a mock-transfected control and a non-targeting control of cells transfected with 10 nM Qiagen Allstars (Qiagen, Crawley, UK) negative control siRNA. Sequences for siRNAs are shown in **Table 2.5**.

2.9.3 Resazurin survival assays

Cells were plated on 96-well plates (1,000-2,000 cells/well depending on cell line and drug dose) and the drug was added for 24 hours (or 72 hours in the case of knockdown experiments). After 7-10 days, resazurin was added to a concentration of 10 μg/mL. Resazurin reduction was measured colourimetrically on an Envision 2103 plate reader (Envision, Waltham, Massachusetts, USA) (560-590nm) after 2 hours of incubation at 37 °C.

2.9.4 Clonogenic survival assays

Clonogenic survival assays were performed using the technique described by Franken *et al.(Franken et al., 2006)* Cells were trypsinised and then suspended in

complete medium for counting. A defined number of cells were plated on 10cm dishes and allowed to attach before being treated with a given dose of the drug. The drug-containing medium was left on for 24 hours before it was removed and replaced with fresh complete medium. Control dishes were treated with and equivalent concentration of DMSO (DMSO was the solvent for suspension of the oxaliplatin). After the cells had grown for a further 10-14 days, the colonies were fixed and stained with 0.4% methylene blue (w/v) in 100% methanol for one hour. After rinsing and drying, the colonies were counted using an automated counter.

After counting, plating efficiency was calculated as the fraction of counted colonies in the untreated control dishes relative to the number of cells plated in those dishes:

$$\text{Plating efficiency} = \frac{\text{number of colonies formed}}{\text{number of cells seeded}} \times 100\%$$

The surviving fraction for each treatment was then calculated as the ratio of the surviving colonies per dish relative to the seeded cell number, corrected for plating efficiency:

$$\text{Surviving fraction} = \frac{\text{number of colonies formed}}{\text{number of cells plated x plating efficiency}}$$

2.9.5 Western blotting

Protein extraction

When cells were ready for harvesting, they were trypsinised and suspended in complete medium. They were then centrifuged and washed in PBS before being centrifuged again to form a cell pellet. The supernatant was then aspirated and the pellet frozen at -80°C. The protein extraction buffer was made up as described in **Table 2.1**, with the protease and phosphatase inhibitors being added immediately before use. The cell pellets were then resuspended in the fresh extraction buffer and the tubes placed on ice for 10 minutes. A further round of centrifuging was performed to harvest the protein-containing supernatant. Quantification of protein concentration in the cell extracts was performed using the Bradford Assay (Bradford, 1976) and a NanoDrop ND-1000 machine.

Gel electrophoresis and blotting

The protein extracts were then diluted with purified water and 5X SDS-PAGE loading buffer (see **Table 2.1**) to achieve 40 µg protein per sample and a 1X SDS-PAGE concentration. Precast 3-8% Tris-Acetate gels were placed in an electrophoresis tank, which was then filled with Tris Glycine SDS running buffer (see **Table 2.1**), taking care to wash out the wells. The samples were boiled in a heat block at 95-100°C for 5 minutes and then loaded into the wells. In addition, 2.5 µl of protein size marker (Bio-Rad Precision Plus Protein Dual Color Standards, Bio-Rad, Hercules, California, USA) was loaded to act as a protein size control.

The gel was then run for 2 hours at 125 V and 25 mAmps. Immediately after running, the gel was removed from the tank and mounted against a PVDF membrane (pre-rinsed in methanol) and placed in a transfer tank, which was filled with transfer buffer (see **Table 2.1**). Transfer was performed for 1.5hrs at 25 V and 250 mAmps. After this, the membrane was removed from the tank and incubated in LI-COR blocking buffer for 1 hour at room temperature.

The primary antibody was diluted to the desired concentration in LI-COR buffer + 0.1% Tween-20 and then incubated with the membrane on a shaker for 1 hr at room temperature (or overnight at 4ºC). The primary antibody was then removed and three five-minute washes of the membrane with washing buffer (see **Table 2.1**) were performed. The membrane was then incubated for 1 hour at room temperature with the fluorescent-labelled secondary antibody on a shaker in a dark box. Three further five-minute washes of the membrane with washing buffer were performed before a final 5-minute wash with PBS. Finally, the membrane was placed on the LI-COR Odyssey scanner for visualisation of binding of the fluorescent secondary antibody.

β-actin was used as a control to check for equal loading of protein samples in each gel. The membranes were re-probed with an antibody using the method described above, with binding to the β-actin again visualised using the LI-COR Odyssey scanner. LI-COR Odyssey software was used to quantify the amount of target protein and β-actin, and all proteins of interest were normalised to the loading control.

Stripping of membranes

When it was necessary to reprobe membranes with a different primary antibody, they were first stripped of binding of the previous antibody. The membranes were washed with PBS for 5-minutes and then shaken in stripping solution (see **Table 2.1**) for 30 minutes. The stripping solution was then removed and the membrane had two further PBS washes, each for 10-minutes, on the shaker. The membrane was then incubated again in LI-COR blocking buffer for 1 hour at room temperature, to prevent non-specific antibody binding. The membrane was then ready for incubation with the primary antibody against the protein of interest as described above.

2.10 IMMUNOHISTOCHEMISTRY PROCEDURES

All immunohistochemistry was performed by the author.

2.10.1 Formalin fixation and paraffin embedding of tissue

Cell pellets for use as immunohostochemistry controls were grown in accordance with the cell culture methods described above. Cells were scraped from plates, washed twice in PBS and spun at 1200 rpm for 5 minutes. Excess PBS was aspirated and 10% neutral buffered formalin was added (twice the volume of the cell pellet) (Sigma-Adrich). Pellets were left in formalin solution at 4°C for 24 hours.

The formalin was aspirated from the pellets, and which were then suspended in an approximately equal volume of 2% agarose (Sigma-Aldrich). The agarose was set for an hour and the agarose/cell mixture was placed in cassettes for tissue processing.

Tissue processing was carried out on the Leica ASP200 S (Leica Microsystems, Wetzlar, Germany) in accordance with the manufacturer's standard protocol. The samples were dehydrated by passage through graded ethanol solutions, starting at 70% ethanol and rising to 100% ethanol. They were then cleared with xylene, before being infiltrated with liquid paraffin. Paraffin wax embedding was performed on the Histostar embedding workstation (ThermoFisher, Waltham, Massachusetts, USA).

2.10.2 General immunohistochemistry methods

Sections were cut with a microtome from formalin-fixed, paraffin-embedded blocks to a thickness of 5μM. They were floated in a water bath (filled with distilled water at 40°C) and mounted on Xtra positively charged glass slides (Leica Microsystems, Wetzlar, Germany). Slides were dried for 36 hours at room temperature. They were stored at 4°C prior to use, and used within 4 weeks of cutting.

Immunohistochemistry was carried out using the Leica BOND-MAX immunostainer (Leica Microsystems, Wetzlar, Germany). This system allowed automated immunostaining of the specimens, except for the final steps of dehydration and coverslipping. Antigen retrieval was performed with either pH 6.0 or pH 9.0 antigen retrieval solutions at 99°C, with the retrieval time and pH optimised for individual tissue types.

Primary antibodies were diluted in Novocastra Bond Primary Antibody Diluent (Leica Microsystems, Wetzlar, Germany). Incubation times were optimised for the different primary antibodies. All incubations took place at room temperature.

Binding of primary antibody was visualized using the Novocastra Bond Polymer Refine Detection Kit (Leica Microsystems, Wetzlar, Germany). This contained a peroxide block, post primary polymer, polymer reagent, 3, 3'-diaminobenzidine (DAB) chromogen and haematoxylin counterstain.

Following staining, specimens were dehydrated though graded ethanol (5 minutes in each of 50%, 70%, 100% and 100% ethanol) before being cleared in Histo-Clear (National Diagnostics, Atlanta, Georgia, USA) and cover-slipped with DPX mountant (Sigma-Aldrich, St Louis, Missouri, USA).

2.10.3 Immunohistochemistry methods for staining DNA damage repair proteins

ERCC1 immunohistochemistry

Experiments were carried out to determine whether immunohistochemical staining of sufficient quality could be obtained with the rabbit polyclonal antibody FL297 (Santa Cruz Biotechnology, Dallas, Texas, USA) on the Leica BOND-MAX automated immunostainer. The results of these experiments are discussed in **Chapter 4**. It was decided that the considerable non-specific cytoplasmic staining occurring with the FL-297 antibody made it a poor choice for detection of level of the XPF-ERCC1 heterodimer, and it was therefore not used further in these studies.

XPF immunohistochemistry

Initially, staining for XPF was carried out by manual immunohistochemistry using the SPM228 mouse monoclonal antibody (Abcam, Cambridge, MA, USA). Gastric mucosa of known high XPF expression was used as positive control, and also as a negative control with the primary antibody omitted. An additional

negative control was performed by staining sections of AGS cell pellets in which XPF expression had been knocked down by RNA interference. Antigen retrieval was performed in a waterbath at 99°C, using 1 mM ethylene diaminetetraacetic acid (EDTA) buffer (pH 8.0). Primary antibody was diluted 1:100 and incubated for 2 hours at room temperature. A horseradish peroxidase based system (EnVision +; Dako, Glostrup, Denmark) was used for detection of primary antibody binding.

To improve reproducibility of XPF staining and for future translational use in clinical laboratories, automated staining for XPF using the Leica BOND-MAX immunostainer was developed as described in **Chapter 4**. Antigen retrieval was carried out for 10 minutes at pH 9.0, and the primary antibody was diluted 1:200.

MUS81 immunohistochemistry

Immunohistochemical staining for MUS81 was performed using the MTA30 2G10/3 MUS81 mouse monoclonal antibody (Abcam, Cambridge, MA, USA). Staining on the Leica BOND-MAX immunostainer was developed as described in **Chapter 4**. Antigen retrieval was carried out for 20 minutes at pH 6.0, with the primary antibody diluted 1:1000. Testicular tissue was used as a positive control, and also as a negative control with omission of the primary antibody. An additional negative control was provided by staining sections of AGS cell pellets in which MUS81 expression had been knocked down by RNA interference. Scoring was undertaken on the basis of nuclear staining for the protein.

2.10.4 Immunohistochemistry methods for staining cell cycle proteins

Cyclin A immunohistochemistry

Cyclin A expression was initially assayed by manual staining with the mouse monoclonal antibody 6E6 (Leica Microsystems, Wetzlar, Germany). Antigen retrieval was carried out in 10mM citrate buffer, pH 6.0, for 17 minutes in a microwave. Primary antibody was diluted 1:50 and incubated for 60 minutes at room temperature. A horseradish peroxidase based system (ABC, Vector Laboratories, Burlingame, CA, USA) was used for detection of primary antibody binding. Tonsil tissue was used as a positive control, and also as a negative control with omission of the primary antibody.

Subsequently, staining was carried out on the Leica BOND-MAX automated stainer. Antigen retrieval was carried out using the standard pH 9.0 buffer for 20 minutes and the antibody was diluted 1:50. Scoring was undertaken looking for nuclear expression of the protein.

Cyclin B1 immunohistochemistry

Cyclin B1 immunohistochemistry was carried out on the Leica BOND-MAX automated immunostainer using the mouse monoclonal antibody 7A9 (Leica Microsystems, Wetzlar, Germany) at 1:100 dilution. Antigen retrieval was performed using the standard pH 9.0 buffer for 30 minutes and staining was carried out in accordance with the manufacturer's protocol. Tonsil tissue was used as a positive control, and also as a negative control with omission of the primary antibody. Scoring was undertaken looking for cytoplasmic expression of the protein.

Cyclin D1 immunohistochemistry

Staining for Cyclin D1 was carried out using the mouse monoclonal antibody P2D11F11 (Leica Microsystems) on the Leica BOND-MAX automated immunostainer at 1:200 dilution. Antigen retrieval was performed using the standard pH 9.0 buffer for 20 minutes and staining was carried out in accordance with the manufacturer's protocol. WI-38 cell line pellets were used as a positive control, and as a negative control with omission of the primary antibody. Scoring was undertaken for nuclear expression of the protein.

Cyclin E immunohistochemistry

Cyclin E immunohistochemistry was carried out on the Leica BOND-MAX automated immunostainer using the mouse monoclonal antibody 13A3 (Leica Microsystems, Wetzlar, Germany) at 1:200 dilution. Antigen retrieval was performed using the standard pH 9.0 buffer for 20 minutes and staining was carried out in accordance with the manufacturer's protocol. Placenta tissue was used as a positive control, and also as a negative control with omission of the primary antibody. Scoring was undertaken for nuclear expression of the protein.

Ki-67 immunohistochemistry

Immunohistochemistry for Ki-67 was initially performed manually using the mouse monoclonal antibody MIB-1 (Dako, Glostrup, Denmark). Antigen retrieval was carried out in 10mM citrate buffer, pH 6.0, for 17 minutes in a microwave. Primary antibody was diluted 1:100 and incubated for 60 minutes at room temperature. A horseradish peroxidase based system (ABC, Vector Laboratories, Burlingame, CA, USA) was used for detection of primary antibody binding. Tonsil tissue was used as a positive control, and also as a negative control with omission of the primary antibody.

Subsequently, staining was carried out on the Leica BOND-MAX automated stainer. Antigen retrieval was carried out using the standard pH 9.0 buffer for 20 minutes and the antibody was diluted 1:200. Scoring was undertaken looking for nuclear expression of the protein.

2.10.5 Scoring of protein expression

Scoring of protein expression was performed by two assessors, one of whom was the author and the other was a Consultant Histopathologist (Professor Runjan Chetty, Dr Ketan Shah or Dr Lai-Mun Wang, Oxford University Hospitals NHS Trust). Slides were scored independently and then the results of scoring compared. In cases of disagreement, both assessors reviewed the slide using a double-headed microscope and a consensus score was reached.

Slides were scored for either nuclear or cytoplasmic expression, depending on the cellular sub-location of the protein in question. The scoring system used varied depending on the type of protein being assayed. For DNA damage repair proteins, a semi-quantitative scoring system was used incorporating both intensity of nuclear expression and percentage of nuclei staining. For the cell cycle markers, scoring was on the percentage of cells expressing the protein of interest.

Scoring of DNA damage repair protein expression

The whole section was assessed, and the area showing the strongest staining was scored at X20 magnification. Two independent assessors scored slides for intensity of nuclear staining, scoring them as absent (0), low (1), moderate (2) or high (3); and percentage of nuclei staining positively - 0% (0), 1-9% (1), 10-49%

(2) or 50-100% (3). These two scores were multiplied together to give a semi-quantitative composite score (0-9). In cases of disagreement, the assessors examined the slide together using a double-headed microscope and a consensus was reached. Due to the relatively small number of patients available for analysis, high expression was defined *a priori* as a greater than median composite score.

Scoring of cell cycle protein expression

The whole section was assessed and the area showing the strongest staining was scored at X20 magnification. Slides were scored by two independent assessors and the percentage of cells staining positive for the protein of interest was counted and rounded to the nearest 5%. Sections containing less than 5% positive cells were considered negative for expression of the protein of interest. In cases of disagreement, the assessors examined the slides using a double-headed microscope and a consensus was reached. Due to the relatively small number of patients available for analysis, high expression was defined *a priori* as a greater than median percentage of cells staining positive for the antibody of interest.

2.11 MICROARRAY ANALYSIS OF GENE EXPRESSION LEVELS

Fresh tumour specimens (either from biopsies or from whole resection specimens) were obtained and RNA was extracted using the Qiagen AllPrep DNA/RNA Micro Kit (Qiagen Inc., Valencia, CA) in accordance with the manufacturer's instructions. Contiguous tissue was taken for formalin fixation and paraffin embedding to allow histological confirmation of the presence of

tumour. RNA purity was verified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). mRNA extraction was performed by Mr Richard Gillies.

Microarray analysis was carried out by the Microarray Facility of the Oxford Genomics Centre, under the supervision of Dr Dilair Baban. Extracted mRNA samples were hybridised to Illumina HumanHT-12-v3 Expression BeadChips (Illumina, San Diego, CA) to obtain raw gene expression data. These were processed using the Illumina GenomeStudio software (v1.6), to generate expression intensities. Analysis of gene expression data was performed by Dr Natasha Saghal and Dr Christiana Kartsonaki in the Department of Oncology, Oxford University.

2.12 STATISTICAL METHODS

Disease-free survival (DFS) was defined as time from date of consent until disease recurrence, or death without recurrence. Overall survival (OS) was taken from date of consent until date of death from any cause. Censoring of survivors was performed at the last date they were known to be alive and disease-free (DFS) or alive (OS). DFS and OS curves were calculated using Kaplan-Meier methods and tested for significance of IHC results by logrank (Mantel Cox) test. In cases where the shape of the Kaplan-Meier curve indicated a possible change in proportionality of hazards with time, the Generalised Wilcoxon test was performed to test for early differences in survival that might

not be detected by the logrank test (Leton and Zuluaga, 2005). Effects of gene expression on survival were assessed using a Cox proportional hazards model.

Data were analysed using R statistical software (v2.15) (http://www.R-project.org)(R development Core Team, 2012) with BioConductor packages (Gentleman et al., 2004), limma (v3.12.3)(Smyth, 2005), vsn (v3.24) and survival (v2.36.13), for microarray analyses and SPSS software version 19.0 (IBM Inc., Armonk, NY, USA) for all other analyses. A *P*-value of <0.05 was considered statistically significant.

For analysis of mRNA microarray results, differential expression between the experimental groups was assessed by generating relevant contrasts corresponding to the two-group comparison. Hazard ratios for OS and DFS according to gene expression level were calculated using Cox regression (Cox proportional hazards model). For functional pathway analysis, the Gene Set Analysis (GSA) R package was used to determine the significance of pre-defined sets of genes (the biocarta gene sets) with respect to an outcome variables.

For analysis of IHC results, categorical outcomes (one-year DFS and pathological response) were assessed for relationship with level of DNA repair protein expression (high or low) using Pearson Chi-squared test. Odds ratios (OR) were also calculated. Inter-observer variability was tested using the kappa statistic.

3. MICROARRAY ANALYSIS OF GENE EXPRESSION IN OESOPHAGEAL ADENOCARCINOMA TREATED WITH OXALIPLATIN-FLUOROURACIL CHEMOTHERAPY

3.1 INTRODUCTION

In this chapter, the association between expression of DNA damage repair (DNAR) genes and response to oxaliplatin-fluorouracil chemotherapy is examined. Analysis of gene expression as a predictor of clinical response to oxaliplatin-fluorouracil chemotherapy was performed in 38 patients with oesophageal adenocarcinoma who either proceeded to tumour resection or had progressive disease. Expression of 280 DNAR genes (**TABLE 3.1**) was tested for association with pathological response, one-year disease free survival (DFS), DFS and overall survival (OS).

The hypothesis to be tested was that high levels of expression of genes encoding the key ICL repair proteins XPF, ERCC1, MUS81 and EME1 is associated with worse outcomes following neoadjuvant oxaliplatin-fluorouracil chemotherapy in oesophageal cancer.

In addition, functional pathway analysis of the gene expression data was used to identify other potential pathways associated with response to oxaliplatin-fluorouracil chemotherapy in oesophageal cancer.

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3.2 PATIENT CHARACTERISTICS

Microarray analysis of gene expression was performed on tumour samples from 38 patients with oesophageal adenocarcinoma who received two cycles of neoadjuvant oxaliplatin and infusional 5-FU as part of a prospective trial. Patients received two cycles of chemotherapy. A cycle of treatment lasted 21 days and consisted of oxaliplatin (130 mg/m^2 IV) on day 1, followed by fluorouracil (1000 mg/m^2/day IV) on days 1-4. Initiation of cycle 2 was dependent on an absolute neutrophil count ≥1500/mm^3, platelets ≥100,000/mm^3, and creatinine clearance >50 ml/min. In the event that these criteria were not met, treatment was delayed by one week for up to three weeks. Dose reduction was permitted, based upon the most severe grade of any single toxicity observed. Restaging was performed by PET-CT. Responding or stable patients were operated on 4-6 weeks after completion of chemotherapy.

Thirty-five patients went on to have resection of their tumours and thus had post-treatment resection specimens. The characteristics of the patients are shown in **Table 3.2** and the treatment received by the patients is summarised in **Figure 3.1**. Response to treatment in these patients was determined by metabolic response (measured by CT-PET), pathological response, disease free survival (DFS) and overall survival (OS).

Pathological response was determined using the method described by Mandard *et al*. This method grades pathological response from Grades 1 to 5. In Grade 1

response, no residual tumour cells are seen on examination of resected tumour specimens; in Grade 2 response, scanty tumour cells are seen within the fibrosis; in Grade 3 response, there is an increase in the number of residual cancer cells, but fibrosis still predominates; in Grade 4 response, residual cancer outgrows the fibrosis; and Grade 5 response shows a complete absence of regressive changes (Mandard et al., 1994). Patients exhibiting Grades 1-3 were considered to be pathological responders, and those exhibiting Grade 4 and 5 responses were classified as pathological non-responders (Fareed et al., 2010; Reynolds et al., 2007). Of the 35 tumours that underwent resection, and for whom pre-treatment biopsy samples were available, 19 showed pathological response. The three patients that had progressive disease and did not undergo resection were considered to be pathological non-responders for the purpose of analyses.

	Age	Sex	Tumour site	Tumour type	Tumour distance from incisors	Stage at trial entry	Differentiation
Patient 1	67	M	Lower third	Adenocarcinoma	36cm	IIA	G3
Patient 2	69	F	Lower third	Adenocarcinoma	30cm	III	G1
Patient 3	52	M	Lower third	Adenocarcinoma	32-42cm	III	G2
Patient 4	70	M	OGJ type 1	Adenocarcinoma	36-42cm	III	G2
Patient 5	68	F	OGJ type 1	Adenocarcinoma	36-39cm	IIB	G3
Patient 6	63	M	Lower third	Adenocarcinoma	33cm	IIA	G3
Patient 7	71	M	OGJ type 1	Adenocarcinoma	35cm	IIA	G3
Patient 8	53	M	Lower third	Adenocarcinoma	32-38cm	IIB	G2
Patient 9	68	M	OGJ type 1	Adenocarcinoma	38-45cm	III	G2
Patient 10	60	M	OGJ type 1	Adenocarcinoma	40cm	IIA	G3
Patient 11	73	M	Lower third	Adenocarcinoma	36cm	IIA	G3
Patient 12	77	M	Lower third	Adenocarcinoma	35cm	III	G3
Patient 13	29	M	OGJ type 2	Adenocarcinoma	45cm	III	G2
Patient 14	57	M	Lower third	Adenocarcinoma	37cm	IIB	G3
Patient 15	59	F	Lower third	Adenocarcinoma	31-38cm	III	G2
Patient 16	65	F	Lower third	Adenocarcinoma	30-35cm	III	G3
Patient 17	61	M	Lower third	Adenocarcinoma	40cm	IIA	G3
Patient 18	54	M	OGJ type 1	Adenocarcinoma	38cm	IIA	G3
Patient 19	60	M	Lower third	Adenocarcinoma	37cm	III	G2
Patient 20	69	F	Lower third	Adenocarcinoma	37cm	III	G3
Patient 21	76	M	Lower third	Adenocarcinoma	35cm	IIA	G2
Patient 22	73	M	OGJ type II	Adenocarcinoma	41cm	IIA	G2
Patient 23	78	F	Lower third	Adenocarcinoma	32cm	III	G1
Patient 24	58	F	Lower third	Adenocarcinoma	35cm	IIA	G2
Patient 25	65	M	Lower third	Adenocarcinoma	31cm	IIA	G2
Patient 26	70	M	Middle third	Adenocarcinoma	28cm	IIB	G2
Patient 27	67	M	Lower third	Adenocarcinoma	36cm	IIA	G3
Patient 28	78	M	Lower third	Adenocarcinoma	35cm	IIA	G3
Patient 29	65	M	OGJ type 1	Adenocarcinoma	30-35cm	III	G3
Patient 30	69	M	OGJ type 1	Adenocarcinoma	40cm	IIB	G2
Patient 31	77	M	Lower third	Adenocarcinoma	31cm	III	G2
Patient 32	67	M	OGJ type 2	Adenocarcinoma	41cm	IIB	G3
Patient 33	65	M	OGJ type 1	Adenocarcinoma	39cm	III	G3
Patient 34	56	F	OGJ type 2	Adenocarcinoma	35cm	III	G3
Patient 35	75	M	Lower third	Adenocarcinoma	36cm	III	G3
Patient 36	78	M	Lower third	Adenocarcinoma	30cm	III	G3
Patient 37	63	M	Middle third	Adenocarcinoma	30cm	III	G3
Patient 38	74	M	Lower third	Adenocarcinoma	38cm	IIA	G3

Table 3.2. Characteristics of the patients who received neoadjuvant oxaliplatin-fluorouracil chemotherapy in the clinical trial.

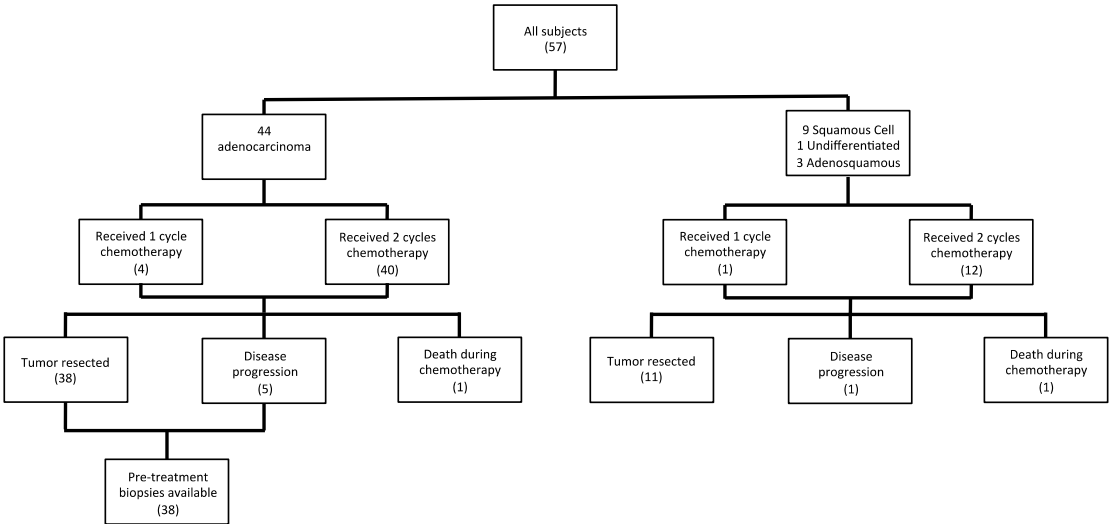


Figure 3.1 Treatment received by patients entering the trial of neoadjuvant oxaliplatin-fluorouracil chemotherapy in oesophageal cancer. Patients are grouped by tumour type and tissue available for analysis. Five patients failed to complete protocol chemotherapy – three patients experienced toxicity during the first cycle, and so only received one cycle and there were two deaths during chemotherapy. One patient, who was not neutropaenic, died from pneumonia on the sixth day of cycle one. The other patient died on the eleventh day of cycle one. Despite a post-mortem, no cause of death was found for this patient.

Median survival for the adenocarcinoma patients treated with oxaliplatin-fluorouracil was 730 days, this compared favourably with the median survival of 512 days seen in the patients treated with chemotherapy followed by surgery (CS) in the OE02 trial. Likewise, slightly better rates of complete (R0) resections were seen, with decreased rates of macroscopically involved (R2) resection margins and equivalent failure to resect tumour after neoadjuvant treatment were seen when the oxaliplatin-fluorouracil treated adenocarcinoma patients were compared with the OE02 patients (**TABLE 3.3**)(Medical Reseach Council Oesophageal Cancer Working Group, 2002; Allum et al., 2009).

Table 3.3. Comparison of resection outcomes between oesophageal adenocarcinoma patients receiving oxaliplatin-fluorouracil chemotherapy and patients in the OE02 trial.

Completeness of resection	Oxaliplatin-fluorouracil patients	OE02 patients
R0 (complete resection)	65.8%	60.0%
R1 (microscopic margin involvement)	21.1%	18.0%
R2 (macroscopic margin involvement)	0.0%	9.0%
Tumour not resected	13.2%	13.0%

Logistic regression was carried out to test for an association between pathological response and the potential prognostic factors of age, sex, tumour stage and nodal status. Lymph node involvement was associated with a significantly lower probability of pathological response (P = 0.012); no other potential prognostic factors were associated with pathological response. The Cox proportional hazards model was used to test for an association between the potential prognostic factors and DFS and OS. There was no association between age, sex or tumour stage and pathological response, DFS or OS (**FIGURES 3.2 – 3.5**). Nodal involvement was associated with worse DFS (HR 2.69, P = 0.023) (**FIGURE 3.6**) and worse OS (HR 3.04, P = 0.024) (**FIGURE 3.7**).

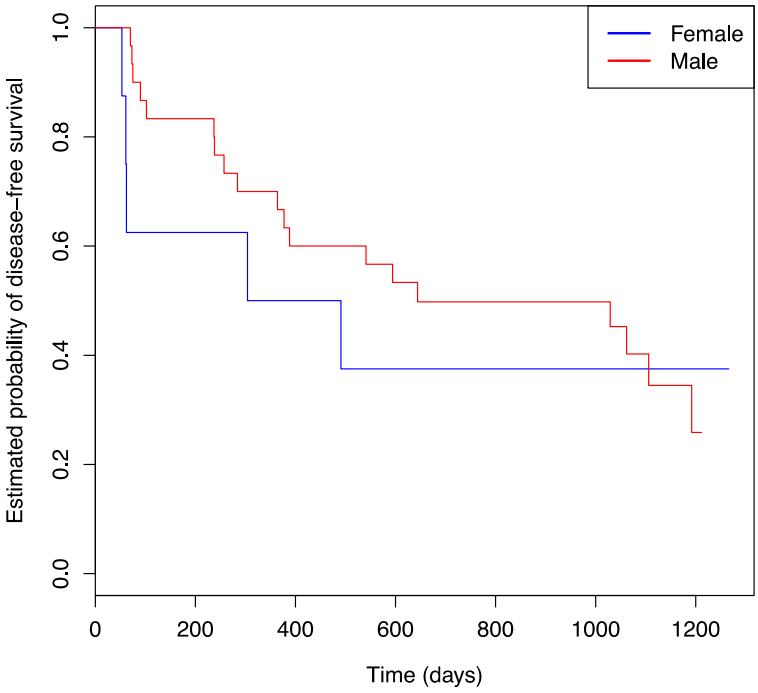


Figure 3.2. Relationship between patient sex and disease free survival in oesophageal adenocarcinoma treated with neoadjuvant oxaliplatin-fluorouracil chemotherapy. No association was found between patient sex and DFS (HR = 0.765, *P* = 0.594).

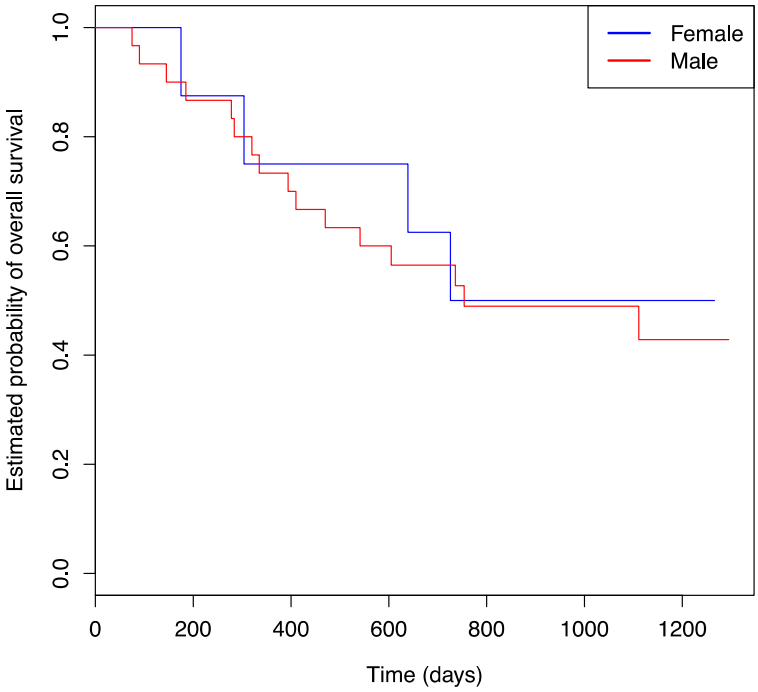


Figure 3.3. Relationship between patient sex and overall survival in oesophageal adenocarcinoma treated with neoadjuvant oxaliplatin-fluorouracil chemotherapy. No association was found between patient sex and OS (HR = *P* =) (HR = 1.123, *P* = 0.836).

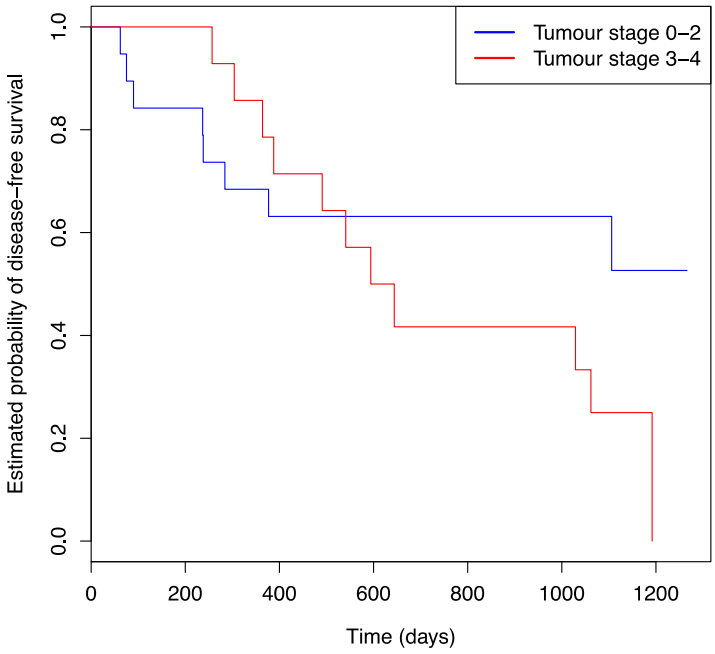


Figure 3.4. Relationship between tumour stage and disease free survival in oesophageal adenocarcinoma treated with neoadjuvant oxaliplatin-fluorouracil chemotherapy. No association was found between tumour stage and DFS (HR = 1.938, *P* = 0.161).

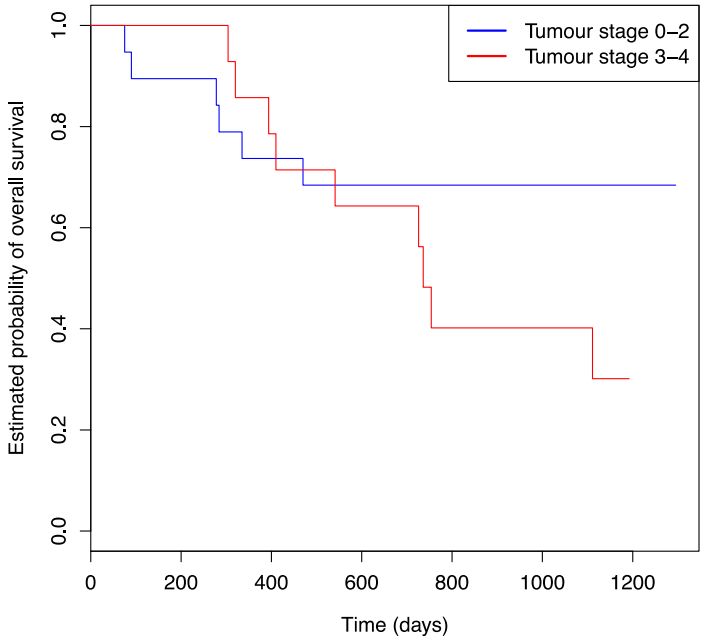


Figure 3.5. Relationship between tumour stage and overall survival in oesophageal adenocarcinoma treated with neoadjuvant oxaliplatin-fluorouracil chemotherapy. No association was found between patient sex and DFS (HR 1.986, *P* = 0.195).

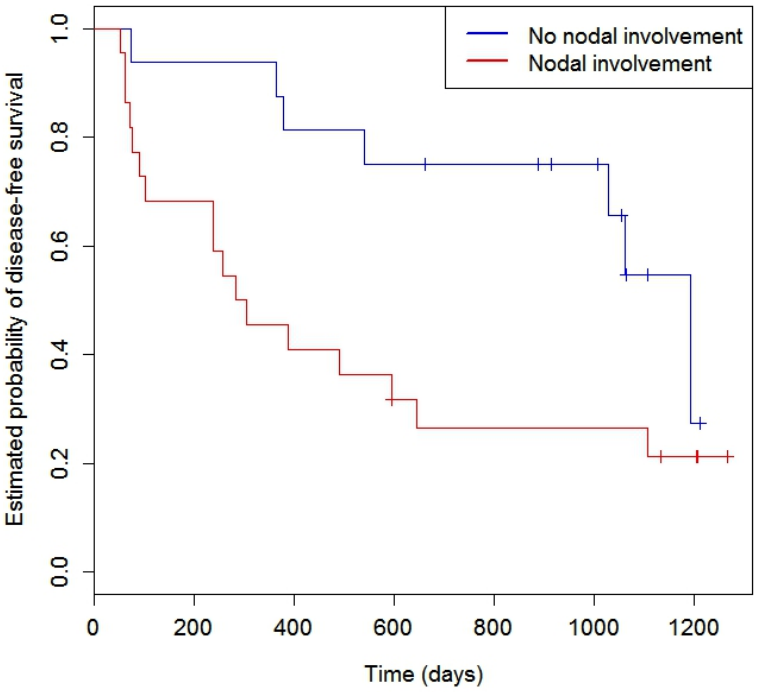


Figure 3.6. Association between lymph node involvement and disease free survival in oesophageal adenocarcinoma treated with neoadjuvant oxaliplatin-fluorouracil chemotherapy. Lymph node involvement was associated with worse DFS (HR 2.69, *P* = 0.023).

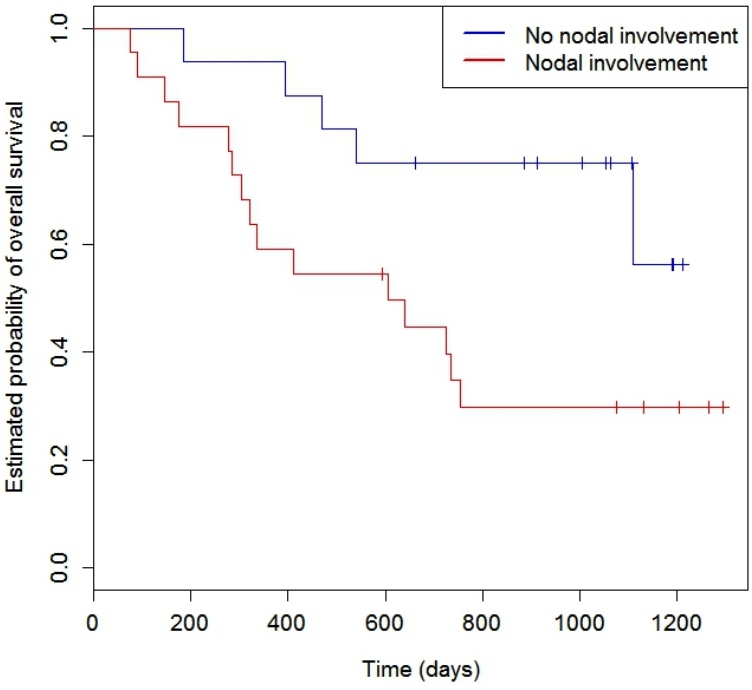


Figure 3.7. Association between lymph node involvement and overall survival in oesophageal adenocarcinoma treated with neoadjuvant oxaliplatin-fluorouracil chemotherapy. Lymph node involvement was associated with worse OS (HR 3.04, *P* = 0.024).

3.3 QUALITY CONTROL OF MICROARRAY RESULTS

The samples from tumour and normal tissue from oesophageal adenocarcinoma patients before and after oxaliplatin-based treatment were hybridized to Illumina Human HT-12 v3 Expression BeadChip to obtain gene expression data. The dataset was then filtered to remove probes not detected (detection score ? <= 0.05) in at least 5% of the samples, resulting in a final dataset of 27555 probes. The data were normalized using the vsn package (Huber et al., 2002). An empirical Bayes (COMBAT) method was used for batch correction (Johnson et al., 2007).

3.4 STATISTICAL CONSIDERATIONS

Due to the relatively small number of individuals in the study, the results of analyses for a relationship between pre-treatment expression levels for individual genes and clinical outcomes were not corrected for multiple testing, and a raw *P*-value of <0.05 was considered significant.

3.5 PRE-TREATMENT DNA DAMAGE REPAIR GENE EXPRESSION IS ASSOCIATED WITH PATHOLOGICAL RESPONSE FOLLOWING OXALIPLATIN-FLUOROURACIL CHEMOTHERAPY IN OESOPHAGEAL CANCER

To test the hypothesis that high levels of expression of DNAR genes linked with ICL repair are associated with lack of pathological response to oxaliplatin-

fluorouracil chemotherapy in oesophageal cancer, pre-treatment expression of DNAR genes in pathological responders was compared to that in non-responders. The definition of pathological response is described in Section 3.2.

Differential expression of DNAR genes between pathological non-responders and pathological responders was compared by linear regression. High expression of 7 DNAR genes was associated with lack of pathological response (P <0.05) following oxaliplatin-fluorouracil chemotherapy (**TABLE 3.4**). None of these results were significant when corrected for multiple testing.

Table 3.4. DNA repair genes associated with lack of pathological response to oxaliplatin-fluorouracil chemotherapy.

Gene	LogFC	*P*-value
High levels associated with lack of pathological response		
MPG	-4.035	0.010
MEN1	-3.024	0.010
USP3	-2.880	0.020
ALKBH1	-3.288	0.025
MGMT	-1.383	0.028
PARP3	-2.543	0.035
KIN	-2.154	0.041

3.6 PRE-TREATMENT DNA DAMAGE REPAIR GENE EXPRESSION IS ASSOCIATED WITH ONE YEAR DISEASE FREE SURVIVAL FOLLOWING OXALIPLATIN-FLUOROURACIL CHEMOTHERAPY IN OESOPHAGEAL CANCER

The relationship between DNAR gene expression and early outcomes following oxaliplatin-fluorouracil chemotherapy was further explored by looking at

whether high levels of expression of DNAR genes linked with ICL repair was associated with death or disease recurrence within one year. One year was used as a cut-off as there is evidence that it takes a year before the health-related quality-of-life scores for patients undergoing oesophagectomy return to baseline, thus it can be argued that patients should only undergo oesophagectomy if a DFS of more than one year can be expected(Lagergren et al., 2007; Parameswaran et al., 2010)

In keeping with the hypothesis, high levels of expression of 14 DNAR genes were associated with disease recurrence or death within a year (P <0.05) (**TABLE 3.5**). These included two key ICL repair genes, *ERCC1* (logFC -0.624, P = 0.025) and *EME1* (logFC -0.573, P =0.037). None of these results were significant when adjusted for multiple testing.

Table 3.5. DNA repair genes associated with worse one-year disease free survival oxaliplatin-fluorouracil chemotherapy.

Gene	LogFC	*P*-value
High levels associated with DFS <1 year		
CHAF1B	-0.648	0.004
BCCIP	-0.704	0.005
RAD51L1	-0.597	0.014
ESCO2	-0.639	0.015
RDM1	-0.689	0.020
MEN1	-0.599	0.022
ERCC1	-0.624	0.025
H2AFX	-0.503	0.027
NEIL3	-0.581	0.027
EME1	-0.573	0.037
ALKBH1	-0.421	0.041
RAD51	-0.561	0.046
GTF2H4	-0.359	0.046

3.7 HIGH LEVELS OF *ERCC1* EXPRESSION ARE ASSOCIATED WITH WORSE DISEASE FREE AND OVERALL SURVIVAL FOLLOWING OXALIPLATIN-FLUOROURACIL CHEMOTHERAPY IN OESOPHAGEAL CANCER

The relationship between DNAR gene expression and long-term disease free survival was assessed with the Cox proportional hazards model (**TABLE 3.6**). High levels of 11 genes were associated with worse disease free survival (P <0.05), the most significant association was found for *ERCC1* (Hazard Ratio 2.72; 95% CI 1.28, 4.62; P = 0.006) (**FIGURE 3.8**).

Table 3.6. DNA repair genes associated with worse disease free survival.

Gene	Hazard ratio	Lower 95% CI	Upper 95% CI	*P*-value
High expression associated with worse DFS				
ERCC1	2.438	1.285	4.625	0.006
BCCIP	2.834	1.271	6.319	0.011
SOD1	4.897	1.305	18.374	0.019
FSBP	2.598	1.134	5.953	0.024
HINFP	7.668	1.260	46.688	0.027
HMGB1	3.375	1.127	10.107	0.030
ERCC6	5.688	1.173	27.588	0.031
PMS2L2	6.584	1.144	37.879	0.035
ALKBH1	3.769	1.088	13.059	0.036
ESCO2	2.505	1.030	6.091	0.043
FANCA	2.207	1.010	4.822	0.047

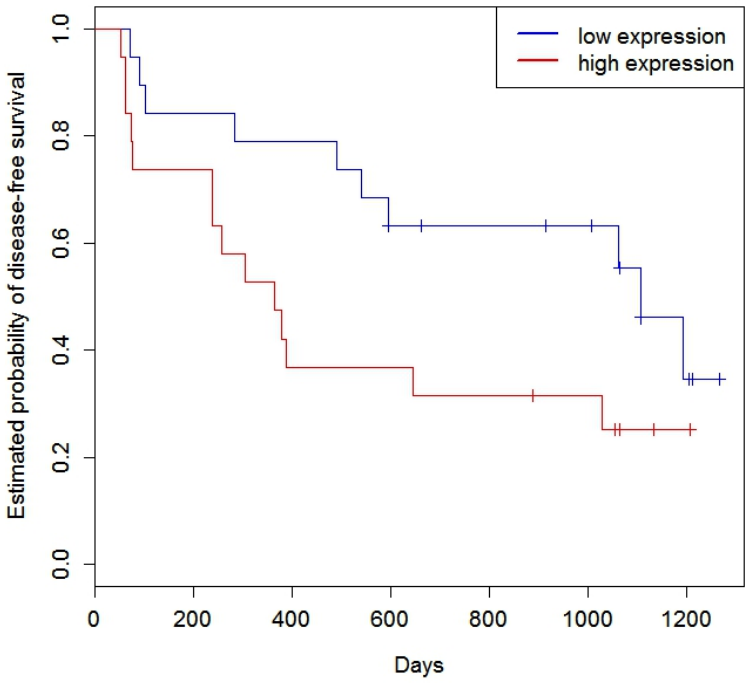


Figure 3.8. Association between high *ERCC1* expression and disease free survival in oesophageal adenocarcinoma treated with neoadjuvant oxaliplatin-fluorouracil chemotherapy. High levels of *ERCC1* expression were associated with worse disease free survival (hazard Ratio 2.72; 95% CI 1.28, 4.62; *P* = 0.006).

Cox proportional hazards analysis also revealed that higher levels of expression of 11 DNAR genes were associated with worse OS (*P* <0.05) (**TABLE 3.7**), with the most significant association again being for *ERCC1* expression (Hazard Ratio 3.21; 95% CI 1.55, 6.22; *P* = 0.001) (**FIGURE 3.9**).

Table 3.7. DNA repair genes associated with worse overall survival.

Gene	Hazard Ratio	Lower 95% CI	Upper 95% CI	*P*-value
High expression associated with worse OS				
ERCC1	3.113	1.557	6.223	0.001
ERCC6	10.505	1.710	64.550	0.011
HINFP	13.008	1.522	111.162	0.019
RAD51L1	3.611	1.232	10.583	0.019
HMGB1	4.381	1.258	15.262	0.020
ALKBH1	5.174	1.276	20.975	0.021
FSBP	3.097	1.182	8.113	0.021
NEIL1	4.161	1.192	14.520	0.025
GTF2H2	3.865	1.164	12.840	0.027
ESCO2	2.796	1.054	7.415	0.039
BCCIP	2.634	1.005	6.903	0.049

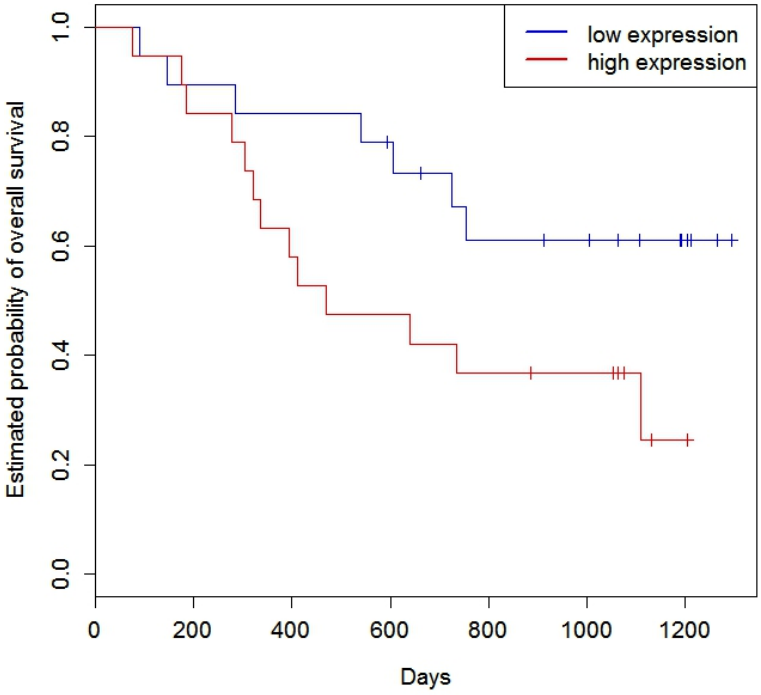


Figure 3.9. Association between high *ERCC1* expression and overall survival in oesophageal adenocarcinoma treated with neoadjuvant oxaliplatin-fluorouracil chemotherapy. High levels of *ERCC1* expression were associated with worse overall survival (Hazard Ratio 3.21; 95% CI 1.55, 6.22; *P* = 0.001).

3.8 INCREASED EXPRESSION OF GENES ENCODING KEY ICL REPAIR PROTEINS, ERCC1 AND MUS81, FOLLOWING OXALIPLATIN-FLUOROURACIL CHEMOTHERAPY IN OESOPHAGEAL CANCER

It was hypothesised that expression of DNAR genes conferring a survival advantage in the presence of oxaliplatin-fluorouracil chemotherapy would be higher in post-treatment specimens when compared with pre-treatment specimens. To ascertain if there was an alteration in DNAR gene expression following two cycles of oxaliplatin-fluorouracil chemotherapy, gene expression in pre-treatment tumour biopsies was compared with expression in post-treatment tumour resection specimens. 33 patients had pre- and post-treatment specimens available for analysis. Differential expression between the groups was assessed using linear regression and empirical Bayes methods, adjusting for multiple testing.

Expression of 15 DNAR genes was significantly increased following oxaliplatin-flurouracil (adjusted *P*-value <0.05) (**TABLE 3.8**). Consistent with the hypothesis, these included genes encoding two key ICL repair proteins, *ERCC1* (logFC 0.270, adj. *P* = 0.043) (**FIGURE 3.10**) and *MUS81* (log FC = 0.482, adj. *P* = 0.009) (**FIGURE 3.11**).

Table 3.8. DNA repair genes showing significantly increased expression following oxaliplatin-fluorouracil chemotherapy.

Gene	logFC	Adj. P-value
SIRT1	0.487	0.002
CRY1	0.479	0.006
ABL1	0.326	0.009
MUS81	0.482	0.009
ATM	0.493	0.010
CDC14B	0.527	0.013
KAT5	0.244	0.016
EEPD1	0.390	0.022
SRBD1	0.292	0.025
RPAIN	0.269	0.033
HMGB1	0.315	0.033
APITD1	0.439	0.037
UVRAG	0.298	0.041
ERCC1	0.270	0.043
RAD17	0.357	0.043

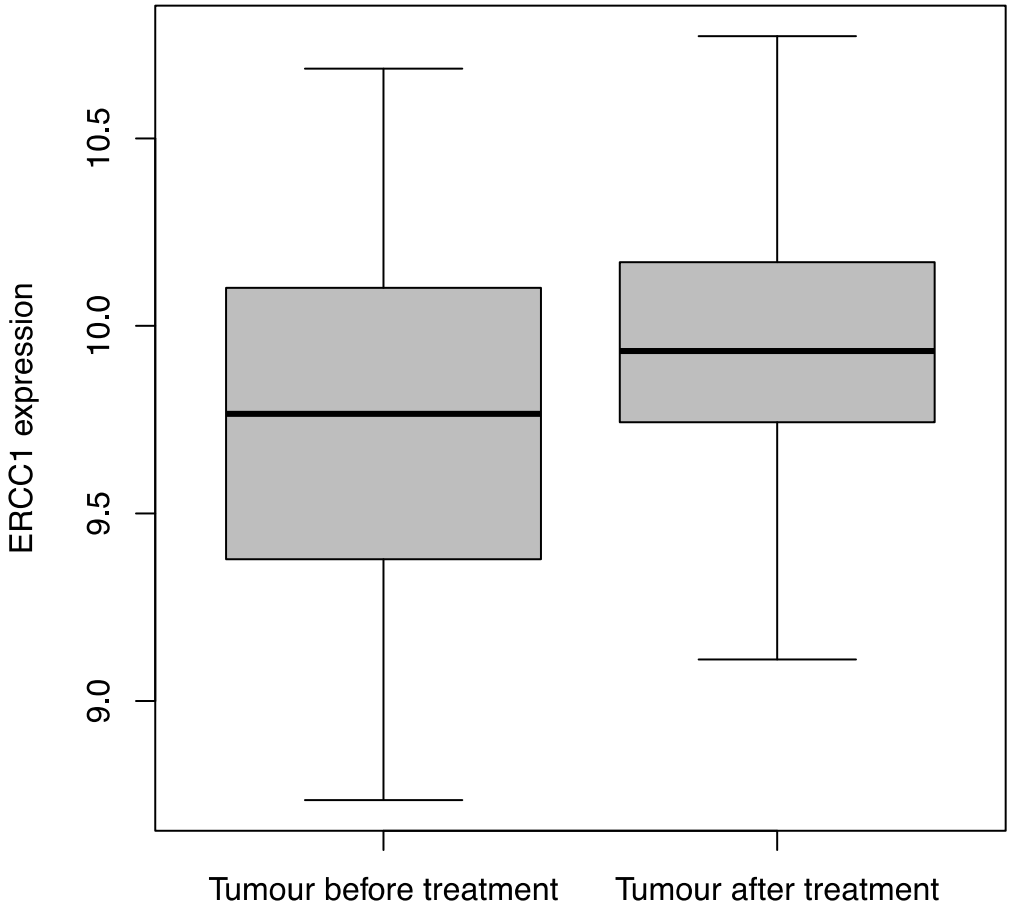


Figure 3.10. Tumour *ERCC1* expression increases following oxaliplatin-fluorouracil chemotherapy. There was a significant increase in *ERCC1* expression in tumours following two cycle of neoadjuvant oxaliplatin-fluorouracil chemotherapy (logFC 0.270, adj. *P* = 0.043).

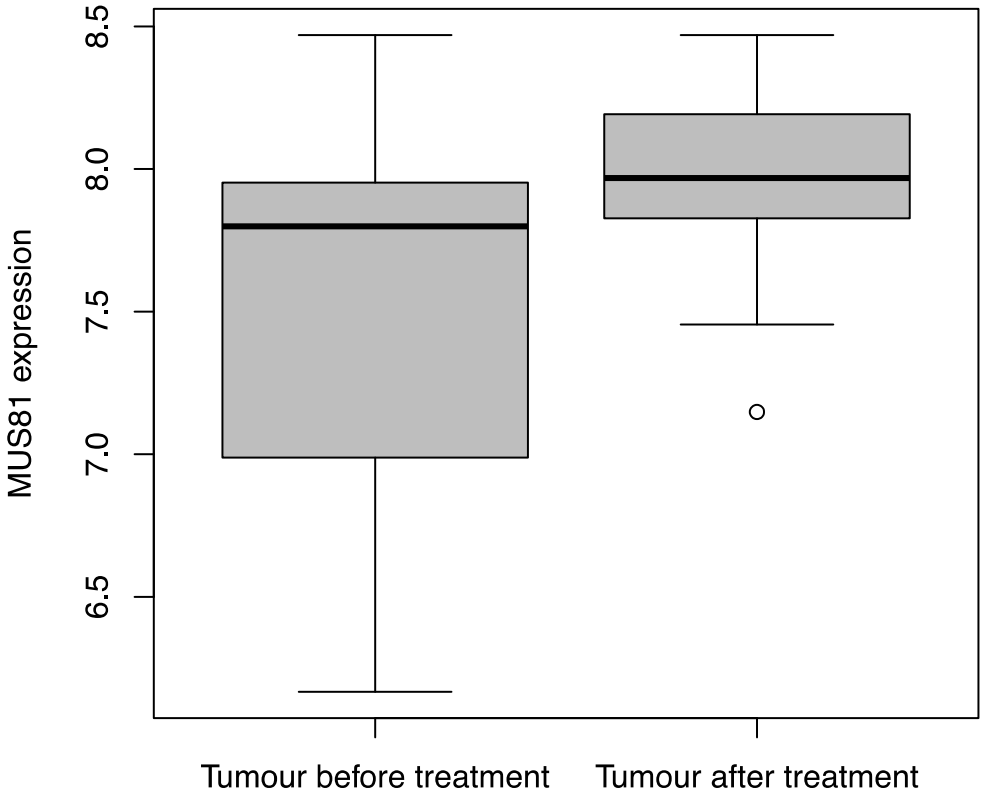


Figure 3.11. Tumour *MUS81* expression increases following oxaliplatin-fluorouracil chemotherapy. There was a significant increase in *MUS81* expression in tumours following two cycle of neoadjuvant oxaliplatin-fluorouracil chemotherapy (log FC = 0.482, adj. *P* = 0.009).

3.9 NO RELATIONSHIP BETWEEN EXPRESSION OF *XPF*, *ERCC1*, *MUS81* AND *EME1* AND LYMPH NODE INVOLVEMENT

The results of the microarray analysis of expression of DNAR protein genes appears to lend some support to our hypothesis that high levels of expression of the XPF/ERCC1 and MUS81/EME1 protein heterodimers are associated with worse outcomes following neoadjuvant oxaliplatin-fluorouracil chemotherapy in oesophageal adenocarcinoma. Expression for each of the genes was tested for association with prognostic features of the tumour (stage and lymph node status) or patient characteristics (age and sex). No relationship was found

between expression of *XPF*, *ERCC1*, *MUS81* or *EME1* and age, sex, tumour stage or lymph node involvement.

3.10 FUNCTIONAL PATHWAY ANALYSIS OF GENE EXPRESSION IN OESOPHAGEAL ADENOCARCINOMA TREATED WITH OXALIPLATIN-FLUOROURACIL CHEMOTHERAPY

Functional pathway analysis was performed on pre- and post-treatment tumour and adjacent normal tissue specimens (squamous epithelium biopsied 10 cm proximal to the tumour). Pathological response (Mandard regression grades 1-3) was used to define response to treatment. There was no significant difference in pre-treatment pathway expression in either tumour or normal tissue between pathological responders and non-responders. Likewise, there was no significant difference in post-treatment pathway expression between pathological responders and non-responders in tumour or normal tissue.

When pathway expression in post-treatment tumour was compared with pathway expression in tumours pre-treatment in those patients who exhibited pathological response to treatment, there was a significant (FDR < 0.001) decrease in expression of the cell cycle pathway (**TABLE 3.9**) and the HSP27 pathway (**TABLE 3.10**). There was also increased expression of the HDAC pathway (**TABLE 3.11**). There were no significant pathway expression alterations in tumours from patients who failed to exhibit pathological response following treatment.

Table 3.9. Genes in the Biocarta Cell Cycle Pathway.

CCNA1	*CDK1*	*CDKN2B*
CCNB1	*CDK2*	*CDKN2C*
CCND1	*CDK4*	*CDKN2D*
CCND2	*CDK6*	*E2F1*
CCND3	*CDK7*	*RB1*
CCNE1	*CDKN1A*	*RBL1*
CCNH	*CDKN1B*	*TFDP1*
CDC25A	*CDKN2A*	

Table 3.10. Genes in the Biocarta HSP27 Pathway.

ACTA1	*CYCS*	*HSPB2*
APAF1	*DAXX*	*IL1A*
BCL2	*FAS*	*MAPKAPK2*
CASP3	*FASLG*	*MAPKAPK3*
CASP9	*HSPB1*	*TNF*

Table 3.11. Genes in the Biocarta HDAC pathway.

AKT1	*IGF1*	*MEF2A*
CABIN1	*IGF1R*	*MEF2BNB-MEF2B*
CALM1	*INS*	*MEF2C*
CALM2	*INSR*	*MEF2D*
CALM3	*LOC124827*	*MYOD1*
CAMK1	*LOC147908*	*NFATC1*
CAMK1G	*MAP2K6*	*NFATC2*
CYCSP35	*MAPK14*	*PIK3CA*
HDAC5	*MAPK7*	*PIK3CG*

Pathway analysis was also performed comparing gene expression in normal oesophageal tissue before and after oxaliplatin-fluorouracil treatment. This showed significantly (FDR <0.001) increased expression of the NF-κB pathway (**TABLE 3.12**) and the IL1R pathway (**TABLE 3.13**) and decreased expression of the EIF4 pathway (**TABLE 3.14**).

Table 3.12. Genes in the Biocarta NF-κB Pathway.

CHUK	*MAP3K14*	*TLR4*
FADD	*MAP3K7*	*TNF*
IKBKB	*MYD88*	*TNFAIP3*
IKBKG	*NFKB1*	*TNFRSF1A*
IL1A	*NFKBIA*	*TNFRSF1B*
IL1R1	*RELA*	*TRADD*
IRAK1	*RIPK1*	*TRAF6*
MAP3K1	*TAB1*	

Table 3.13. Genes in the Biocarta IL1R Pathway.

CHUK	*IL6*	*MAPK14*
ECSIT	*IRAK1*	*MAPK8*
IFNA1	*IRAK2*	*MYD88*
IFNB1	*IRAK3*	*NFKB1*
IKBKB	*JUN*	*NFKBIA*
IL1A	*MAP2K3*	*RELA*
IL1B	*MAP2K6*	*TAB1*
IL1R1	*MAP3K1*	*TGFB1*
IL1RAP	*MAP3K14*	
IL1RN	*MAP3K7*	

Table 3.14. Genes in the Biocarta EIF4 Pathway

AKT1	*GHR*	*PDK2*
EIF4A1	*IRS1*	*PDPK1*
EIF4A2	*MAPK1*	*PIK3CA*
EIF4E	*MAPK14*	*PIK3R1*
EIF4EBP1	*MAPK3*	*PRKCA*
EIF4G1	*MKNK1*	*PRKCB*
EIF4G2	*MTOR*	*PTEN*
EIF4G3	*PABPC1*	*RPS6KB1*

3.11 DISCUSSION OF RESULTS OF MICROARRAY ANALYSES

It is known that oxaliplatin chemotherapy exerts its effects on cells by forming adducts with purine residues on DNA strands. Three major adducts are formed – monofunctional adducts, intrastrand cross-links and interstrand cross-links

(ICLs) (Woynarowski et al., 2000). It is thought that, although ICLs represent only 1-3% of the DNA adducts induced by oxaliplatin, they are the most toxic lesion (Chaney et al., 2005; Eastman, 1987; Palom et al., 2002). A number of DNA damage repair pathways have been implicated in cellular response to oxaliplatin. Intrastrand cross-links are repaired by nucleotide excision repair (NER), mismatch repair (MMR) and translesion synthesis (TLS). Repair of ICLs is a far more complex process, as both DNA strands must be incised to remove the lesion. ICL repair may be either replication-dependent, or replication-independent, and relies on components of a number of other DNA damage repair pathways, including elements of NER, TLS and homologous recombination (HR). It is thought that resolution of DSBs is essential to ICL repair.

Expression levels of DNA repair genes were assayed in samples of oesophageal adenocarcinomas taken before and after two cycles of oxaliplatin-fluorouracil chemotherapy. Tumour expression levels of individual DNA repair genes before treatment were tested for association with pathological response, one year DFS, DFS and OS. None of these results reached significance when adjusted for multiple testing, but a number of interesting results were yielded when they were tested for significance using a raw *P*-value of less than 0.05. The effect of treatment on DNA repair gene expression was tested by comparing gene expression in pre- and post-treatment tumour specimens.

3.11.1 High pre-treatment levels of DNA repair gene expression is associated with lack of pathological response in oesophageal adenocarcinoma

When differential expression of DNAR genes in pre-treatment tumour samples was compared in pathological responders (Mandard tumour regression grades 1 – 3) was compared with those in pathological non-responders (Mandard grades 4 and 5), high levels of expression of seven genes were associated with lack of pathological response (**TABLE 3.4**). One of these, *MEN1*, encodes a protein that interacts with the key ICL repair protein FANCD2; it has been shown that *MEN1* knockout cells are more sensitive to the ICL-forming agent mitomycin C (Jin et al., 2003), supporting a role for MEN1 in ICL repair. Another of these genes, *MGMT*, encodes a protein that has been shown to inhibit the formation of DNA cross-links (Paranjpe et al., 2014). Of the products of the other genes, two have a role in BER, MPG (Hedglin and O'Brien, 2008) and ALKBH1 (Sedgwick, 2004)); PARP3 is involved in DSB repair (Rulten et al., 2011); KIN is involved in the detection of DNA damage (le Maire et al., 2006); and USP3 is a regulator of ubiquitination signalling (Sharma et al., 2014).

3.11.2 High pre-treatment levels of DNA repair gene expression is associated with worse disease free survival in oesophageal adenocarcinoma treated with oxaliplatin-fluorouracil chemotherapy

Differences in expression of DNA repair genes were compared in those patients experiencing death or disease recurrence within one year and those with a DFS of greater than a year. High levels of expression of 13 DNAR genes were associated with a DFS of less than one year (**TABLE 3.5**). Four of these are

known to play a role in interstrand cross-link repair – *ERCC1*, *EME1*, *MEN1* and *RAD51*. Two of the other genes – *RDM1* and *GTF2H4* – also encode proteins that may have a role in ICL repair.

As has been discussed in the introduction, XPF-ERCC1 is a key endonuclease for both NER (Matsunaga et al., 1995) and ICL repair (Rahn et al., 2010). It makes incisions in DNA strands adjacent to ICLs and is critical for the "unhooking" step of ICL repair (Kuraoka et al., 2000; Niedernhofer et al., 2004; Wang et al., 2011). Cells that are deficient in XPF-ERCC1 are hypersensitive to cross-linking agents (Andersson et al., 1996; De Silva et al., 2000). *EME1* also encodes a protein that forms a heterodimer to act as a structure-specific endonuclease. It binds with MUS81 to form MUS81-EME1. MUS81-EME1 is capable of cleaving a variety of complex structures, including replication forks, 3' DNA flaps and Holliday Junctions (Ciccia et al., 2003; Ogrunc and Sancar, 2003) and it may represent an alternative pathway for ICL repair in cases where XPF-ERCC1 has failed (Sengerova et al., 2011). Embryonic stem cells that are null for *EME1* are hypersensitive to cross-linking agents (Abraham et al., 2003). RAD51 has recently been demonstrated to have a role in ICL repair that is separate to its known role in HR (Wang et al., 2015). In addition, the product of another of the genes, *RDM1*, may play a role in the initiation of ICL repair by the detection of ICLs – it has been shown to localise to sites of cisplatin-induced DNA damage (Hamimes et al., 2005).

GTF2H4 encodes a protein that is part of the NER pathway, and is thought to be required for XPF-ERCC1 to function as an endonuclease in NER (Bessho et al.,

1997), via its role in detecting bulky adducts (such as cisplatin) on DNA (Li et al., 2015). No role for GTF2H4 in stimulating ICL repair has yet been demonstrated, but it is known that XPA and XPC are involved in the detection of ICLs for replication-independent ICL repair and that GTF2H4 interacts with these two proteins to detect cisplatin-DNA adducts for NER (Zhao et al., 2009). It is therefore possible that GTF2H4 may be involved in the detection of platinum-induced ICLs for replication-independent ICL repair

Of the products of the other genes, H2AFX is phosphorylated in response to DNA damage; it is a major signaller for the recruitment of DNA repair proteins to sites of DNA damage and is involved in the regulation of DSB repair (Stucki et al., 2005). ; RAD51L1 (Lio et al., 2003) and BCCIP (Lu et al., 2007) are involved in HR and ESCO2 is also required for DSB repair (Whelan et al., 2012). ALKBH1 (Sedgwick, 2004) and NEIL3 (Bandaru et al., 2002) have a role in BER; and CHAF1B aids chromatin reassembly after DNA repair (Gaillard et al., 1996). It is possible that these proteins known to be involved in DSB repair also play a role in the final stages of ICL repair.

When over-expression of DNAR genes was tested for association with DFS, high levels of expression of 11 genes were found to be associated with worse DFS (**TABLE 3.6**). Four of these are known to have a role in ICL repair. Again, these included the key ICL repair gene *ERCC1*, which encodes part of the key XPF-ERCC1 heterodimer. Another gene, *FANCA*, encodes a protein that, via its role in the Fanconi anaemia core complex, it critical for the detection of ICLs and the stimulation of replication-dependent ICL repair (Matsushita et al., 2005;

Pichierri and Rosselli, 2004). *HMGB1* encodes a protein that binds to DNA that has been distorted by ICLs and has been shown, via its interaction with XPA, to induce ICL repair (Lange et al., 2009; Lange and Vasquez, 2009). The product of *ERCC6*, CSB, has been shown coordinate the resolution of ICLs, possibly via its interaction with SNM1A in replication-independent ICL repair (Iyama et al., 2015). In addition to these four genes, the product of *FSBP*, the RAD54B protein, may also play a role in ICL repair via its interaction with WRN (Otterlei et al., 2006). Deficiency of RAD54B renders cell sensitive to the ICL-inducing agent mitomycin C (Wesoly et al., 2006).

The association of high expression of the DSB repair genes *BCCIP* and *ESCO2* and the BER gene *ALKBH1*, with poor one year DFS is described above, and they were also associated with worse DFS. *SOD1* encodes another protein that has been implicated in HR-mediated DSB repair, although its precise function remains to be demonstrated (Sajesh and McManus, 2015). The product of *HINFP* activates transcription of ATM and plays a role in DNA damage detection (Medina et al., 2007). *PMS2L2* has homology with the PMS2 gene, which is involved in mismatch repair, but its precise role in DNA repair is unclear.

3.11.3 High pre-treatment levels of DNA repair gene expression associated with worse overall survival in oesophageal adenocarcinoma treated with oxaliplatin-fluorouracil chemotherapy

Over-expression of eleven DNA repair genes was associated with worse OS (**TABLE 3.7**). Five of these genes encode proteins with a known role in ICL repair – *ERCC1*, *ERCC6*, *HMGB1*, *NEIL1* and *GTF2H2* – and the potential role of the

protein product of a further gene, *FSBP*, in ICL repair has been discussed above. NEIL1 is involved in the recognition of ICLs, although it appears that it binds competitively with XPC at the sites of ICLs. It may represent an alternative pathway to XPC for recognition of ICLs (McNeill et al., 2013). The product of *GTF2H2*, P44, is a component of the human transcription/repair factor TFIIH – thus it is critical for NER, and is thought to also play a role in replication-independent ICL repair (Rahn et al., 2010; Wang et al., 2001). The role of ERCC1, ERCC6 and HMGB1 in ICL repair is discussed above. It should be noted that, again, high of expression of *ERCC1*, which encodes part of the key ICL repair endonuclease XPF-ERCC1, was associated with a worse clinical outcome.

Of the other five genes, two encode proteins involved in HR (RAD51L1 and BCCIP); the product of *ALKBH1* is part of the BER pathway; *ESCO2* plays a role in DSB repair (and may therefore be involved in the resolution of DSBs as part of ICL repair); and *HINFP* helps with damage detection and ATM activation.

3.11.4 DNA repair genes showing increased levels of expression following two cycles of oxaliplatin-fluorouracil chemotherapy in oesophageal adenocarcinoma

Fifteen DNA repair genes showed significantly increased levels of expression, when adjusted for multiple testing, in oesophageal adenocarcinomas following two cycles of oxaliplatin-fluorouracil chemotherapy (**TABLE 3.8**). Four of these - *ERCC1*, *MUS81*, *HMGB1* and *APITD1* – are known to encode proteins that are directly involved in ICL repair. The product of *APITD1*, MHF1, is recruited to sites of ICLs along with FANCM, and the two proteins facilitate assembly of the FA

core complex and stabilisation of stalled replication forks (Yan et al., 2010). It is notable that genes encoding components of the key ICL repair endonucleases XPF-ERCC1 and MUS81-EME1 show a significant increase in their expression following oxaliplatin-fluorouracil chemotherapy.

A fifth gene, *RPAIN*, encodes RPA interacting protein, which is responsible for the transfer of RPA into the nucleus (Park et al., 2005). RPA is involved in many DNA damage repair pathways (Marechal and Zou, 2015). It is a key component of HR, in which RPA coating of the ssDNA fragment allows RAD51 to be loaded to catalyse the DNA strand invasion and exchange required for HR (Sugiyama et al., 1997). There is also evidence that RPA is directly involved in the detection of cisplatin-induced ICLs and that it promotes the endonuclease activity of XPF-ERCC1, so increased expression of RPAIN may help in the early triggering of ICL repair (Matsunaga et al., 1996; Patrick et al., 2008). The product of a further gene that shows increased expression following treatment, *SRBD1*, is also known to interact with the RPA proteins, but the precise nature of its role in DNA damage repair remains to be elucidated (Marechal et al., 2014).

Of the other genes, the products of seven of them are known to be involved in DSB repair – *ATM*, *ABL1*, *KAT5*, *RAD17*, *CDC14B EEPD1* and *UVRAG*. ATM is one of the key regulators of cellular response to DSBs – it is attracted to DSBs by the MRN (MRE11-RAD50-NBS1) complex (Lee and Paull, 2005). RAD17 is involved in the early recruitment and retention of the MRN complex to DSBs. The interaction of RAD17 with NBS1 is enhanced by the ATM-mediated phosphorylation of RAD17, suggesting an ATM-Rad17-MRN-ATM amplification

loop (Wang et al., 2014). For ATM to be activated, it must undergo acetylation by TIP60 – this is the protein product of another of the genes showing increased expression following treatment, *KAT5* (Sun et al., 2005). Activation of TIP60, in turn, is accomplished by its tyrosine phosphorylation by c-Abl, which is encoded for by another of the up-regulated genes, *ABL1* (Kaidi and Jackson, 2013).

CDC14B is a phosphatase that is required for both HR- and NHEJ-mediated DSB repair (Lin et al., 2015). EEPD1, Exonuclease/Endonulease/Phosphatase domain 1, has recently been shown to be involved in the HR-mediated rescue of stalled replication forks. Depletion of EEPD1 in lung adenocarcinoma cell lines sensitises them to cisplatin (Wu et al., 2015). The protein product of *UVRAG* is known to interact with the key DSB repair protein KU80, promoting NHEJ (Zhao et al., 2012). It should be noted, however, that UVRAG is also known to be important in autophagy, and it possible that increased autophagy is part of the tumoral response to oxaliplatin-fluorouracil (Huang et al., 2012; Liang et al., 2006).

CRY1 encodes the crytochrome protein, CRY1, which is well known for its role in maintaining circadian rhythm (Miyamoto and Sancar, 1998). It has been shown that CRY1 controls expression of the key NER protein XPA (Kang et al., 2010). In addition, CRY1 is known to modulate the activity of ATR(Kang and Leem, 2014).

SIRT1 encodes a protein that is a promoter of DNA damage repair by protein deacetylation. It has been shown to regulate the activity of proteins in a number of key DNA repair pathways, including XPA in NER (Fan and Luo, 2010); NBS1 in

DSB repair (Yuan et al., 2007); and WRN which plays a role in both DSB repair and ICL repair (Li et al., 2008).

3.12 FUNCTIONAL PATHWAY ANALYSIS IN OESOPHAGEAL ADENOCARCINOMAS TREATED WITH OXALIPLATIN-FLUOROURACIL CHEMOTHERAPY

Functional pathway analysis was carried out on the microarray data of gene expression in oesophageal adenocarcinomas before and after two cycles of oxaliplatin-fluorouracil chemotherapy. This showed that patients who exhibited pathological response had down-regulation of cell cycling pathways and down-regulation of the anti-apoptotic HSP27 pathway in their tumours following oxaliplatin-fluorouracil chemotherapy.

The down regulation of cell cycling pathways seen in pathological responders may be due to oxaliplatin-induced cell cycle arrest, and is therefore a marker of response to treatment. An alternative explanation is that pathological non-responders have a different tumour cell cycle profile, and that cell cycle stage dictates the strategy used by a cell to repair platinum-DNA adducts. In support of this idea, repair of platinum-induced interstrand cross-links (ICLs) occurs in both a replication-dependent and replication-independent fashion (see **Section 1.1.7**). Replication-dependent ICL repair is though to be more efficient, with futile cycles of incision and ligation possible in replication-independent ICL repair (Mu et al., 2000). The hypothesis that a different cell cycle profile may occur be seen in in patients responding to treatment is supported by the finding

that over-expression of cell cycle pathways pre-treatment in a small series of colorectal cancer patients correlated with lack of response to oxaliplatin-fluorouracil chemotherapy (Stevenson et al., 2012), although no differential pathway expression was found when pre-treatment gene expression was compared in pathological responders and non-responders in the oesophageal patients.

It is known that high levels of HSP27 expression increase cisplatin resistance in ovarian and oesophageal squamous cell cancer (Arts et al., 1999; Miyazaki et al., 2005), and that overexpression of HSP27 is associated with worse outcomes in rectal cancer treated with radiotherapy (Tweedle et al., 2010). No studies have thus far looked at the role of HSP27 in response to oxaliplatin, but it has been demonstrated that, in keeping with our data, overexpression of HSP27 is associated with resistance to cisplatin in colorectal cancer cell lines (Garrido et al., 1997).

There are two possible explanations for the association between down regulation of the HSP27 pathway and pathological response to oxaliplatin-fluorouracil chemotherapy. The first is that HSP27 has an anti-apoptotic role by inhibiting cytochrome c and TNF-mediated cell death (Garrido et al., 2006); therefore down-regulation of the HSP27 pathway predisposes tumours to response by promoting apoptosis.

Another possibility is that HSP27 has a role in modulating DNA repair pathways. It has been demonstrated in head and neck cell line models that decreasing

levels of HSP27 impairs repair of radiation-induced double strand breaks (DSBs), and that this radiosensitisation is ATM-dependent (Guttmann et al., 2013). It has also been shown that, following treatment with cisplatin, HSP27 co-localizes with MLH1 and MSH2, in HCT116 (colorectal cancer) cell lines (Sottile et al., 2015). This implies a role for HSP27 in the recognition of platinum-DNA adducts. These findings raise the possibility that the HSP27 pathway also plays a role in facilitating the activation of DNA damage repair pathways, and that down-regulation of the pathway promotes response to oxaliplatin by decreasing the ability of tumours to repair platinum-DNA adducts.

Tumours that showed pathological response also had increased expression of a histone deacetylase (HDAC) pathway after treatment. The role of HDAC in response to oxaliplatin treatment in cancer remains to be defined. It has been shown that HDAC inhibitors increase sensitivity of human colorectal cancer cells to oxaliplatin, but this runs contrary to our finding here that increased expression of the HDAC pathway is seen in tumours that respond to oxaliplatin-fluorouracil chemotherapy (Kim et al., 2009a; Na et al., 2010). On the other hand, HDAC inhibition by sodium valproate causes increase in levels of XPC in bladder cancer cell lines (Xu et al., 2011). It may, therefore be that increased expression of the HDAC pathway results in suppression of XPC expression. XPC is involved in the recognition of platinum-DNA adducts for global genome nucleotide excision repair (GG-NER) (Masutani et al., 1994; Nishi et al., 2005). Therefore, increased expression of HDAC pathways may result in decreased levels of XPC, limiting tumour cells abilities to detect oxaliplatin-DNA adducts and so increasing sensitivity to oxaliplatin.

Following treatment, normal oesophageal squamous epithelium exhibited up-regulation of the NF-κB pathway, which is a key component of cellular response to harmful stimuli, and the IL1R pathway, another damage response pathway that results in activation of NF-κB. These findings are in keeping with the response of normal tissue to chemical insult. Interestingly, there was also decreased expression of the EIF4 pathway in normal squamous mucosa following treatment. The EIF4 complex is responsible for the initiation of translation in eukaryotes (Topisirovic et al., 2011) and it is thought that this is the key point at which control of translation takes place (Jackson et al., 2010).

Tumour growth is determined not just by the cells of the tumour itself, but also by the stroma surrounding the tumour, including fibroblasts and epithelial cells, and the effect of infiltrating immune cells. Characteristics of the tumour microenvironment (TME) include angiogenesis and the presence of activated fibroblasts (cancer associated fibroblasts – CAF).

CAFs are present in many TMEs and may be derived from a number of resident tissue types, including endothelial cells and smooth muscle cells. It has been shown that the tumour-promoting effects of CAFs are abolished by inhibition of NF-κB (Erez et al., 2010). It has also been demonstrated that the endothelial to mesenchymal transition to generate CAFs in oesophageal adenocarcinoma is mediated by IL-1β, a key component of the IL1R pathway (Nie et al., 2014). Furthermore, this induction of endothelial to mesenchymal transition by IL-1β is known to be NF-κB-dependent (Maleszewska et al., 2013; Nie et al., 2014).

It is unlikely that biopsies taken from macroscopically normal mucosa 10cm proximal to the tumour are representative of the TME, but it is possible that changes in pathway expression in adjacent normal mucosa affect the TME. This raises the possibility that the increased expression of the NF-κB and IL1R pathways as part of the normal mucosa's response to oxaliplatin-fluorouracil chemotherapy results in a paradoxical promotion of TME development.

The down-regulation of translational initiation factors in normal mucosa following treatment is interesting in the context of the increased expression of a number of key DNA repair genes seen in tumours. It may be that is represents a global decrease in gene expression following oxaliplatin-induced cell cycle arrest in normal mucosa.

3.13 SUMMARY

In this chapter, microarray analysis of gene expression was used to test the hypothesis that high levels of expression of genes encoding the key ICL repair proteins XPF, ERCC1, MUS81 and EME1 is associated with worse outcomes following neoadjuvant oxaliplatin-fluorouracil chemotherapy in oesophageal cancer. In support of this hypothesis, high levels of *ERCC1* mRNA were associated with worse DFS and OS following treatment. In addition, there was a significant increase in expression of *ERCC1* following two cycles of oxaliplatin-fluorouracil chemotherapy. High levels of *EME1* expression were associated with worse DFS at one year following treatment, and levels of expression of its binding partner, *MUS81*, increased significantly following treatment.

It should be noted that no association was found between expression of *SLX4* and clinical outcomes. It is known that SLX4 is involved in ICL repair, with deletion of it causing increased sensitivity to ICLs (Castor et al., 2013). It has binding domains for interacting with both XPF-ERCC1 and MUS81-EME1 (Fekairi et al., 2009; Munoz et al., 2009; Svendsen et al., 2009). It may form a scaffold for the recruitment of XPF-ERCC1 and MUS81-EME1 to ICLs and it has been demonstrated that SLX4 potentiates the nuclease activity of XPF-ERCC1 (Hodskinson et al., 2014). A possible explanation for this is that both heterodimers have been shown to have roles that are independent of their interaction with SLX4. SLX4 is not part of the NER pathway (in which XPF-ERCC1 plays a key role); and MUS81-EME1 has been shown to be able to maintain its nuclease ability and stability when its interaction with SLX4 has been disrupted, bringing about the possibility that MUS81-EME1 has an SLX4-independent role in ICL repair (Nair et al., 2014). These different roles independent of the SLX4-XPF-ERCC1-MUS81-EME1 super-complex may also explain why the gene expression results for XPF-ERCC1 and MUS81-EME1 do not exactly correlate.

Taken together, these results support the hypothesis that high levels of the genes encoding the DNA repair protein heterodimers XFP-ERCC1 and MUS81-EME1 are associated with worse outcomes following oxaliplatin-based chemotherapy. It was therefore decided to develop immunohistochemical assays to detect protein levels of the two heterodimers in pathological specimens to further test for an association between their levels and clinical outcomes. The results of this work are presented in the next two chapters.

In addition, functional pathway analysis was carried out. This showed that patients who exhibited pathological response had down-regulation of cell cycling pathways and down-regulation of the anti-apoptotic HSP27 pathway in their tumours following oxaliplatin-fluorouracil chemotherapy. These tumours also showed increased expression of a histone deacetylase pathway after treatment. Following treatment, normal oesophageal squamous epithelium exhibited up-regulation of the NF-κB pathway, which is a key component of cellular response to harmful stimuli, and the IL1R pathway, another damage response pathway that results in activation of NF-κB. The normal tissue also showed down-regulation of the EIF4 pathway, which is involved in translational regulation.

The findings of alterations in inflammatory and transcriptional regulation pathways in normal mucosa raise questions about the interaction between the TME and adjacent tissue following oxaliplatin-fluorouracil chemotherapy, and raise the possibility that this response may paradoxically promote development of the TME.

The association between down regulation of cell cycle pathways and pathological response to oxaliplatin-fluorouracil chemotherapy suggests that cell cycle profile of oesophageal adenocarcinomas may be associated with likelihood of response to oxaliplatin-fluorouracil chemotherapy. In particular, it is known that a major source of toxicity from ICLs is replication fork stalling in S-phase of the cell cycle, and that these lesions are repaired by replication-dependent ICL repair (Niedernhofer, 2007). There is also a more poorly understood replication-

independent ICL repair pathway (Guainazzi and Scharer, 2010; Muniandy et al., 2010). It may be, therefore, the ability of cells to repair oxaliplatin-induced ICLs depends on the phase of the cell cycle in which they are generated. The relationship between tumour cell cycle profile and response to oxaliplatin-fluorouracil chemotherapy in oesophageal cancer was tested, and the results are presented in Chapter 7.

4. DNA REPAIR PROTEIN EXPRESSION AND RESPONSE TO PLATINUM-BASED CHEMOTHERAPY IN PATIENTS WITH OESOPHAGEAL CANCER

4.1 INTRODUCTION

As discussed in detail in Chapter 1, XPF-ERCC1 and MUS81-EME1 are endonucleases that are known to play a role in the processing of platinum-DNA lesions, and ICLs in particular. In Chapter 3, microarray analysis of gene expression in patients with adenocarcinoma of the oesophagus treated with neoadjuvant oxaliplatin-fluorouracil chemotherapy showed a correlation between genes encoding proteins involved in ICL repair, including the key ICL repair proteins XPF-ERCC1 and MUS81-EME1, and clinical outcomes. This chapter tested the hypothesis that high levels of expression of XPF protein and MUS81 protein in oesophageal adenocarcinomas are associated with lack of response to platinum-based chemotherapy.

Firstly, the IHC techniques for detection of ERCC1, XPF and MUS81 were optimised, and the specificity of the MTA30 2G10/3 MUS81 antibody was tested. Secondly, the validity of a semi-quantitative scoring system for IHC staining of protein expression was examined. Finally, pre-treatment levels of XPF and MUS81 proteins were tested for association with clinical outcomes in three cohorts of patients with operable oesophageal adenocarcinoma – one cohort received neoadjuvant oxaliplatin-fluorouracil; one cohort received neoadjuvant

cisplatin-fluorouracil chemotherapy; and the final cohort was treated with surgery alone.

4.2 CHOICE OF ANTIBODY TO ASSESS EXPRESSION OF THE XPF-ERCC1 HETERODIMER

Immunohistochemistry for ERCC1 has been used extensively in an attempt to predict response to platinum chemotherapy. The most commonly used antibody, 8F1, has been shown to be non-specific for immunodetection of ERCC1 (Niedernhofer et al., 2007). It was subsequently demonstrated that the only commercially available ERCC1 antibody that is specific for ERCC1 on immunohistochemistry is the rabbit polyclonal FL-297 antibody (Bhagwat et al., 2009); however, strong cytoplasmic staining occurs with this antibody.

To determine if the cytoplasmic staining seen in the published literature could be eliminated sufficiently to give confidence in the FL-297 antibody for semi-quantitative immunodetection of ERCC1 expression, samples were stained on the Leica BOND-MAX immunostainer. Normal human tonsil was stained, as it is known to express ERCC1. Four different antigen retrieval methods were tested:

- pH6 for 10 minutes
- pH6 for 20 minutes
- pH9 for 10 minutes
- pH9 for 20 minutes

For each of these methods, four primary antibody dilutions were attempted (1:50, 1:100, 1:500 and 1:100). The rest of the staining was carried out according to the laboratory standard IHC protocol (Protocol F).

The slides were then reviewed, representative images of the staining are shown in **FIGURE 4.1,** and a summary of the comments on each slide are shown in **TABLE 4.1**. The best nuclear staining was obtained at pH9 with 20 minutes of antigen retrieval and a 1:100 dilution of primary antibody. The cytoplasmic staining was, however, still felt to be too strong.

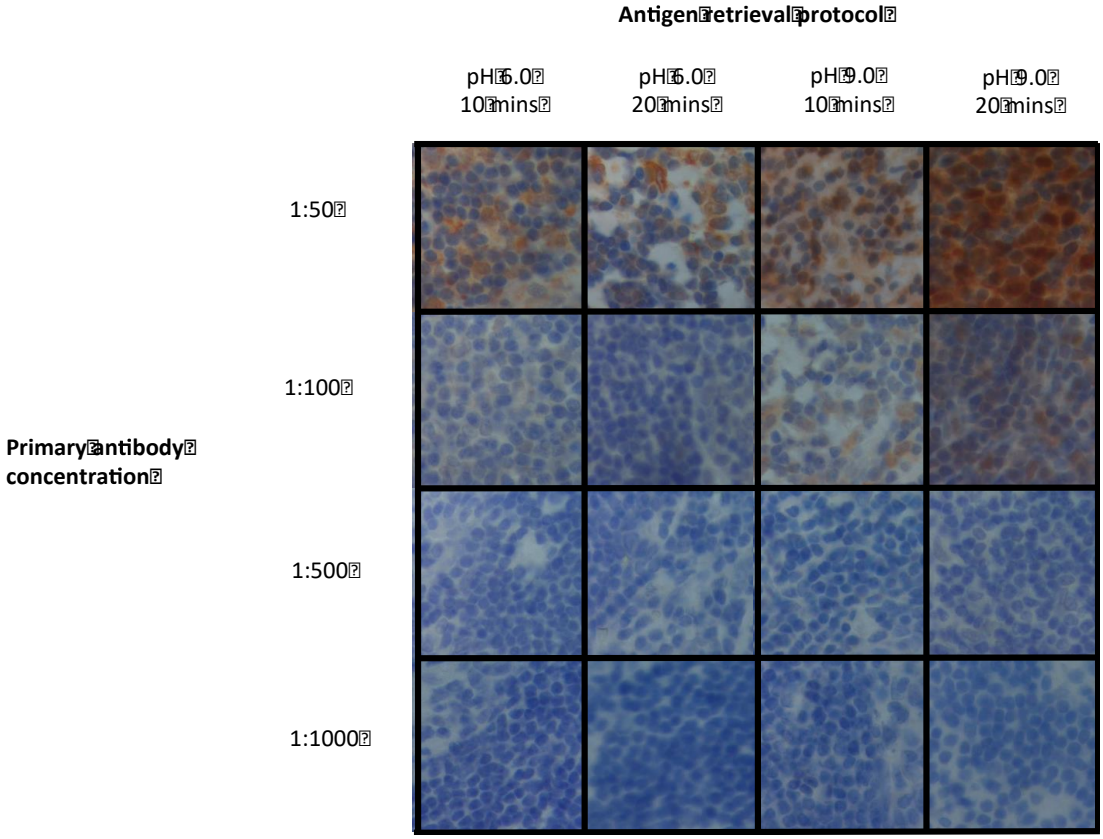


Figure 4.1. Staining of human tonsil with the FL-297 antibody. A variety of different antigen retrieval methods and primary antibody concentrations were attempted. All photographed at X20 magnification.

Table 4.1. Comments on staining of human tonsil with FL-297 antibody.

	Antigen retrieval			
Antibody dilution	pH6 for 10 minutes	pH6 for 20 minutes	pH9 for 10 minutes	pH9 for 20 minutes
1:50	Nuclear + weak Cytoplasmic ++ strong	Nuclear + weak Cyctoplasmic ++ strong	Cytoplasmic ++ strong Background ++ strong	Cytoplasmic ++ strong Background ++ strong
1:100	Cytoplasmic ++ weak	Cytoplasmic ++ weak	Nuclear + weak Cytoplasmic ++ moderate	Nuclear ++ moderate Cytoplasmic ++ moderate
1:500	No staining	No staining	No staining	No staining
1:1000	No staining	No staining	No staining	No staining

A second series of staining was therefore carried out using the pH9 antigen retrieval solution for 20 or 30 minutes, and primary antibody dilutions of 1:100, 1:200 and 1:400. Images of the staining are shown in **FIGURE 4.2**, and the comments on each slide are in **TABLE 4.2**. There was still considerable cytoplasmic staining in all slides that showed reasonable nuclear staining.

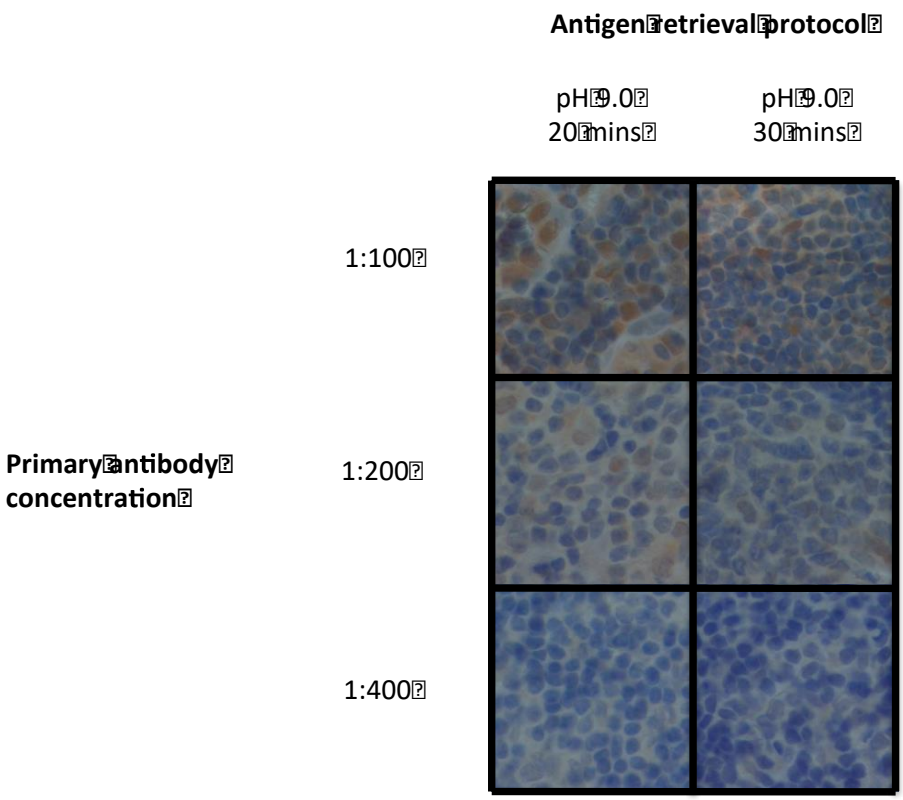


Figure 4.2. Staining of human tonsil with the FL-297 antibody – second series. All photographed at X20 magnification.

Table 4.2. Comments on second series of staining of human tonsil with FL-297 antibody.

	Antigen retrieval	
Antibody dilution	pH9 for 20 minutes	pH9 for 30 minutes
1:100	Nuclear + moderate Cytoplasmic + moderate	Nuclear + moderate Cytoplasmic + moderate
1:200	Cytoplasmic + moderate	Nuclear + weak Cytoplasmic + weak
1:400	No staining	Cytoplasmic + occasional, weak

It was decided that the considerable non-specific cytoplasmic staining occurring with the FL-297 antibody made it a poor choice for detection of levels of ERCC1 protein. After these initial experiments were performed, Friboulet *et al*. showed that the cytoplasmic staining seen with the FL-297 antibody was due to it detecting the ERCC1-203 isoform of the protein. This has a cytoplasmic location and appears to be inactive in DNA damage repair (Friboulet et al., 2013b). FL-

297 was demonstrated to detect all four ERCC1 isoforms, although only the ERCC1-202 isoform was active in the repair of platinum-induced DNA lesions (Friboulet et al., 2013a). These findings gave further support to the decision not to use FL-297 to detect the functional XPF-ERCC1 heterodimer. Consideration was given to the in-house manufacture of an ERCC1 monoclonal antibody, but it was decided not to pursue this on the basis of time, cost, and the difficulties of generating an antibody specific to the ERCC1-202 isoform.

It has been demonstrated previously that XPF expression correlates with ERCC1 expression and that the two proteins provide mutual stabilization in a heterodimer (Biggerstaff et al., 1993; Hatch et al., 2014). Friboulet *et al*. went on to demonstrate that ERCC1-202 is the only isomer of ERCC1 capable of interacting with XPF, and that it is the 202 isoform that stabilizes XPF allowing its immunodetection (Friboulet et al., 2013b). In the absence of a reliable antibody for immunohistochemical detection of ERCC1, XPF immunohistochemistry provided a potential alternative method of detection of the XPF-ERCC1 heterodimer, avoiding the chance of detecting non-functioning ERCC1 isoforms. Therefore, the SPM228 antibody to XPF was used for immunodetection of XPF, and thus the functional XPF-ERCC1 heterodimer.

4.3 OPTIMISATION OF XPF IMMUNOHISTOCHEMISTRY

Automated immunohistochemistry using the Bond-Max automated immunostainer was optimised for XPF staining. The methods for automated staining are described in the Materials and Methods chapter. Optimisation of

staining was carried out in oesophageal tumour specimens of known high and low XPF expression status and tonsillar tissue. Antigen retrieval was carried out for different times (10 and 20 minutes) and at different pH (pH 6.0 and pH 9.0). Each combination of antigen retrieval was tried with primary antibody diluted at 1:50, 1:100, 1:200 and 1:1000.

Slides were reviewed with a Consultant Pathologist, assessing for quality of staining and comparable intensity of staining with control sections. Based on these criteria, antigen retrieval was carried out for 10 minutes at pH 9.0 and the primary antibody was diluted 1:200 (**FIGURE 4.3**). In IHC runs, gastric mucosa sections with known high and low XPF levels and also AGS cell pellets with and without knockdown of XPF expression were used as positive and negative controls.

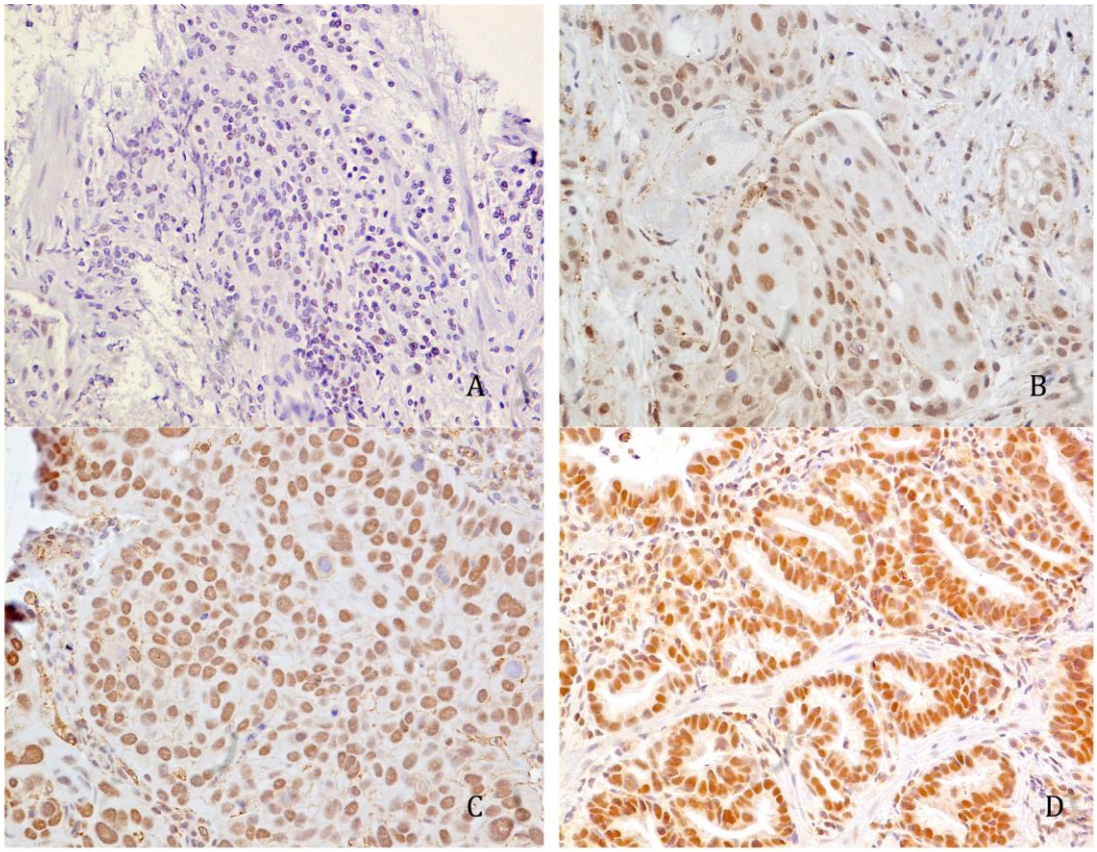


Figure 4.3. XPF staining of oesophageal adenocarcinomas. Oesophageal adenocarcinoma pre-treatment biopsies stained for XPF protein showing intensity scores of 0(A), 1(B), 2(C) and 3(D). All photographed at X20 magnification.

4.4 CHOICE OF ANTIBODY FOR IMMUNODETECTION OF THE MUS81-EME1 HETERODIMER

At the time of experimental design, only one EME1 antibody suitable for IHC was available, a rabbit polyclonal antibody (catalogue number orb100254, Biorbyt, Cambridge, UK). EME2 is half the length of EME1, but shows 44% identity with EME1, mainly aligning at the C-terminal end (Ciccia et al., 2003). There was concern that, given the considerable sequence homology between EME1 and EME2, this polyclonal antibody might cross-react with EME2. As for ERCC1,

consideration was given to the in-house manufacture of an EME1-specific antibody suitable for IHC. Again, this option was rejected on the basis of cost, time and risk of failure. It was therefore decided to try to identify and validate a commercially available MUS81 antibody for immunodetection of the MUS81-EME1 heterodimer.

The MTA30 2G10/3 MUS81 antibody (Abcam, Cambridge, MA, USA) had previously been successfully used within the group for Western blotting. It had not previously been used for IHC, and, therefore, no protocol was available from the manufacturers. For initial work-up, testicular tissue was used as a positive control. Antigen retrieval was carried out for different times (10 and 20 minutes) and at different pH (pH 6.0 and pH 9.0). Each combination of antigen retrieval was tried with primary antibody diluted at 1:50, 1:100, 1:200 and 1:1000. Sections were reviewed with a Consultant Pathologist and it was decided that optimal staining was achieved with antigen retrieval in the standard pH 6.0 buffer for 20 minutes and incubation with the primary antibody at 1:1000 dilution. Testicular tissue was used as a positive control, and also as a negative control with omission of the primary antibody. Cell pellets generated from AGS cell lines with knockdown of MUS81 expression were also used as negative controls. Examples of MUS81 staining of oesophageal adenocarcinomas are shown in **FIGURE 4.4**.

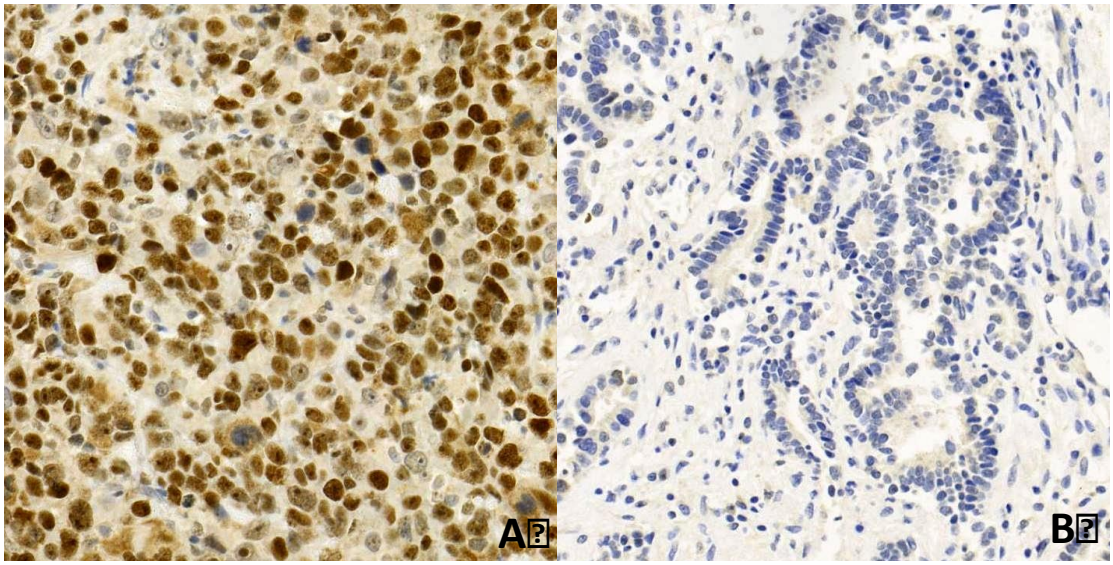


Figure 4.4 MUS81 staining of oesophageal adenocarcinomas. Oesophageal adenocarcinoma pre-treatment biopsies stained for MUS81 showing high (**A**) and low (**B**) expression, both photographed at X20 magnification.

To confirm antibody specificity, cell pellets from AGS and ST16 gastric cancer cell lines were used with and without MUS81 expression knockdown by RNA interference. These pellets were formalin fixed and paraffin embedded for sectioning. Western blotting of cell extracts was also undertaken. The Western blot and IHC of these samples is shown in **FIGURE 4.5** and demonstrates good antibody specificity.

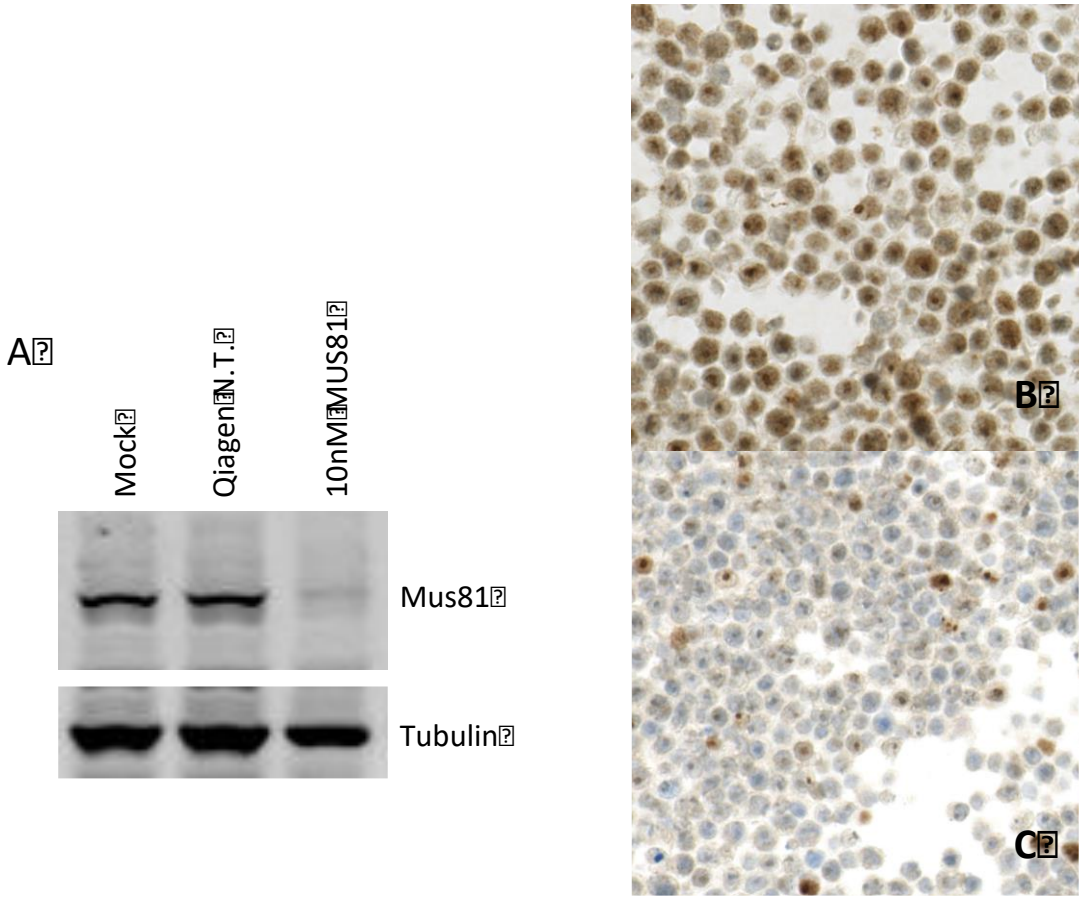


Figure 4.5. Specificity of MTA30 2G10/3 MUS81 antibody. Specificity of the MTA30 2G10/3 MUS81 antibody was confirmed in AGS gastric cancer cell lines with knockdown of MUS81 with (**A**) Western blotting; and immunohistochemical staining of (**B**) non-targeting and (**C**) knockdown cell pellets.

4.5 VALIDATION OF SEMI-QUANTITATIVE SCORING SYSTEM FOR NUCLEAR EXPRESSION OF DNA REPAIR PROTEINS

To validate the reproducibility of the semi-quantitative, composite scoring system for nuclear expression of DNA repair proteins (as detailed in the Material and Methods Chapter), 84 slides of oesophageal cancer previously stained and scored for XPF expression were rescored. A pair of independent scorers scored the slides separately. In cases where scores for an individual slide differed, the slide was reviewed together using a double-headed microscope and a consensus

score reached. When the consensus scores of the second pair of observers were compared with those of the first pair of observers, good correlation in the scores was found (**TABLE 4.3**), with an overall kappa statistic for the composite score of 0.69.

Table 4.3. Inter-observer variability for semi-quantitative scoring of nuclear expression of DNA repair proteins.

Intensity score	Weighted kappa = 0.81
Frequency of nuclei staining	Weighted kappa = 0.88
Composite score	Unweighted kappa = 0.69

It was decided that, due to the relatively small number of patients in the cohorts studied, high expression of a DNA repair protein would be defined *a priori* as greater a greater than median composite score.

4.6 OESOPHAGEAL ADENOCARCINOMA PATIENT CHARACTERISTICS

The relationship between tumour expression of XPF and MUS81 and outcomes following oxaliplatin-fluorouracil chemotherapy in oesophageal adenocarcinoma was assessed in two cohorts of patients.

The first cohort comprised 38 patients, who received two cycles of neoadjuvant oxaliplatin and infusional 5-FU as part of a prospective trial. This trial recruited 57 patients in total. The treatment received and samples available are

summarised in **FIGURE 3.1**, and a full description of the treatment is given in Section 1.2 Thirty-eight patients with adenocarcinoma had pre-treatment biopsies available for analysis, of whom 35 went on to have resection of their tumours and thus had post-treatment resection specimens. The characteristics of these patients are shown in **TABLE 3.2**. Response to treatment in these patients was determined by metabolic response (measured by CT-PET), pathological response, disease free survival (DFS) and overall survival (OS).

CT-PET was performed before the start of chemotherapy, and repeated 24-28 days after the start of cycle two. Response was assessed using the PERCIST criteria – complete metabolic response (CMR) was defined as complete resolution of ^{18}F-FDG uptake within the primary tumour (ie to below the level of mean liver activity); partial metabolic response (PMR) was defined as a greater than 30% decrease in SUV_{max} (Wahl et al., 2009). Twenty-two patients (57.9%) exhibited PMR, and there were 14 patients (36.8%) who showed a CMR.

Pathological response was assessed on the post-treatment resection specimens using the method described by Mandard *et al*.(Mandard et al., 1994) Patients exhibiting Mandard grades 1-3 were classified as pathological responders; those with Mandard grades 4 and 5 were classified as non-responders (Fareed et al., 2010; Reynolds et al., 2007). Of the 35 tumours that underwent resection, and for whom pre-treatment biopsy samples were available, 19 showed pathological response. The three patients that had progressive disease and did not undergo resection were considered to be pathological non-responders for the purpose of analyses.

A second cohort of 54 oesophageal adenocarcinoma patients treated with surgery alone between 1997 and 2004 was used to explore the prognostic role of ICL repair protein expression in the absence of neoadjuvant chemotherapy. Inclusion criteria were as per the oxaliplatin-fluorouracil patients except that Stage 1 patients were also included. The outcome measured in these patients was OS. A comparison of the characteristics of these patients with those of the patients receiving neoadjuvant oxaliplatin-fluorouracil is shown in **TABLE 4.4**.

Table 4.4. Characteristics of the cohorts of patients with oesophageal adenocarcinoma treated with neoadjuvant oxaliplatin-fluorouracil chemotherapy and with surgery alone.

Characteristic	Oxaliplatin-fluorouracil cohort (*N*=38)		Prognostic (no chemotherapy) cohort (*N*=54)	
Age				
Median	67		64	
Range	49-78		40-80	
Sex				
Male	30	(73.3%)	46	(85.2%)
Female	8	(26.7%)	8	(14.8%)
Clinical stage				
Ib	9	(23.7%)	17	(31.5%)
IIa	6	(15.8%)	7	(13.0%)
IIb	6	(15.8%)	5	(19.4%)
III	17	(44.7%)	25	(46.3%)
IV	0	(0%)	0	(0%)

4.7 CORRELATION BETWEEN TUMOUR *ERCC1* AND *XPF* MRNA LEVELS AND XPF PROTEIN EXPRESSION IN OESOPHAGEAL ADENOCARCINOMA

In Chapter 3, it was shown that high levels of *ERCC1* mRNA expression were associated with worse disease free and overall survival following neoadjuvant

oxaliplatin-fluorouracil chemotherapy in oesophageal adenocarcinoma. As has been described above, no suitable antibody for immunohistochemical detection of ERCC1 protein could be found, and XPF IHC was proposed as an alternative for measurement of expression of the XPF-ERCC1 heterodimer.

To confirm the previously published *in vitro* findings that XPF and ERCC1 levels correlate (Hatch et al., 2014), and support the use of XPF as an alternative marker to ERCC1 for measuring expression of the XPF-ERCC1 heterodimer, the correlation between level of tumour *ERCC1* mRNA expression and composite score for tumour XPF protein expression was assessed in pre-treatment samples from the oxaliplatin-fluorouracil cohort of patients. There was a positive correlation between *ERCC1* mRNA expression detected on microarray analysis and XPF expression as measured by semi-quantitative immunohistochemistry (z value 2.043, P=0.041). No correlation was found between *XPF* mRNA expression and XPF expression (z value 0.918, P = 0.359).

4.8 EXPRESSION OF XPF IN OESOPHAGEAL ADENOCARCINOMA BEFORE AND AFTER OXALIPLATIN-FLUOROURACIL CHEMOTHERAPY

To assess the expression of XPF in oesophageal adenocarcinoma, samples for 38 patients were accessed. Thirty-four of these patients went on to have surgical resection of the primary tumour, and so post-treatment samples were also available. After two cycles of oxaliplatin-fluorouracil chemotherapy there was a significantly increased expression of XPF in the tumours (median pre-treatment

2, IQR 0-4; median post-treatment 4.5, IQR 3-6; *P* = 0.004, Wilcoxon's sign rank test) (**FIGURE 4.6**).

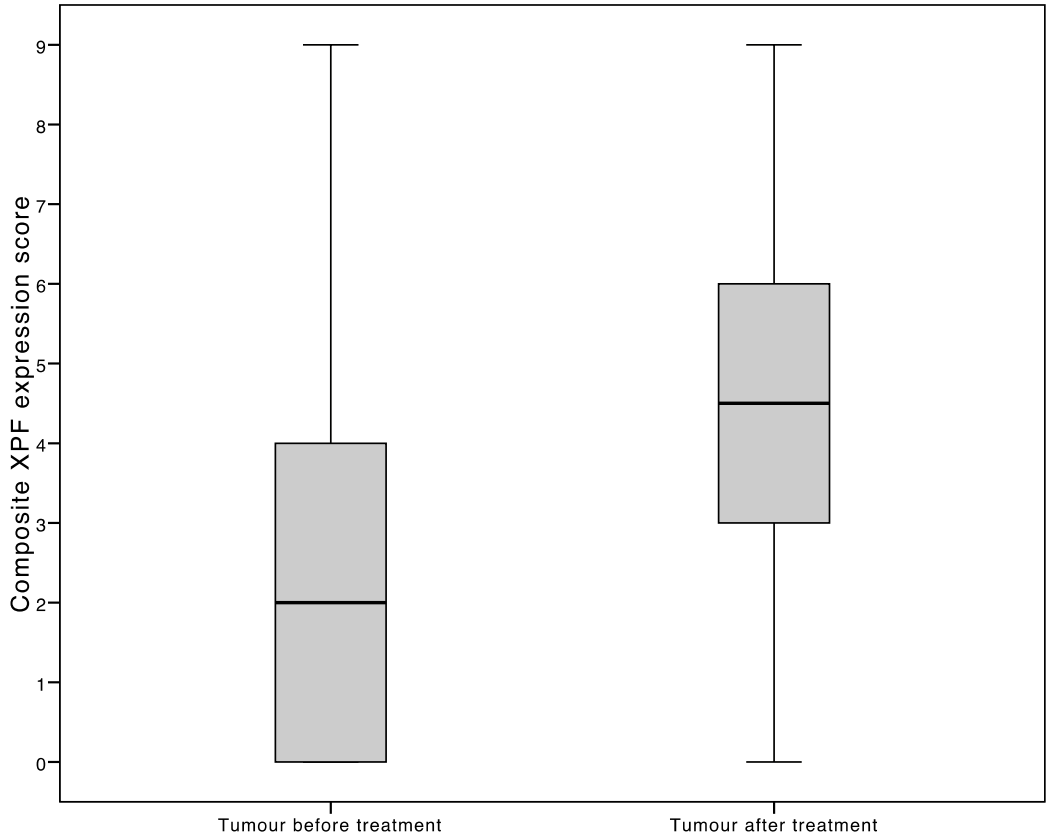


Figure 4.6. Alteration in tumour XPF levels following oxaliplatin-fluorouracil chemotherapy. There was a significant increase in levels of XPF levels when pre-treatment biopsies were compared with post-treatment resection samples (median pre-treatment 2, IQR 0-4; median post-treatment 4.5, IQR 3-6; *P* = 0.004, Wilcoxon's sign rank test).

4.9 CORRELATION BETWEEN XPF EXPRESSION AND CLINICAL OUTCOMES FOLLOWING OXALIPLATIN-FLUOROURACIL CHEMOTHERAPY IN OESOPHAGEAL ADENOCARCINOMA

To assess the relationship between XPF expression and clinical outcomes following oxaliplatin-fluorouracil chemotherapy, patients were divided into high and low expressers. High expression had been previously defined *a priori* as a greater than median composite score, thus a composite score of 3 or more was deemed high expression. Fifteen patients (39.5%) were scored as high expressers. High and low expressers were compared for differences in metabolic response, pathological response, one-year DFS, DFS and OS.

To evaluate whether XPF expression was related to potential prognostic factors, the relationship between XPF expression and patient age, sex and pre-operative stage was assessed in the cohort of patients who received neoadjuvant oxaliplatin-fluorouracil chemotherapy. There was no significant relationship between high XPF expression and age (t = 1.210, P = 0.214, independent samples t-test), sex (χ^2 =2.521, P = 0.112), or stage (I and II *vs* III and IV) (χ^2 = 1.400, P = 0.237).

Low XPF expression was associated with pathological response (χ^2 = 4.332; P = 0.049; OR = 3.846) (**FIGURE 4.7**). No significant association was found between XPF expression and PMR (χ^2 = 0.707, P = 0.401), CMR (χ^2 = 1.666, P = 0.197), one year DFS (χ^2 = 0.030; P = 0.862), DFS (high *vs* low expression, median 394 days *vs*

594 days; $\chi^2 = 0.024$; $P = 0.876$) (logrank statistic), or OS (high *vs* low expression, median 639 days *vs* 754 days; $\chi^2 = 0.098$; $P = 0.755$).

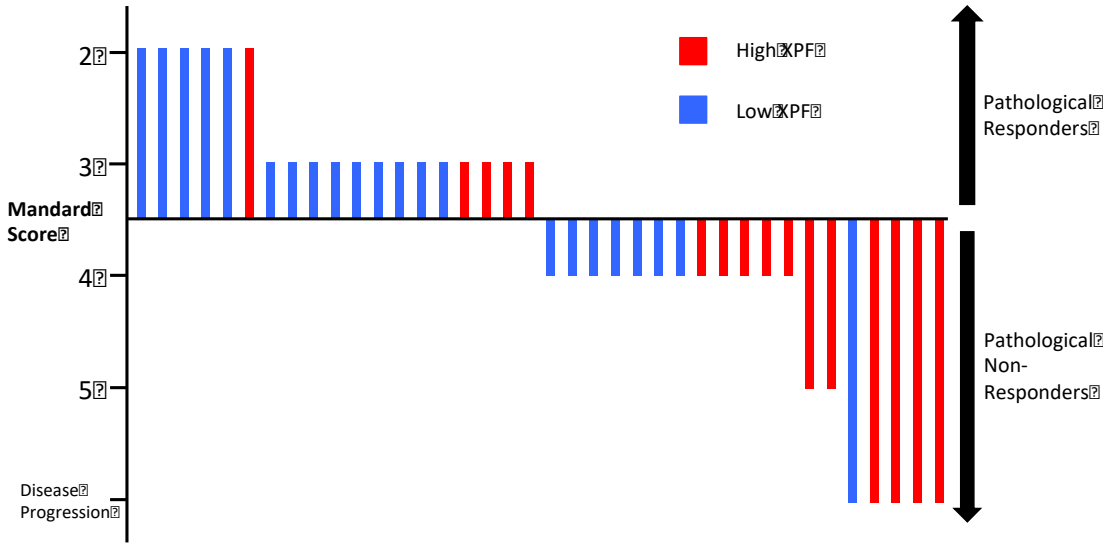


Figure 4.7. A waterfall plot of the association between pre-treatment tumour XPF level and pathological response following oxaliplatin-chemotherapy. Each line represents the degree of Mandard regression in an individual patient; low XPF expression was associated with pathological response (odds ratio 3.846, *P*=0.041).

4.10 XPF EXPRESSION AND OVERALL SURVIVAL IN PATIENTS WITH OESOPHAGEAL ADENOCARCINOMA TREATED WITH SURGERY ALONE

To assess the potential that XPF expression is a prognostic biomarker in the absence of chemotherapy, the relationship between XPF expression and overall survival in the series of 54 patients whose oesophageal adenocarcinoma was managed with surgery alone was assessed. Forty-eight tumours were evaluable

for XPF expression. Twenty-six patients (55.3%) had a composite score of 6 or more and were classified as high expressers of XPF. There was no significant association between XPF expression level and OS in these patients (high *vs* low expression, median 1095 days *vs* 669 days; $\chi^2 = 0.912$; $P = 0.340$; Logrank).

4.11 CORRELATION BETWEEN TUMOUR *MUS81* AND *EME1* MRNA LEVELS AND MUS81 PROTEIN EXPRESSION IN OESOPHAGEAL ADENOCARCINOMA

Following the results above showing that level of expression of *ERCC1* mRNA correlated with XPF protein expression, the tumour MUS81 expression was tested for correlation with tumour expression of *MUS81* and *EME1* mRNA. No significant association was found between level of MUS81 expression in a tumour and expression of *MUS81* mRNA (z value 0.627, $P = 0.53$) or *EME1* mRNA (z value 1.441, $P = 0.149$).

4.12 EXPRESSION OF MUS81 IN OESOPHAGEAL ADENOCARCINOMA BEFORE AND AFTER OXALIPLATIN-FLUOROURACIL CHEMOTHERAPY

The influence of two cycles of oxaliplatin-fluorouracil chemotherapy on tumour MUS81 expression was assessed. Thirty-three patients had pre-treatment biopsies for which MUS81 levels could be scored, and 31 patients also had evaluable post-treatment biopsies. After two cycles of oxaliplatin-fluorouracil chemotherapy there was a trend to increased levels of MUS81 in the tumours, but this failed to reach statistical significance (median pre-treatment 6, IQR 3-9;

median post-treatment 9, IQR 6-9; *P* = 0.051, Wilcoxon's sign rank test) (**FIGURE 4.8**).

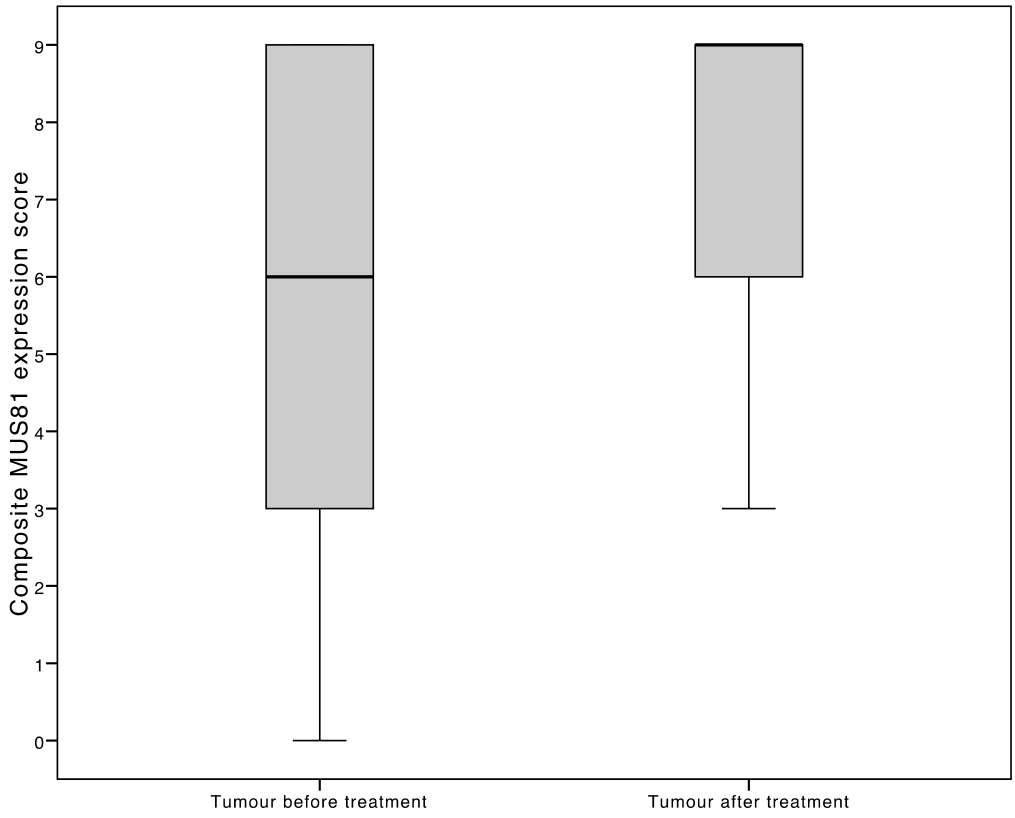


Figure 4.8. Alteration in tumour MUS81 levels following oxaliplatin-fluorouracil chemotherapy. There was not a significant increase in levels of MUS81 levels when pre-treatment biopsies were compared with post-treatment resection samples (median pre-treatment 6, IQR 3-9; median post-treatment 9, IQR 6-9; *P* = 0.051, Wilcoxon's sign rank test).

4.13 CORRELATION OF MUS81 EXPRESSION AND CLINICAL OUTCOMES FOLLOWING OXALIPLATIN-FLUOROURACIL CHEMOTHERAPY IN OESOPHAGEAL ADENOCARCINOMA

The relationship between pre-treatment tumour MUS81 levels and outcomes was assessed. As the pre-treatment median score of 6 for MUS81 expression was relatively high, this posed a problem for the *a priori* defined cut-off of greater than median expression. It was therefore decided that high expression should be defined as staining of strong intensity (intensity 3) in more that 10% of tumour nuclei (frequency score of 2-3). Eighteen (54.5%) patients were defined as having high MUS81 expression.

To exclude a relationship between level of MUS81 expression and potential prognostic factors, the MUS81 expression level was tested for association with patient age, sex and pre-operative stage in the cohort of patients who received neoadjuvant oxaliplatin-fluorouracil chemotherapy. There was no significant relationship between high MUS81 expression and age (t = 0.148, P = 0.883, independent samples t-test), sex (χ^2 = 0.061, P = 0.805), or stage (I and II *vs* III and IV) (χ^2 = 0.550, P = 0.458).

MUS81 level was assessed for correlation with metabolic response, pathological response, one year DFS, DFS and OS. There was no correlation between MUS81 expression and PMR (χ^2 = 1.245, P = 0.265), CMR (χ^2 = 2.672, P = 0.102) or pathological response (χ^2 = 0.203; P = 0.653). High MUS81 expression was associated with worse one-year DFS (χ^2=4.332, P=0.037, OR=5.000) and worse

early disease recurrence or death (high *vs* low expression, median 257 days *vs* 1,107 days; χ^2 = 4.377; *P* = 0.036; Generalised Wilcoxon) (**FIGURE 4.9**). High MUS81 expression also correlated with worse OS (high *vs* low expression, median 470 days *vs* not reached; χ^2 = 6.216; *P* = 0.013; Logrank) (**FIGURE 4.10**).

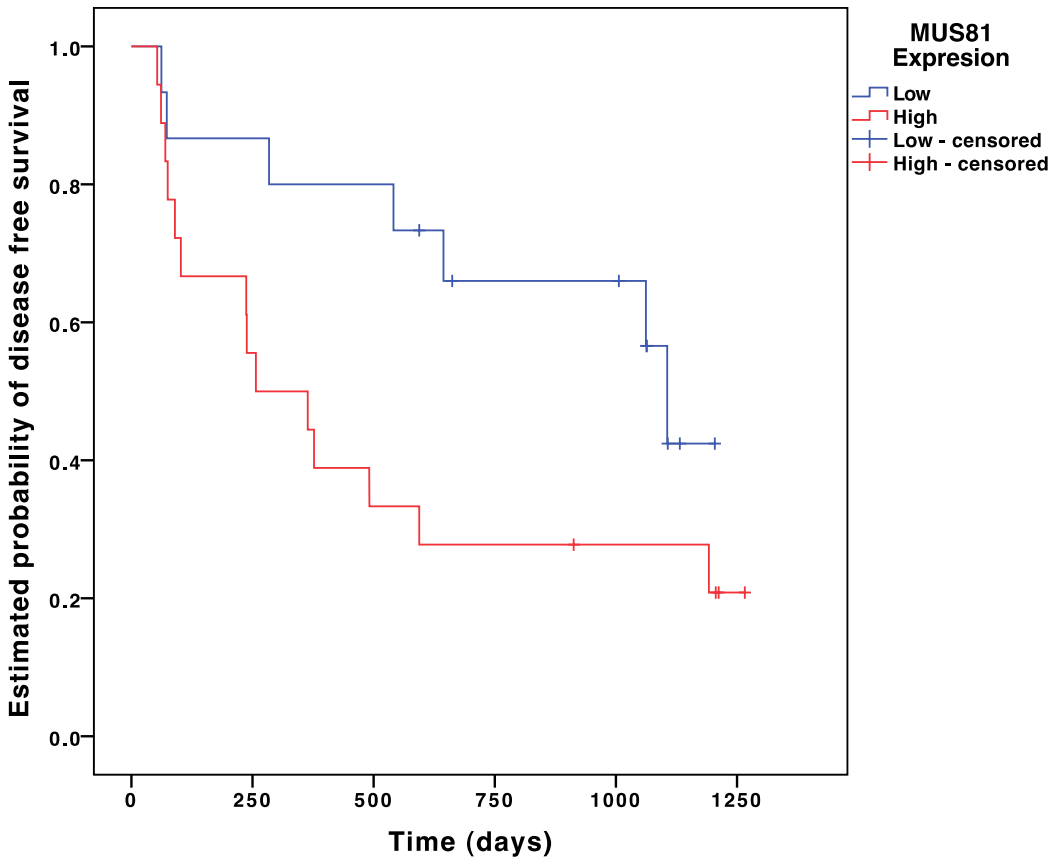


Figure 4.9. Kaplan-Meier analysis of disease free survival following oxaliplatin-fluorouracil chemotherapy based on pre-treatment level of MUS81 protein. Patients with low levels of MUS81 had improved DFS (high *vs* low expression, median 257 days *vs* 1,107 days; χ^2 = 4.377; *P* = 0.036; Generalised Wilcoxon).

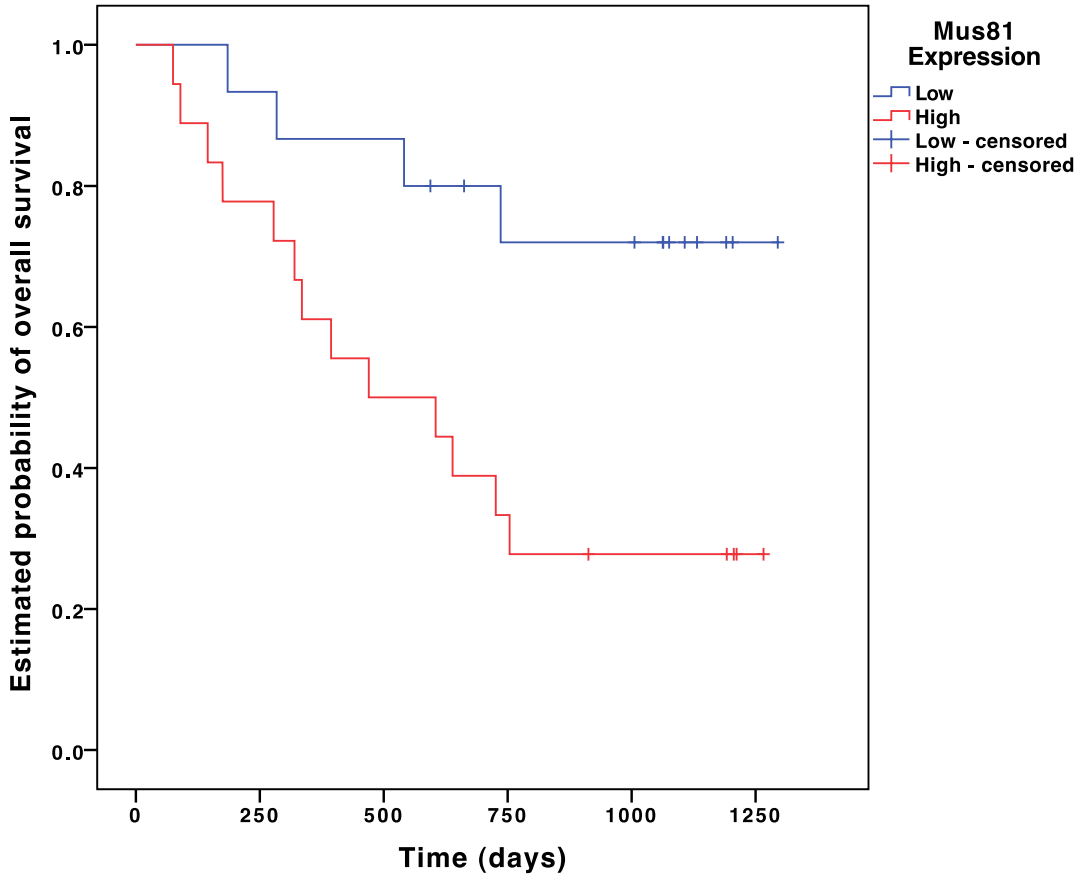


Figure 4.10. Kaplan-Meier analysis of overall survival following oxaliplatin-fluorouracil chemotherapy based on pre-treatment level of MUS81 protein. Patients with low levels of MUS81 had improved OS (high *vs* low expression, median 470 days *vs* not reached; $\chi^2 = 6.216$; $P = 0.013$; Logrank).

4.14 MUS81 EXPRESSION AND OVERALL SURVIVAL IN PATIENTS WITH OESOPHAGEAL ADENOCARCINOMA TREATED WITH SURGERY ALONE

MUS81 expression was measured in the series of 54 patients whose oesophageal adenocarcinoma was managed with surgery alone with the aim of determining if MUS81 was a biomarker for prognosis in the absence of chemotherapy. 48

tumours were evaluable for MUS81 expression. Thirteen patients (28.3%) were classified as high expressers of MUS81. There was no significant association between MUS81 expression level and OS in these patients (high *vs* low expression, median 1095 days *vs* 700 days; $\chi^2 = 0.904$; $P = 0.342$; Logrank).

4.15 CHARACTERISTICS OF COHORT OF PATIENTS WITH OESOPHAGEAL ADENOCARCINOMA TREATED WITH EPIRUBICIN-CISPLATIN-FLUOROURACIL CHEMOTHERAPY

Following the results above, indicating that high levels of XPF and MUS81 were associated with worse outcomes following neoadjuvant oxaliplatin-fluorouracil, it was decided to test the hypothesis that expression of these two DNA repair proteins was also associated with outcomes following neoadjuvant cisplatin-fluorouracil chemotherapy in oesophageal adenocarcinoma. Pre-treatment tumour biopsy samples were obtained for 46 patients with oesophageal adenocarcinoma who had received three cycles of neoadjuvant epirubicin-cisplatin-fluorouracil (ECF) or epirubicin-cisplatin-capecitabine (EXC) chemotherapy. Each 3-week cycle consisted of epirubicin (50 mg per square metre of body-surface area) by intravenous bolus on day 1, cisplatin (60 mg per square metre) intravenously with hydration on day 1, either and fluorouracil (200 mg per square metre) daily for 21 days by continuous intravenous infusion or oral capecitabine. The characteristics of these patients are shown in **TABLE 4.5**. Measures of response to treatment available for these patients were pathological response, DFS and OS. Pathological response was again defined as Mandard regression grades 1-3.

Table 4.5. Characteristics of the cohort of patients with oesophageal adenocarcinoma treated with neoadjuvant epirubicin-cisplatin-fluorouracil chemotherapy.

Characteristic	Cisplatin-fluorouracil cohort (*N*=46)
Age	
Median	62
Range	28-78
Sex	
Male	43 (73.9%)
Female	12 (26.1%)
Clinical stage	
Ib	6 (13.0%)
IIa	11 (23.9%)
IIb	2 (4.4%)
III	27 (48.7%)
IV	0 (0%)

4.16 CORRELATION OF XPF EXPRESSION AND CLINICAL OUTCOMES FOLLOWING EPIRUBICIN-CISPLATIN-FLUOROURACIL CHEMOTHERAPY IN OESOPHAGEAL ADENOCARCINOMA

Tumour expression of level XPF protein was tested for association with clinical outcomes following neoadjuvant ECF chemotherapy. As previously, a composite score of three or more was used to define high expression for XPF. Twenty-seven patients (58.7%) were scored as high expressers. High and low expressers were compared for differences in pathological response, DFS and OS.

There was no association between XPF expression and pathological response (χ^2 = 0.146, P = 0.702). Nor was there a significant association between XPF expression and DFS (high *vs* low expression, median 1233 days *vs* 669 days; χ^2 =

3.360; P = 0.067; Logrank) or OS (high *vs* low expression, median 1344 days *vs* 1077 days; χ^2 = 3.546; P = 0.060; Logrank).

4.17 CORRELATION OF MUS81 EXPRESSION AND CLINICAL OUTCOMES FOLLOWING EPIRUBICIN-CISPLATIN-FLUOROURACIL CHEMOTHERAPY IN OESOPHAGEAL ADENOCARCINOMA

MUS81 expression levels were measured in the tumours of the patients receiving neoadjuvant ECF or ECX chemotherapy. High expression was again defined as an intensity score of 3 in more than 10% of tumour nuclei. Thirty-four patients (73.9%) were defined as having high levels of tumour MUS81 expression. The high and low expressers were compared for differences in rates of pathological response, DFS and OS.

No association was found between level of MUS81 expression and rate of pathological response (χ^2 = 0.060, P = 0.806). Nor was there an association between MUS81 expression and DFS (high *vs* low expression, median 974 days *vs* 2808 days; χ^2 = 2.272; P = 0.132; Logrank) or OS (high *vs* low expression, median 1194 days *vs* 2808 days; χ^2 = 1.814; P = 0.178; Logrank).

4.18 DISCUSSION OF RESULTS OF MEASUREMENT OF DNA REPAIR PROTEIN LEVELS IN OESOPHAGEAL ADENOCARCINOMAS

4.18.1 Influence of XPF expression on response and survival following platinum-based chemotherapy in oesophageal adenocarcinoma

XPF expression has been demonstrated to correlate with resistance to oxaliplatin in melanoma cell lines (Hatch et al., 2014). Knockdown of XPF expression has

been shown to sensitise colorectal and osteosarcoma cell lines to oxaliplatin (Graf et al., 2011; Hatch et al., 2014). No studies to date have established the relationship between XPF protein expression and response to oxaliplatin-based chemotherapy in oesophageal adenocarcinoma.

Two studies have tested the relationship between pre-treatment XPF protein expression and outcomes following neoadjuvant cisplatin-containing chemotherapy in oesophageal cancer. Fareed *et al*. investigated the XPF levels in 61 patients receiving neoadjuvant platinum-based chemotherapy for gastro-oesophageal adenocarcinoma (Fareed et al., 2010). This study included both gastric cancers and oesophageal adenocarcinomas, with patients receiving either epirubicin, cisplatin, and 5-fluorouracil (ECF) or epirubicin, cisplatin, and capecitabine (ECX) neoadjuvant chemotherapy. The relative numbers of the two tumour types was not specifically reported in the subgroup analysed for XPF expression, although the larger cohort of patients in the study, 19.4% had gastric cancers.

Fareed *et al*. failed to demonstrate a relationship between XPF expression and pathological response, DFS or OS. Interestingly, high expression of ERCC1, the other component of the XPF-ERCC1 functional heterodimer, was associated with lack of pathological response and worse DFS and OS. The results relating to ERCC1 in this study must, however, be treated with caution, as the non-specific 8F1 antibody used for the immunohistochemistry; the concerns surrounding this antibody have been discussed in **Section 1.4.1**. Potential confounding factors for the failure of this study to demonstrate a relationship between XPF expression

and outcomes include the inclusion of the anthracycline, epirubicin, in the chemotherapy regimen and the heterogeneity of tumour types included.

Alexander *et al*. measured XPF protein expression in gastro-oesophageal cancers treated with neoadjuvant chemoradiotherapy and found an association between low levels of XPF and a shorter time to recurrence (Alexander et al., 2012). This is the converse of our hypothesis that high levels of XPF are associated with worse outcomes. The study, however, is very difficult to interpret as they failed to define their cohort of 79 patients, instead using a subgroup of two other studies. These studies incorporated a heterogenous mixture of adenocarcinomas and squamous cell carcinomas and the treatment received was neoadjuvant paclitaxel, cisplatin and fluorouracil chemoradiotherapy (Choi et al., 2004; Roof et al., 2006). It is, therefore, difficult to extrapolate these results to situations where platinum-based chemotherapy is the major component for the treatment of gastrointestinal adenocarcinomas.

In our study, high levels of tumour XPF expression pre-treatment was associated with lack of pathological response as measured by Mandard tumour regression grading of resection specimens following two cycles of oxaliplatin-fluorouracil chemotherapy. This is in keeping with the hypothesis that high levels of XPF expression are associated with lack of response to platinum chemotherapy. This did not, however, translate into a significant association between high XPF expression and worse OS or DFS. The lack of translation of the association between XPF expression and pathological response into an association with DFS

or OS may be due to the relatively small number of patients in the study (N = 39) or the confounding effects of surgical outcomes and complications.

There was also a significant increase in XPF levels in oesophageal adenocarcinomas after two cycles of oxaliplatin-fluorouracil chemotherapy. This supports the hypothesis that high levels of XPF expression are associated with tumour cell survival following oxaliplatin exposure. There are two possible explanations for this. Firstly, there may be selection for cells expressing high levels of XPF by the chemotherapy. Alternatively, the DNA damage response to oxaliplatin chemotherapy causes an increase in XPF levels. There is evidence in support of the first hypothesis from a study performed in a subset of 24 of the patients in the trial. This demonstrated that, even over the short time period of the two cycles of neoadjuvant chemotherapy followed by a 4-6 weeks of pre-operative recovery, considerable clonal evolution of the tumours occurred (Findlay et al., 2016). It can be hypothesised that the increase in XPF protein levels represents the result of a positive selection pressure due to the protective effect of high levels of XPF-ERCC1 function in the face of oxaliplatin-based chemotherapy.

The potential that XPF is a prognostic biomarker in oesophageal adenocarcinoma was tested in a matched cohort of 48 patients with oesophageal adenocarcinoma treated with surgery alone. No association was found between XPF expression and OS in these patients. This supports the hypothesis that the association between XPF expression and pathological response is related to the

influence of XPF on processing oxaliplatin-induced lesions, rather than XPF being a prognostic biomarker *per se*.

No other cohorts of oxaliplatin-treated oesophageal adenocarcinoma patients were available to validate the association between high XPF expression and lack of response to oxaliplatin-fluorouracil chemotherapy in oesophageal adenocarcinoma. In an attempt to see if this finding was generalizable to platinum-based chemotherapy in oesophageal adenocarcinoma, XPF levels were assayed in pre-treatment biopsies from 46 patients with oesophageal adenocarcinoma who received three cycles of neoadjuvant epirubicin-cisplatin-fluorouracil chemotherapy. In these patients, no association was found between XPF expression and pathological response, DFS or OS following the chemotherapy. This finding is in keeping with the result published by Fareed *et al*. that are discussed above (Fareed et al., 2010).

The lack of association between XPF expression and response to epirubicin-cisplatin-fluorouracil chemotherapy in oesophageal adenocarcinoma does not support our hypothesis. There are three possible explanations for this. Firstly, it may be that XPF plays a specific role in the processing of oxaliplatin-induced lesions, but is not essential for processing cisplatin-induced lesions. It is known that different degrees of DNA strand distortion occur with cisplatin and oxaliplatin crow-links; for example, the minor groove with the oxaliplatin intrastrand cross-link is relatively narrow when compared with that of cisplatin (Wu et al., 2004). It may be that the different DNA shapes generated by individual cross-linking agents alters the choice of structure specific

endonuclease for processing them. Going against this explanation, it has been shown *in vitro* that knockdown of XPF expression sensitises cell lines to both oxaliplatin (Graf et al., 2011; Hatch et al., 2014) and cisplatin (Arora et al., 2010).

Secondly, the inclusion of epirubicin in the chemotherapy regimen may complicate the pattern of response. Epirubicin is a DNA intercalating agent, and there is no *in vitro* evidence of a role for XPF-ERCC1 in repairing epirubicin-induced DNA lesions. Interestingly, high levels of ERCC1 expression have been linked with response to doxorubicin-based chemotherapy in breast cancer (Kolacinska et al., 2012), this is the converse of what might be expected if ERCC1 had a role in the repair of doxorubicin-induced DNA damage. On the basis of the current literature, no role has been demonstrated for XPF-ERCC1 in the repair of epirubicin-induced DNA damage. It may be that the addition of epirubicin to cisplatin-fluorouracil chemotherapy in oesophageal cancer drowns out the association between XPF expression and response to treatment.

Finally, the failure to find an association may relate to the handling and storage of the tumour biopsies. In the oxaliplatin-fluorouracil treated cohort of patients, a strict sampling handling protocol was imposed, with uniform fixation times, tissue processing and storage conditions. The epirubicin-cisplatin-flurouracil cohort of patients' biopsies were taken at a number of different hospitals, with a lack of uniform techniques and equipment for tissue fixation and processing. It has been shown that tissue fixation times have a significant impact on detection of XPF in tumour samples, with measurement of expression level increasing with time spent in formalin before tissue processing (Pierceall et al., 2011). In

addition, the slides for the cisplatin-fluorouracil samples had been transported at room temperature prior to arrival in our laboratory. This is in contrast with the protocol enforced for the oxaliplatin-fluorouracil treated oesophageal samples that slides for IHC should be stored at 4 °C immediately after drying. There is evidence that cut sections stored at room temperature undergo significant deterioration in detectable levels of a variety of proteins, but that sections stored at 4 °C show little deterioration in protein levels for up to one year (DiVito et al., 2004; Karlsson and Karlsson, 2011). The hypothesis that the failure to show an association between high XPF expression and worse outcomes in the epirubicin-cisplatin-fluorouracil treated cohort is due to variable tissue handling is supported by the fact that in this cohort 58.7% of patients were classified as high XPF expressers, whereas 39.5% of patients were classified as high XPF expressers in the oxaliplatin-fluorouracil treated cohort.

Another potential criticism of the finding in support of the hypothesis that high XPF was associated with pathological response is that the patients also received 5-fluorouracil. It is unlikely that DNA repair protein expression determines sensitivity to 5-fluorouracil as pyrimidine analogues function by their irreversible inhibition of thymidylate synthase.

4.18.2 Influence of MUS81 expression on response and survival following platinum-based chemotherapy in oesophageal adenocarcinoma

The endonuclease MUS81-EME1 has been shown to have a key role in replication-dependent ICL repair (Hanada et al., 2006). It is known that deficiency of expression of MUS81 or EME1 renders cells hypersensitive to the

cross-linking agents mitomycin C and cisplatin (Abraham et al., 2003; Hiyama et al., 2006; Tomoda et al., 2009). It has also been demonstrated that levels of EME1 and MUS81 correlate with sensitivity to cisplatin in a variety of human cancer cell lines (Hiyama et al., 2006). No previous studies have examined levels of MUS81 or EME1 as markers of sensitivity to oxaliplatin-based chemotherapy, either *in vitro* or in patients.

In this study, high levels of MUS81 protein in pre-treatment tumour biopsies were found to be associated with worse one year DFS, DFS and OS in 39 patients with oesophageal adenocarcinoma treated with neoadjuvant oxaliplatin-fluorouracil chemotherapy. MUS81 expression in these samples was found to be independent of age, sex or tumour stage. There was also a non-significant (P = 0.051) trend towards increased in levels of MUS81 in tumours following two cycles of oxaliplatin-fluorouracil chemotherapy. These results are in keeping with the published literature on *in vitro* studies, and support the hypothesis that high levels of tumour MUS81 protein expression before treatment is associated with lack of response to oxaliplatin chemotherapy.

In an attempt to exclude the possibility that MUS81 is a prognostic biomarker in oesophageal adenocarcinoma, levels of MUS81 expression were assayed in tumours from 49 matched patients treated with surgery alone. In support of the hypothesis, no association was found between MUS81 expression and OS in this cohort of patients.

Following the results suggesting an association between MUS81 expression and DFS and OS following oxaliplatin-fluorouracil chemotherapy, MUS81 expression was measured in the cohort of 46 patients with oesophageal adenocarcinoma treated with three cycles of neoadjuvant epirubicin-cisplatin-fluorouracil chemotherapy. As was the case for XPF, and going against the hypothesis, no association was found between MUS81 expression and pathological response, DFS or OS in these patients.

The possibilities for the failure to replicate the results from the oxaliplatin-fluorouracil treated cohort in this cohort have been discussed in above for XPF. Similar explanations can be made for MUS81. There is considerable in vitro evidence of a role for MUS81-EME1 in processing cisplatin-induced DNA damage (Abraham et al., 2003; Hanada et al., 2006; Hiyama et al., 2006; Tomoda et al., 2009), making it unlikely that differences in adduct conformation between cisplatin and oxaliplatin explain the failure to replicate the findings from the oxaliplatin-fluorouracil cohort in the epirubicin-cisplatin-fluorouracil cohort.

The addition of epirubicin to cisplatin-fluorouracil is again a possible explanation for the failure of MUS81 to predict clinical response. No studies have examined MUS81 in anthracycline treated patients. One small study has examined the effect of MUS81 depletion in a hepatocellular carcinoma model in nude mice, and found that it increased sensitivity to epirubicin chemotherapy (Wu et al., 2016). Further evidence is required to demonstrate a convincing link between MUS81 expression and response to epirubicin. It may be that, if MUS81 does not affect response to epirubicin in oesophageal cancer, the effect of

epirubicin addition on outcomes overcomes any association between MUS81 levels and cisplatin response.

It is also possible that variations in the handling of the biopsies from the cisplatin-fluorouracil treated patients in the different centres that they were obtained from caused an artificial variance in MUS81 levels in these patients. This idea is supported by the finding that 73.9% of patients in the epirubicin-cisplatin-fluorouracil treated series were defined as having high levels of MUS81 compared with 54.5% of those in the oxaliplatin-fluorouracil cohort.

4.19 SUMMARY

In support of the hypothesis that high levels of expression of XPF protein and MUS81 protein in oesophageal adenocarcinomas are associated with lack of response to platinum-based chemotherapy, it has been shown for the first time that high levels of MUS81 are associated with worse clinical outcomes (DFS and OS) following oxaliplatin-fluorouracil chemotherapy in oesophageal adenocarcinoma. High XPF levels were associated with lack of pathological response in these patients, lending further support to the hypothesis. In addition, there was a significant increase in the level of XPF protein in tumours following two cycles of oxaliplatin-fluorouracil chemotherapy.

No association was found between levels XPF or MUS81 proteins and OS in a cohort of patients treated with surgery alone. This data, combined with a lack of association between levels of the two proteins and age, sex and tumour stage, supports the potential that levels of XPF and MUS81 may be predictive of

response to oxaliplatin-based treatment, rather than simply being prognostic markers. Going against the hypothesis, no association was found between levels of XPF and MUS81 proteins and clinical outcomes following three cycles of neoadjuvant epirubicin-cisplatin-fluorouracil in oesophageal adenocarcinomas.

5. DNA REPAIR PROTEIN AND RESPONSE TO CROSS-LINKING AGENT-BASED CHEMOTHERAPY IN OTHER CANCERS

5.1 INTRODUCTION

This chapter followed on from the findings documented in **Chapter 4** that high levels of XPF and MUS81 proteins in tumours pre-treatment were associated with worse clinical outcomes for patients with oesophageal adenocarcinoma receiving neoadjuvant oxaliplatin-fluorouracil chemotherapy. It tested the hypothesis that high levels of expression of XPF protein and MUS81 protein are associated with lack of response to cross-link generating chemotherapeutic agents. Firstly, pre-treatment levels of XPF and MUS81 proteins were tested in samples from patients treated in the FOCUS2 trial. Half of these patients received oxaliplatin-fluorouracil chemotherapy, and the other half received fluorouracil treatment only. This allowed both the relationship between tumour XPF and MUS81 protein levels and response to oxaliplatin-based chemotherapy in colorectal cancer to be determined and also a potential relationship between levels of the two proteins and response to fluorouracil to be tested for.

Secondly, pre-treatment levels of XPF and MUS81 proteins were measured in biopsies from a series of patients with anal cancer who were treated with mitomycin C-fluorouracil chemoradiotherapy. This aimed to test whether pre-treatment levels of the two proteins were associated with response to the cross-linking agent mitomycin C.

5.2 IMMUNOHISTOCHEMISTRY FOR XPF AND MUS81 IN COLORECTAL CANCER

The rationale for deciding to focus on XPF and MUS81, rather than ERCC1 and EME1, as representatives of the XPF-ERCC1 and MUS81-EME1 heterodimers is discussed in detail in **Sections 4.2** and **4.4**. In order to determine the range of expression levels of the two DNA repair biomarkers in colorectal cancer, sections of colorectal tumours from chemotherapy naïve patients were obtained from the Oxford Biobank. Staining and semi-quantitative scoring were undertaken as described in the Materials and Methods Chapter. No patient characteristics were available for theses samples as they were supplied anonymised by the Biobank. Cut-offs to determine high *vs*. low expression of the two proteins in colorectal cancer were established using this exploratory cohort, with greater than median composite score being used to define high expression of the protein of interest.

One hundred and forty-four full-face sections of colorectal tumours were stained for XPF protein (**FIGURE 5.1)**. **FIGURE 5.2** illustrates the range of scores found – the median score was 3 (IQR 3-6). Using a greater than median cut-off to define high XPF expression (composite score of 4 or more), 44.7% of cases were defined as high XPF expressers.

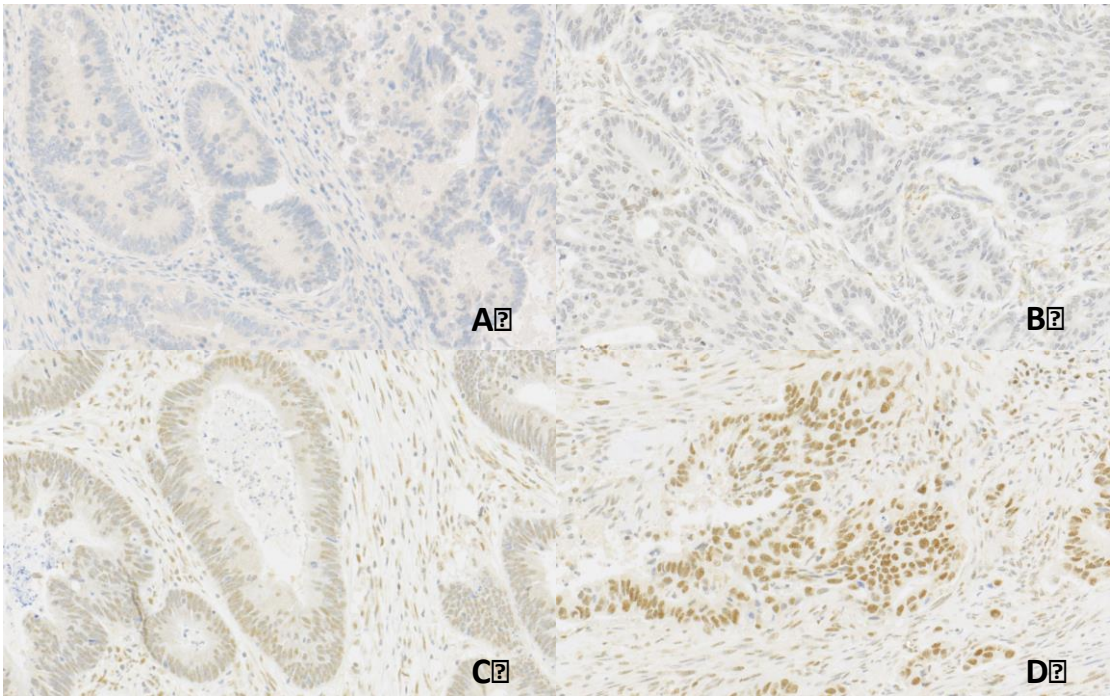


Figure 5.1. XPF staining of colorectal adenocarcinomas. Colorectal adenocarcinomas stained for XPF protein showing intensity scores of 0(A), 1(B), 2(C) and 3(D). All photographed at X20 magnification.

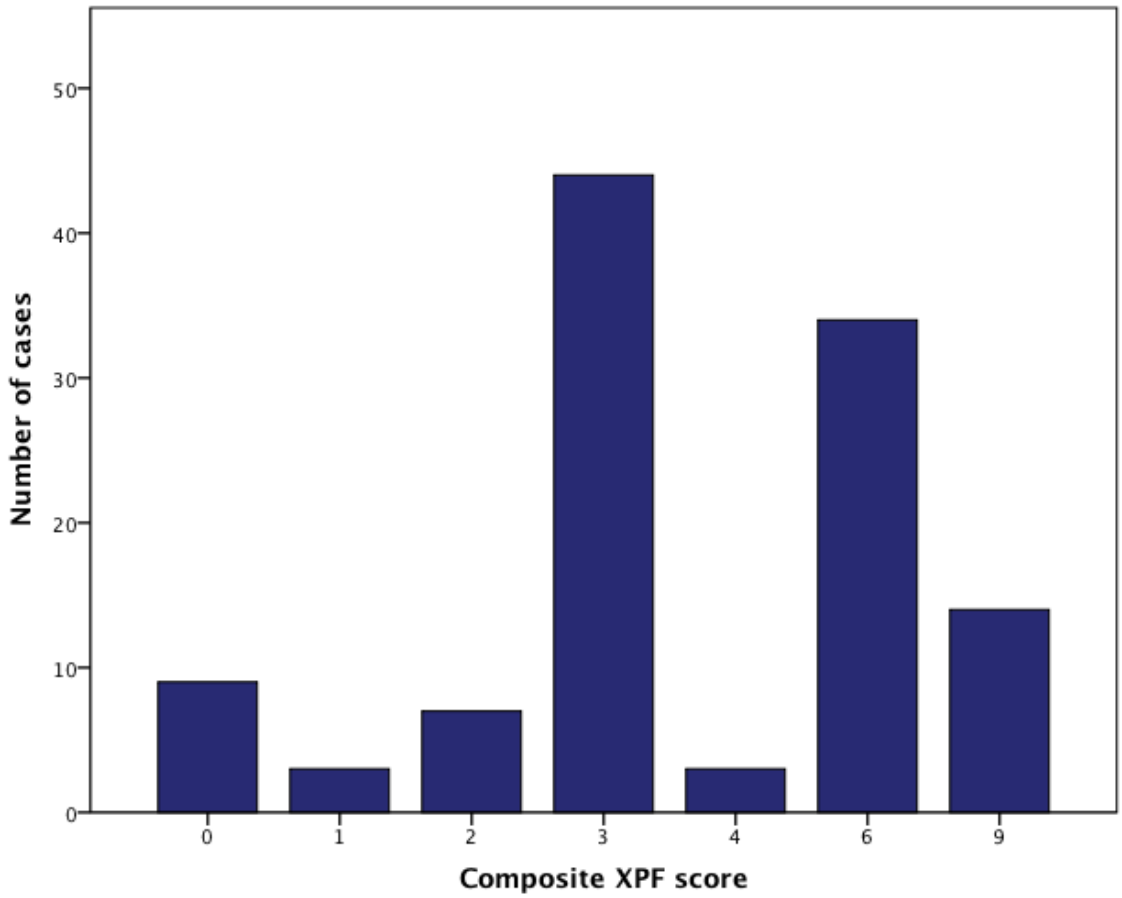


Figure 5.2. Distribution of XPF levels in colorectal adenocarcinomas. One hundred and forty-four full face sections of colorectal adenocarcinomas were stained and scored for levels of XPF protein. The median score was 3 (IQR 3-6).

Ninety-four cases from the same colorectal series were also stained for MUS81 expression (**FIGURE 5.3**). The distribution of the composite MUS81 scores is shown in **FIGURE 5.4**. Using a greater than median score to define high expression (composite score of 6 or more), 38.3% of cases were classified as high expressers.

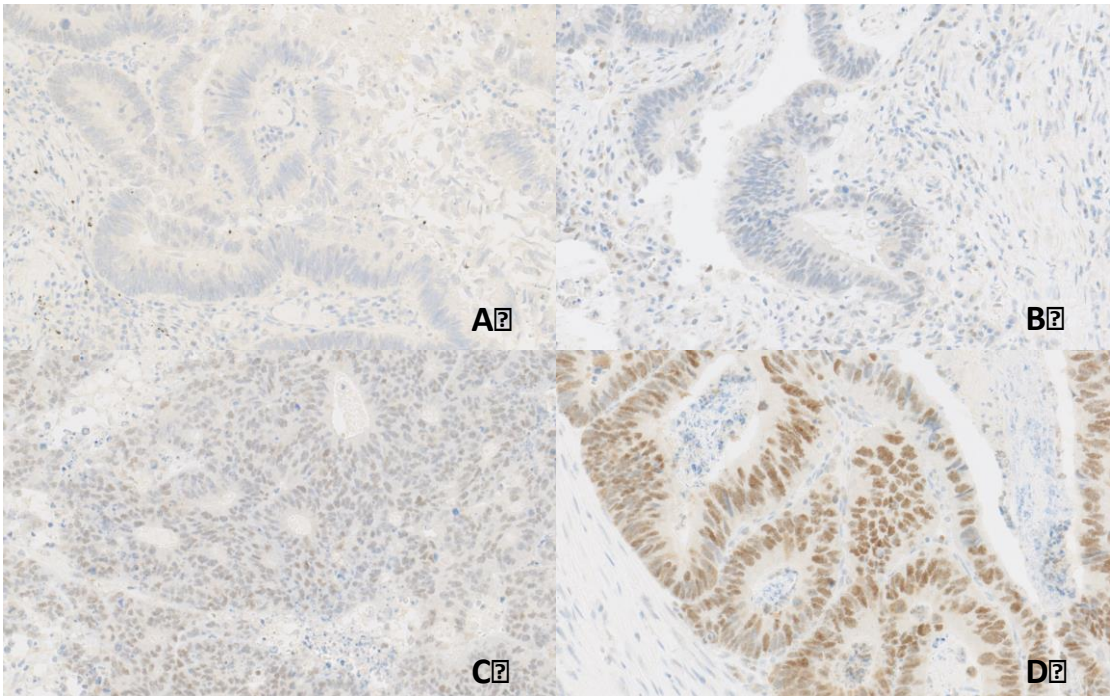


Figure 5.3. MUS81 staining of colorectal adenocarcinomas. Colorectal adenocarcinomas stained for MUS81 protein showing intensity scores of 0(A), 1(B), 2(C) and 3(D). All photographed at X20 magnification.

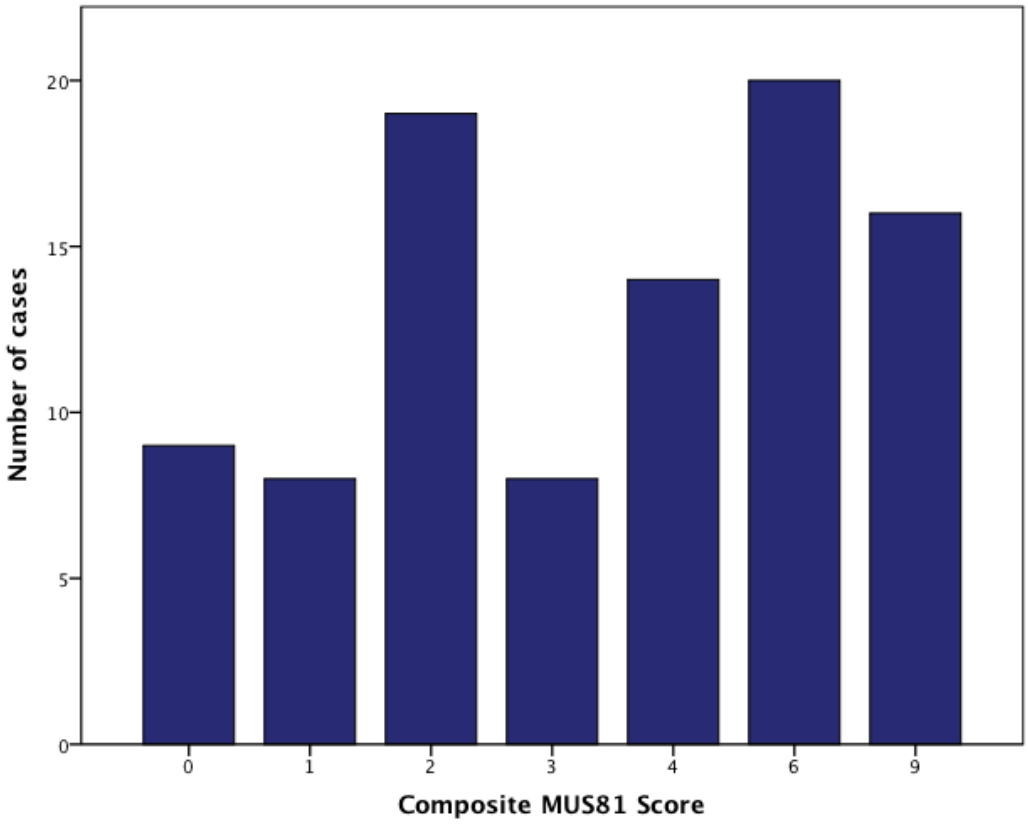


Figure 5.4. Distribution of MUS81 levels in colorectal adenocarcinomas. Ninety-four full face sections of colorectal adenocarcinomas were stained and scored for levels of MUS81 protein. The median score was 4 (IQR 2-6).

5.3 XPF EXPRESSION AND CLINICAL OUTCOMES FOLLOWING OXALIPLATIN-FLUOROPYRIMADINE BASED CHEMOTHERAPY FOR COLORECTAL CANCER

To further evaluate the relationship between pre-treatment XPF expression and response to oxaliplatin-fluoropyrimidine based chemotherapy, seven TMAs containing cores from the FOCUS2 trial (Seymour et al., 2011) were stained for XPF expression. Each patient was represented by three tumour cores, and one core of normal colonic tissue. The TMAs had cores from 209 patients, which were representative of the 459 patients in the original trial.

The patients were randomised to receive one of four chemotherapy regimens: 48 hours of intravenous fluorouracil with levofolinate (group A); oxaliplatin and fluorouracil (group B); capecitabine (group C); or oxaliplatin and capecitabine (group D). Of the 209 patients represented on the TMAs, 53 (25.4%) were in Group A, 52 (24.9%) were in Group B, 51 (24.4%) were in Group C, and 53 (25.4%) were in Group D. The mean age of the patients was 74.5 years (range 46-86 years). There was no significant variation in age between the four treatment arms (F ratio = 0.785, P = 0.503, one-way ANOVA). 114 (54.5%) patients were male and 95 (45.5%) were female; again there was no significant variation of the male:female ratio between the four groups (χ^2 = 1.694, P = 0.638). Other baseline characteristics were also well balanced between the four groups (**TABLE 5.1**).

Patients who received oxaliplatin chemotherapy (Groups B and D) had no significant difference in OS (oxaliplatin *vs* no oxaliplatin, median 389 days *vs* 331 days; χ^2 = 0.290; P = 0.590; Logrank), when compared with those treated with 5-FU based drugs only (**FIGURE 5.6**). The shape of the Kaplan-Meier curves indicated the possibility of a better early PFS in the oxaliplatin-treated patients (**FIGURE 5.5**). Using the rationale outlined in **Section 2.12**, this was tested with the Generalised Wilcoxon test, and an early PFS benefit for the addition of oxaliplatin was confirmed (oxaliplatin *vs* no oxaliplatin, median 180 days *vs* 118 days; χ^2 = 6.165; P = 0.013; Generalised Wilcoxon).

Table 5.1. Characteristics of the colorectal cancer patients. Group A received intravenous fluorouracil with levofolinate; Group B received oxaliplatin and fluorouracil; Group C received capecitabine; and Group D received oxaliplatin and capecitabine.

	Group A *n* = 53	**Group B** *n* = 52	**Group C** *n* = 51	**Group D** *n* = 53	**Total** *n* = 209
Sex					
Male	31 (58.5%)	30 (57.7%)	24 (47%)	29 (54.7%)	114 (54.5%)
Female	22 (41.5%)	22 (42.3%)	27 (53%)	24 (55.3%)	95 (45.5%)
Age (years)					
Median	75	75	74	76	75
IQR	71.5-79	72.25-78.5	70-80	69.5-80	71-79.5
Range	62-86	66-86	55-84	46-86	46-86
WHO performance status					
0	10 (18.9%)	12 (23.1%)	14 (27.5%)	17 (32.1%)	53 (25.4%)
1	30 (56.6%)	27 (51.9%)	26 (51%)	22 (41.5%)	105 (50.2%)
2	13 (24.5%)	13 (25%)	11 (21.6%)	14 (26.4%)	51 (24.4%)
Primary tumour site					
Rectum	9 (17%)	7 (13.5%)	8 (15.7%)	7 (13.2%)	31 (14.8%)
Colon	44 (83%)	45 (86.5%)	43 (84.3%)	46 (86.8%)	178 (85.2%)
Metastatic sites					
No distant metastases	1 (1.9%)	0 (0%)	1 (2%)	0 (0%)	2 (1%)
Liver & other metastases	41 (77.4%)	42 (80.8%)	39 (76.5%)	37 (69.8%)	159 (76.1%)
Liver-only metastases	11 (20.8%)	10 (19.2%)	11 (21.6%)	16 (30.2%)	48 (23%)
Non-liver metastases	21 (39.6%)	28 (53.8%)	22 (43.1%)	27 (50.9%)	98 (46.9%)

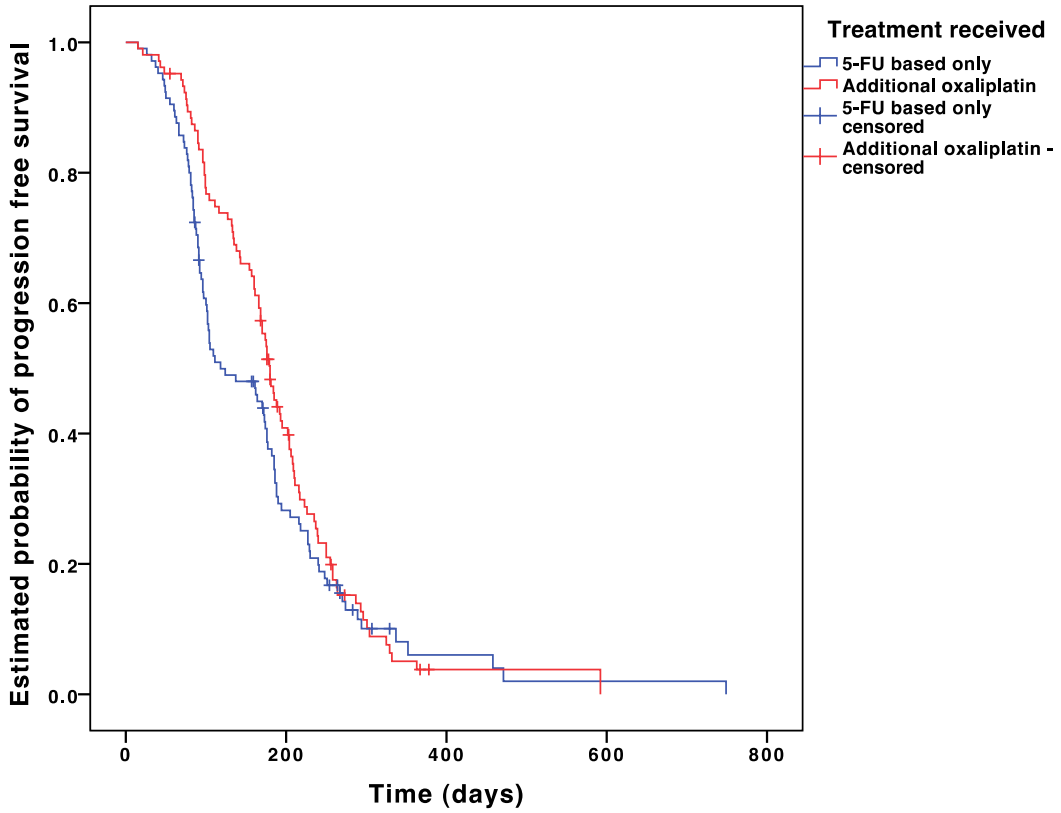


Figure 5.5. Kaplan-Meier analysis of progression free survival in colorectal cancer comparing fluorouracil treatment only with oxaliplatin-fluorouracil treatment. Addition of oxaliplatin was associated with improved early PFS (oxaliplatin *vs* no oxaliplatin, median 180 days *vs* 118 days; $\chi^2 = 6.165$; $P = 0.013$; Generalised Wilcoxon).

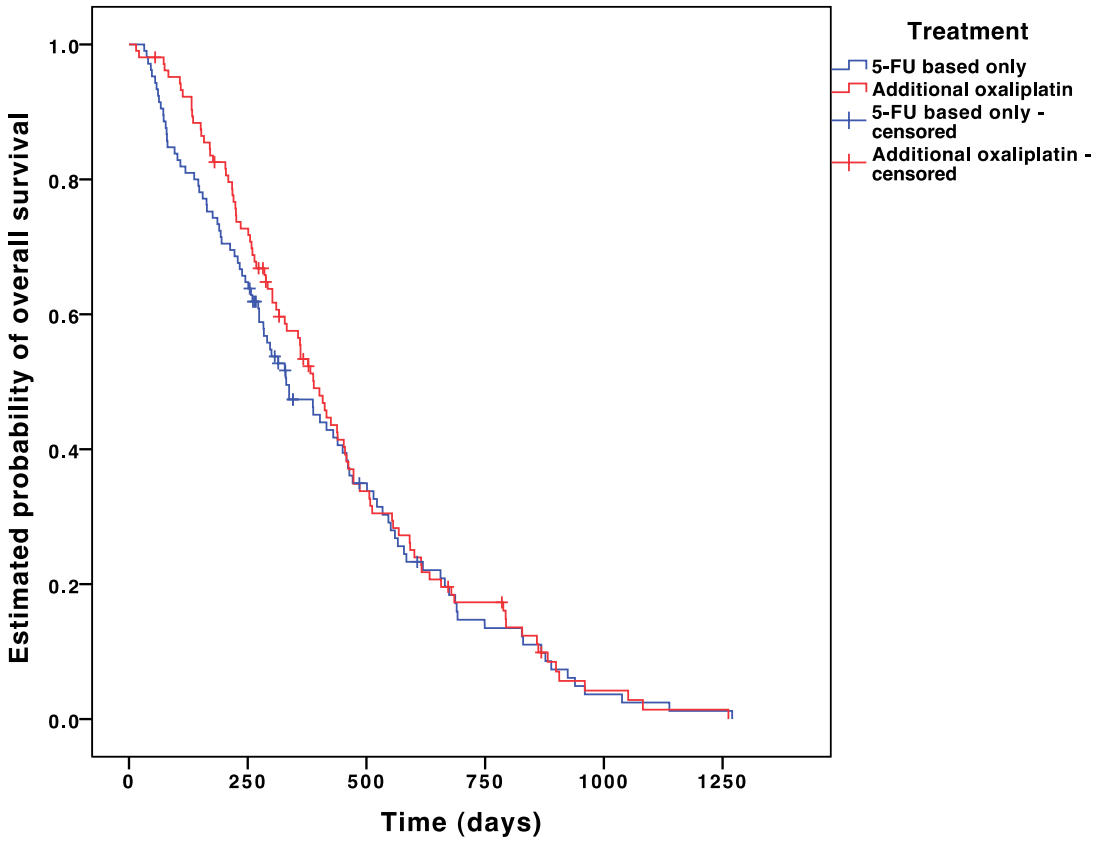


Figure 5.6. Kaplan-Meier analysis of overall survival in colorectal cancer comparing fluorouracil treatment only with oxaliplatin-fluorouracil treatment. Addition of oxaliplatin was associated no significant alteration in OS (oxaliplatin *vs* no oxaliplatin, median 389 days *vs* 331 days; $\chi^2 = 0.290$; $P = 0.590$; Logrank).

Two-hundred and one patients had at least one core of tumour that could be scored. In cases where scores differed between cores for an individual patient, the highest composite score was used for analysis. The median XPF expression score was 3 (IQR 1.75-6), and, using the pre-defined cut-off of a composite score of 4 or more to define high expression, 86 patients (42.6%) had high XPF expression. This produced a comparable proportion of tumours being defined as having high expression to those in the Oxford Biobank series (in which, using the

same cut-off, 44.7% of samples were deemed to have high expression) (**FIGURE 5.7**).

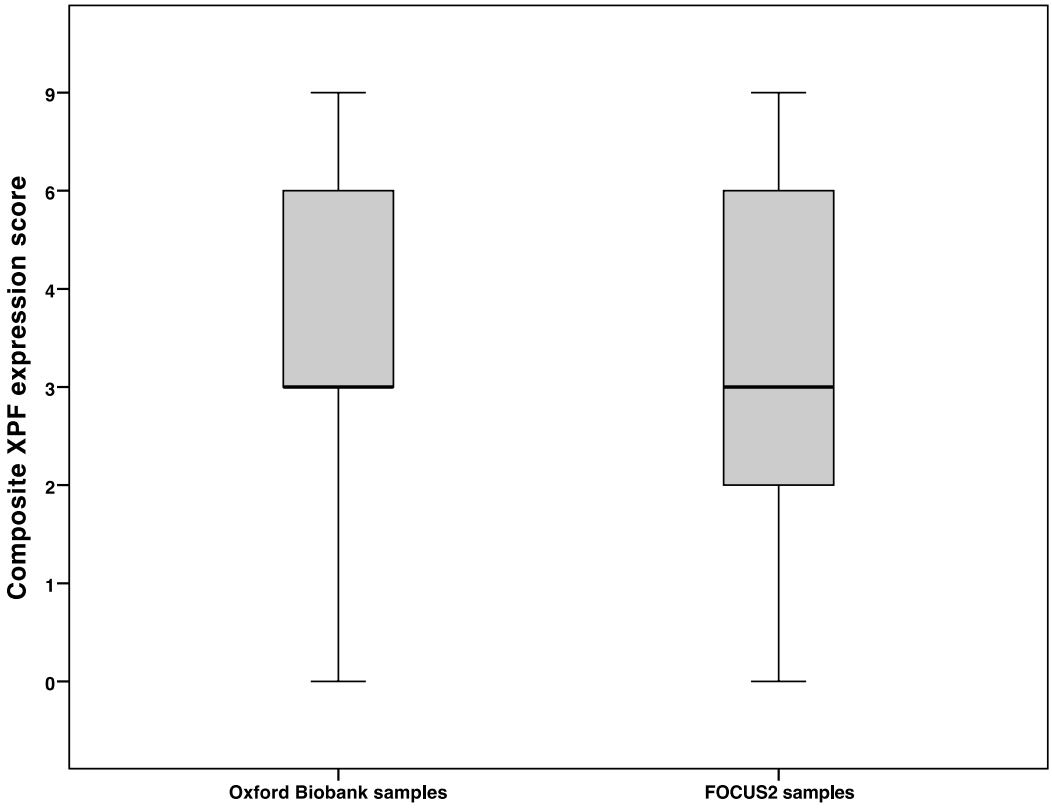


Figure 5.7. Comparison of distribution of XPF levels in samples from Oxford Biobank and FOCUS2 specimens. There was no significant difference in distribution of XPF levels in the two groups (independent samples t-test).

There was no association between XPF protein levels and age (t = 1.140, P = 0.256, independent samples t-test), sex (χ^2 =0.161, P = 0.688), presence of hepatic metastases (χ^2 =0.004, P = 0.949) or primary tumour site (χ^2 =0.476, P = 0.788).

XPF protein levels was tested for association with radiological response, progression free survival (PFS) and overall survival (OS). The hypothesis tested was that high levels of XPF protein would be associated with worse outcomes in the groups receiving oxaliplatin (Groups B and D), but would not be associated with outcome in those treated with 5-fluorouracil (5-FU) based drugs alone (Groups A and C).

Radiological response was measured 12 weeks after the commencement of treatment using the Response Evaluation Criteria In Solid Tumours (RECIST) criteria(Therasse et al., 2000). Radiological response (RR) was defined as complete (CR) or partial response (PR). Disease control (DC) was defined as stable disease (SD), CR or PR. In the whole cohort of patients, there was no association between XPF protein levels and either RR ($\chi^2 = 0.057$, $P = 0.811$) or DC ($\chi^2 = 0.316$, $P = 0.574$). In the patients receiving only 5-FU based treatment, there was no significant relationship between XPF expression and RR ($\chi^2 = 1.836$, $P = 0.175$) or DC ($\chi^2 = 0.346$, $P = 0.556$). Similarly, there was no association in those receiving oxaliplatin between XPF expression and RR ($\chi^2 = 1.57$, $P = 0.210$) or DC ($\chi^2 = 2.359$, $P = 0.125$).

No difference in PFS was detectable in the whole cohort when those expressing high levels of XPF were compared with those expressing low levels (high *vs* low expression, median 174 days *vs* 166 days; $\chi^2 = 0.160$; $P = 0.689$; Logrank). Nor was any association found in the group receiving 5-FU based treatment (high *vs* low expression, median 111 days *vs* 161 days; $\chi^2 = 0.000$; $P = 0.994$; Logrank) or

those receiving oxaliplatin (high *vs* low expression, median 192 days *vs* 176 days; $\chi^2 = 0.238$; $P = 0.625$; Logrank).

XPF protein levels was also tested for association with OS. No correlation between XPF levels and OS was found in the whole cohort (high *vs* low expression, median 388 days *vs* 361 days; $\chi^2 = 0.111$; $P = 0.739$; Logrank); amongst those receiving just 5-FU based treatment (high *vs* low expression, median 337 days *vs* 328 days; $\chi^2 = 0.004$; $P = 0.951$; Logrank); or amongst those receiving oxaliplatin (high *vs* low expression, median 408 days *vs* 382 days; $\chi^2 = 0.280$; $P = 0.596$; Logrank).

5.4 MUS81 EXPRESSION AND CLINICAL OUTCOMES FOLLOWING OXALIPLATIN-FLUOROPYRIMADINE BASED CHEMOTHERAPY IN COLORECTAL CANCER

The seven FOCUS2 TMAs were also stained and scored for MUS81 protein expression, as previously described. 205 patients had at least one core for which a MUS81 protein expression score could be generated. Again, in cases where scores differed between cores for an individual patient, the highest composite score was used for analysis. The median MUS81 expression score was 2 (IQR 1-6), and, using the pre-defined cut-off of a composite score of 6 or more to define high expression, 30.2% of patients had high levels of MUS81 expression. MUS81 expression in the FOCUS2 tumours was lower that that found in the Oxford Biobank samples, in which, using the same cut-off, 38.3% of samples were classified as showing high expression ($t = 3.108$, $P = 0.002$, independent samples t-test) (**FIGURE 5.8**).

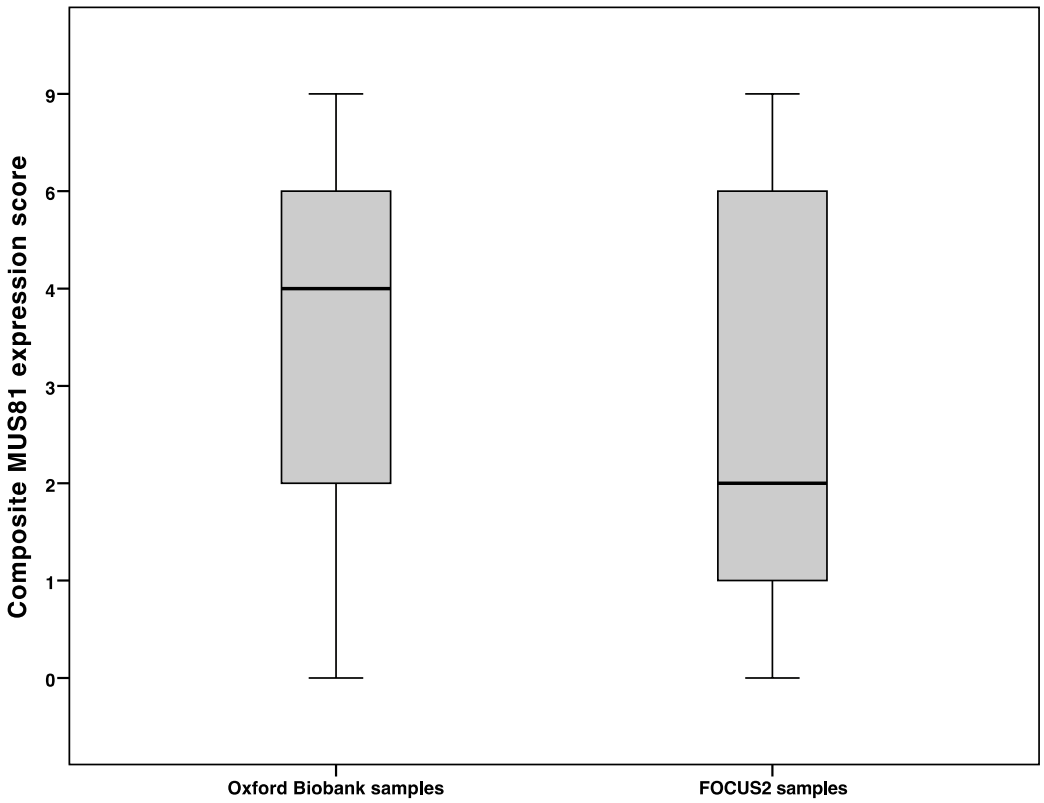


Figure 5.8. Comparison of distribution of MUS81 levels in samples from Oxford Biobank and FOCUS2 specimens. MUS81 levels in the FOCUS2 tumours were lower that those found in the Oxford Biobank samples (t = 3.108, P = 0.002, independent samples t-test).

There was no association between MUS81 levels and age (t = 0.179, P = 0.858, independent samples t-test), sex (χ^2 = 0.912, P = 0.340), presence of hepatic metastases (χ^2 = 0.847, P = 0.358) or primary tumour site (χ^2 = 0.942, P = 0.630).

MUS81 levels were tested for association with the radiological outcomes RR and DC. For the whole cohort, there was no association between MUS81 expression and RR (χ^2 = 0.394, P = 0.530) or DC (χ^2 = 0.316, P = 0.574). In the group receiving 5-FU based treatment alone, there was no significant relationship between MUS81 expression and RR (χ^2 = 2.855, P = 0.091) or DC (χ^2 = 2.328, P =

0.127); nor was there a correlation between MUS81 expression and RR (χ^2 = 0.339, *P* = 0.560) or DC (χ^2 = 1.077, *P* = 0.299) in the group receiving oxaliplatin.

There was no association between MUS81 levels and PFS in the whole cohort (high *vs* low expression, median 160 days *vs* 174 days; χ^2 = 1.513; *P* = 0.219; Logrank), the group receiving only 5-FU based treatment (high *vs* low expression, median 102 days *vs* 161 days; χ^2 = 1.515; *P* = 0.218; Logrank) or the group receiving oxaliplatin (high *vs* low expression, median 174 days *vs* 180 days; χ^2 = 0.289; *P* = 0.591; Logrank).

The relationship between MUS81 levels and OS was also tested. There was a trend towards an association between high levels of MUS81 protein and worse OS in the whole cohort (high *vs* low expression, median 314 days *vs* 416 days; χ^2 = 2.892; *P* = 0.089; Logrank) (**FIGURE 5.9**) and the oxaliplatin treated cohort (high *vs* low expression, median 316 days *vs* 416 days; χ^2 = 2.665; *P* = 0.103; Logrank) (**FIGURE 5.10**), but this did not reach statistical significance. There was no association between MUS81 and OS in the group receiving 5-FU based treatment only (high *vs* low expression, median 297 days *vs* 402 days; χ^2 = 0.567; *P* = 0.451; Logrank) (**FIGURE 5.11**).

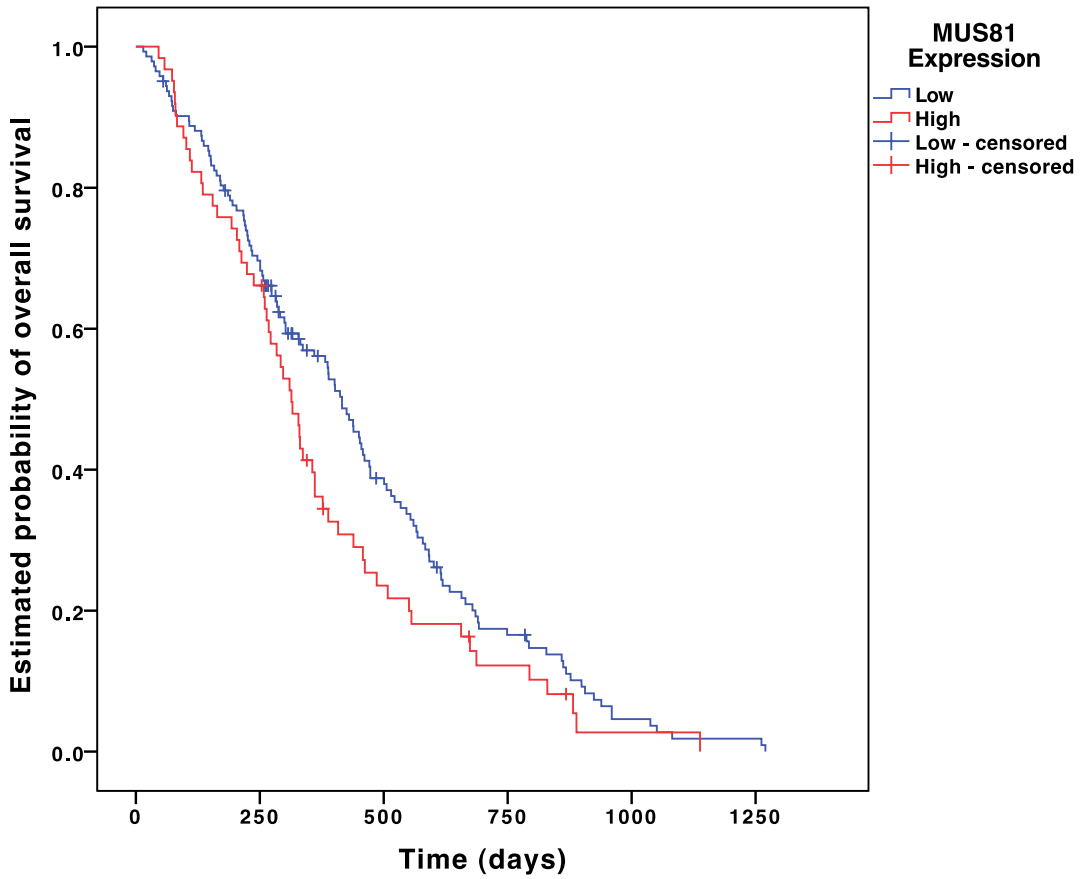


Figure 5.9. Kaplan-Meier analysis of overall survival in colorectal cancer in patients treated with either fluorouracil or oxaliplatin-fluorouracil chemotherapy based on pre-treatment levels of MUS81 protein. There was no significant difference in OS between those with high MUS81 and those with low MUS81 (high *vs* low expression, median 314 days *vs* 416 days; $\chi^2 = 2.892$; $P = 0.089$; Logrank)

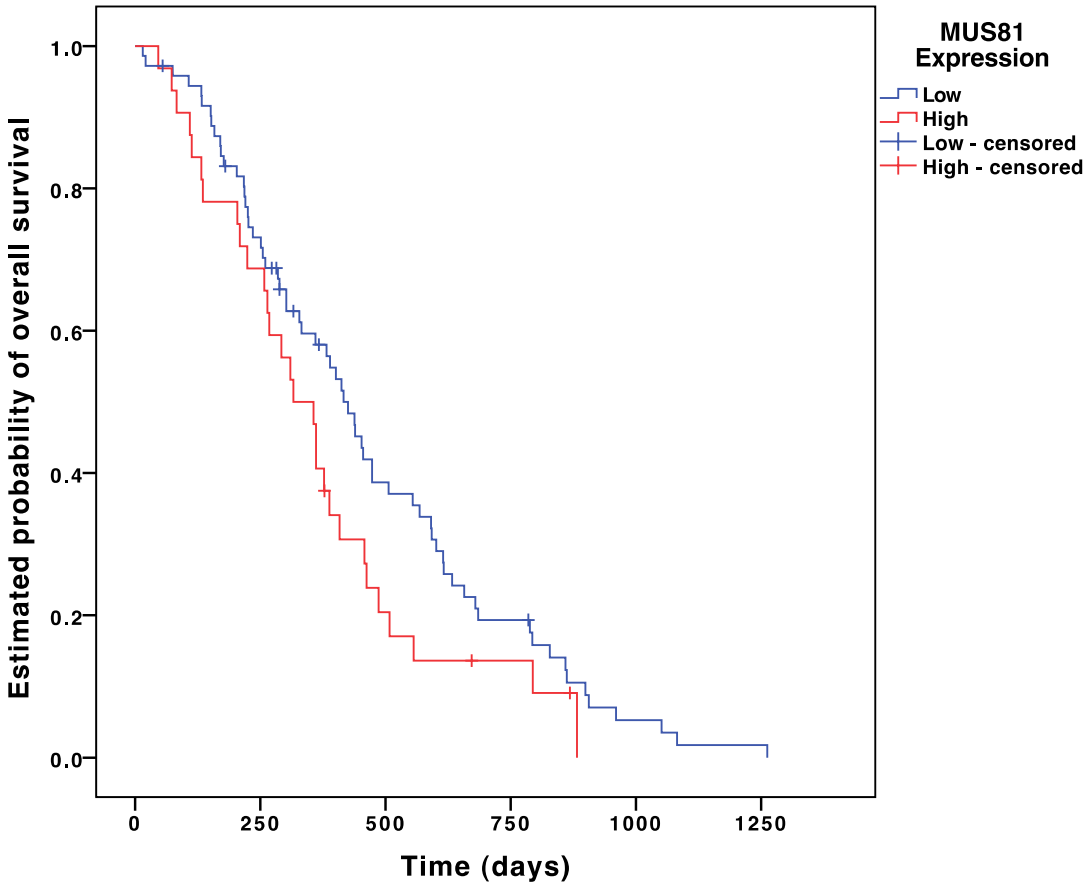


Figure 5.10. Kaplan-Meier analysis of overall survival in colorectal cancer in patients treated with oxaliplatin-fluorouracil chemotherapy based on pre-treatment levels of MUS81 protein. There was no significant difference in OS between those with high MUS81 and those with low MUS81 (high *vs* low expression, median 316 days *vs* 416 days; $\chi^2 = 2.665$; $P = 0.103$; Logrank).

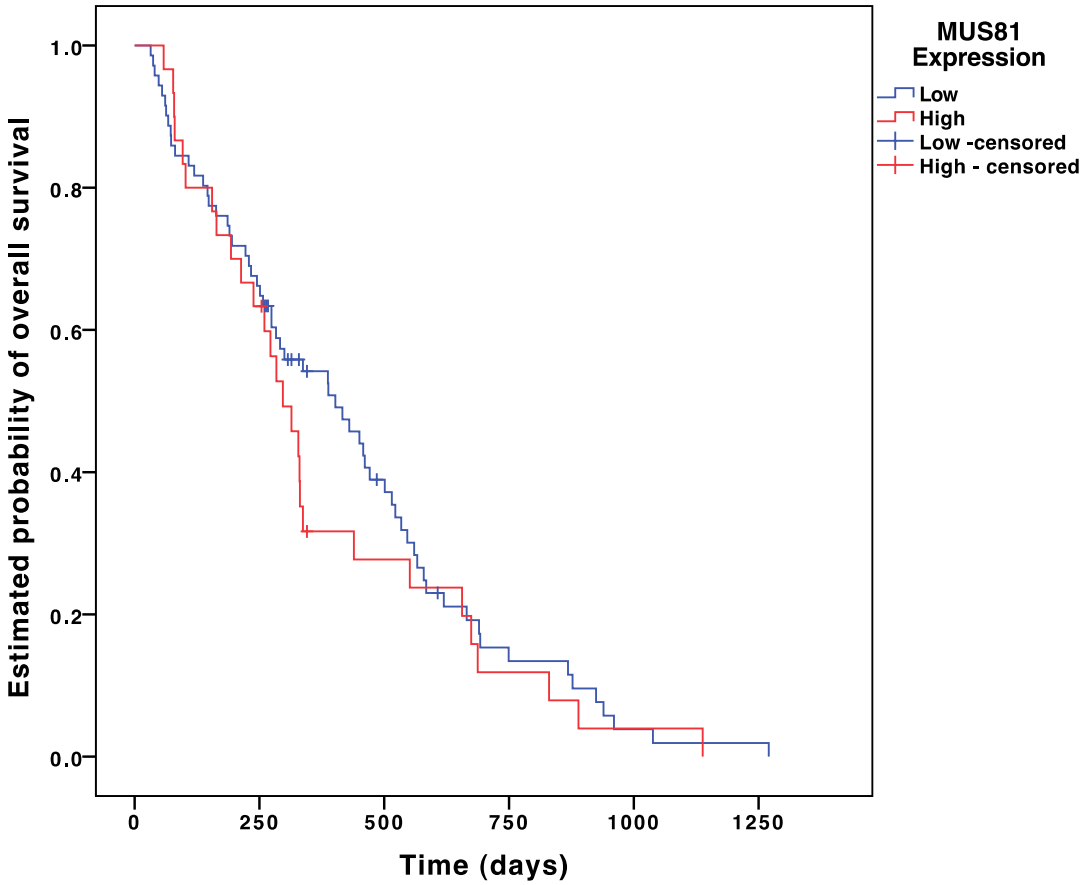


Figure 5.11. Kaplan-Meier analysis of overall survival in colorectal cancer in patients treated with fluorouracil chemotherapy based on pre-treatment levels of MUS81 protein. There was no significant difference in OS between those with high MUS81 and those with low MUS81 (high *vs* low expression, median 297 days *vs* 402 days; $\chi^2 = 0.567$; $P = 0.451$; Logrank).

5.5 ANAL CANCER

Following the results indicating that levels of expression of XPF and MUS81 may be associated with sensitivity to oxaliplatin-based chemotherapy in oesophageal cancer, it was decided to further investigate whether they were associated with sensitivity to the classic DNA cross-linking agent mitomycin C. Pre-treatment tumour biopsies were obtained from 53 patients with anal cancer who went on

to have treatment with mitomycin C and 5-fluorouracil based chemoradiotherapy. Forty-eight of these biopsies had sufficient tumour for immunohistochemical analysis. The characteristics of these patients are shown in **TABLE 5.2**. Response to treatment was assessed as relapse free survival.

Table 5.2. Characteristics of the anal cancer patients.

Characteristic		
Age		
Median	62	years
Range	38-89	years
Sex		
Male	19	(36.5%)
Female	33	(63.5%)
Clinical stage		
I	8	(15.4%)
II	30	(38.5%)
IIIa	12	(23.1%)
IIIb	9	(17.3%)
IV	1	(1.9%)
Stage unknown	2	(3.8%)

5.6 XPF EXPRESSION AND CLINICAL OUTCOMES FOLLOWING MITOMYCIN C-FLUOROURACIL CHEMORADIOTHERAPY IN ANAL CANCER

In the 48 tumours for which XPF protein levels could be analysed, the median composite score was 6 (IQR 4-9), so high expression was defined as a composite score of 9. There was no significant association between high levels of XPF and relapse free survival following mitomycin C-fluorouracil chemoradiotherapy in anal cancer (median survival time not reached in either group; $\chi^2 = 1.621$; $P = 0.203$; Logrank) (**FIGURE 5.12**). There was no association between XPF levels and p16 positivity ($\chi^2 = 1.145$, $P = 0.285$) or p53 positivity ($\chi^2 = 0.553$, $P = 0.487$).

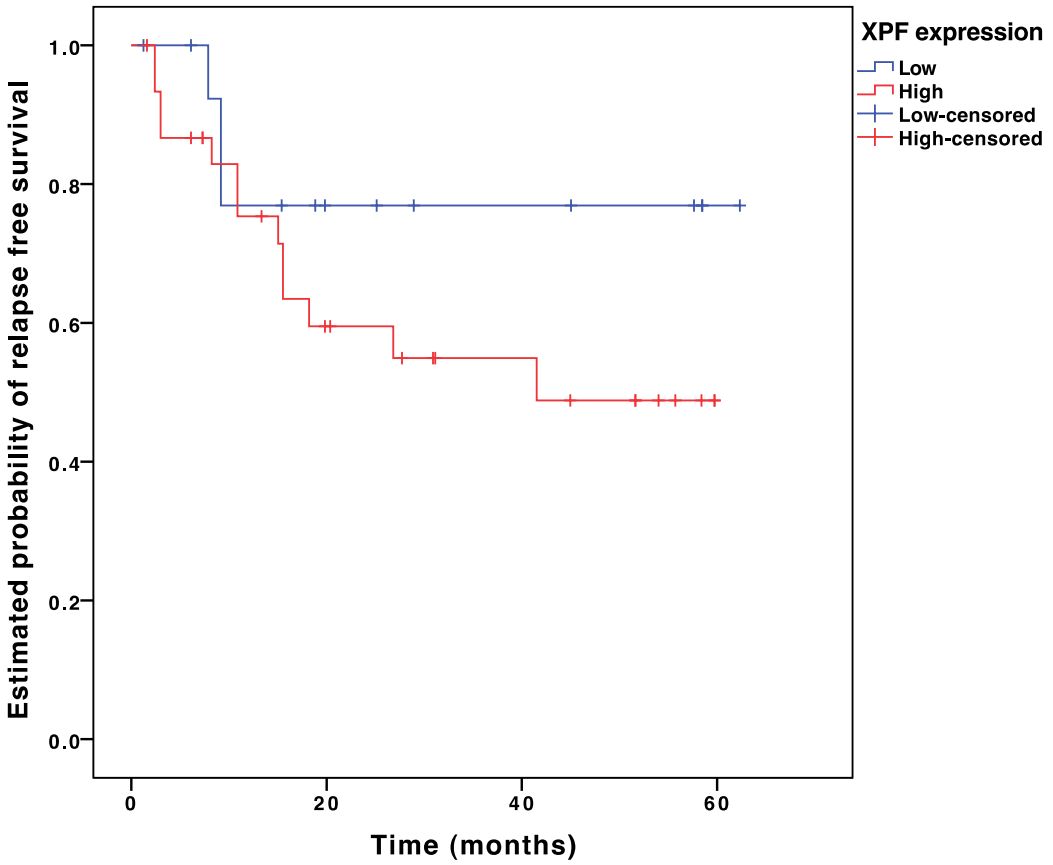


Figure 5.12. Kaplan-Meier analysis of relapse free survival in anal cancer in patients treated with mitomycin C-fluorouracil chemotherapy based on pre-treatment levels of XPF protein. There was no significant difference in RFS between those with high XPF and those with low XPF (median survival time not reached in either group; $\chi^2 = 1.621$; $P = 0.203$; Logrank).

5.7 MUS81 PROTEIN LEVELS AND CLINICAL OUTCOMES FOLLOWING MITOMYCIN C-FLUOROURACIL CHEMORADIOTHERAPY IN ANAL CANCER

The median composite score for MUS81 protein levels in the 48 tumours available for analysis was 4 (IQR 2-6), thus high expression of MUS81 in anal cancer was defined as a composite score of 6 or more. There was no significant

difference in RFS between those with high MUS81 and those with low MUS81 when tested with the Logrank test (median survival time not reached in either group; $\chi^2 = 1.676$; $P = 0.196$). The shape of the Kaplan-Meier curve indicated a possible early RFS survival advantage for patients with low MUS81 levels, and this was tested using the Generalised Wilcoxon test (the rationale for this is discussed in **Section 2.12**). This showed that there was a trend towards high MUS81 being associated with early relapse in anal cancer, although this did not reach statistical significance (median survival time not reached in either group; $\chi^2 = 3.614$; $P = 0.057$; Generalised Wilcoxon) (**FIGURE 5.13**). There was no association between MUS81 levels and p16 positivity ($\chi^2 = 0.043$, $P = 0.836$) or p53 positivity ($\chi^2 = 0.440$, $P = 0.507$).

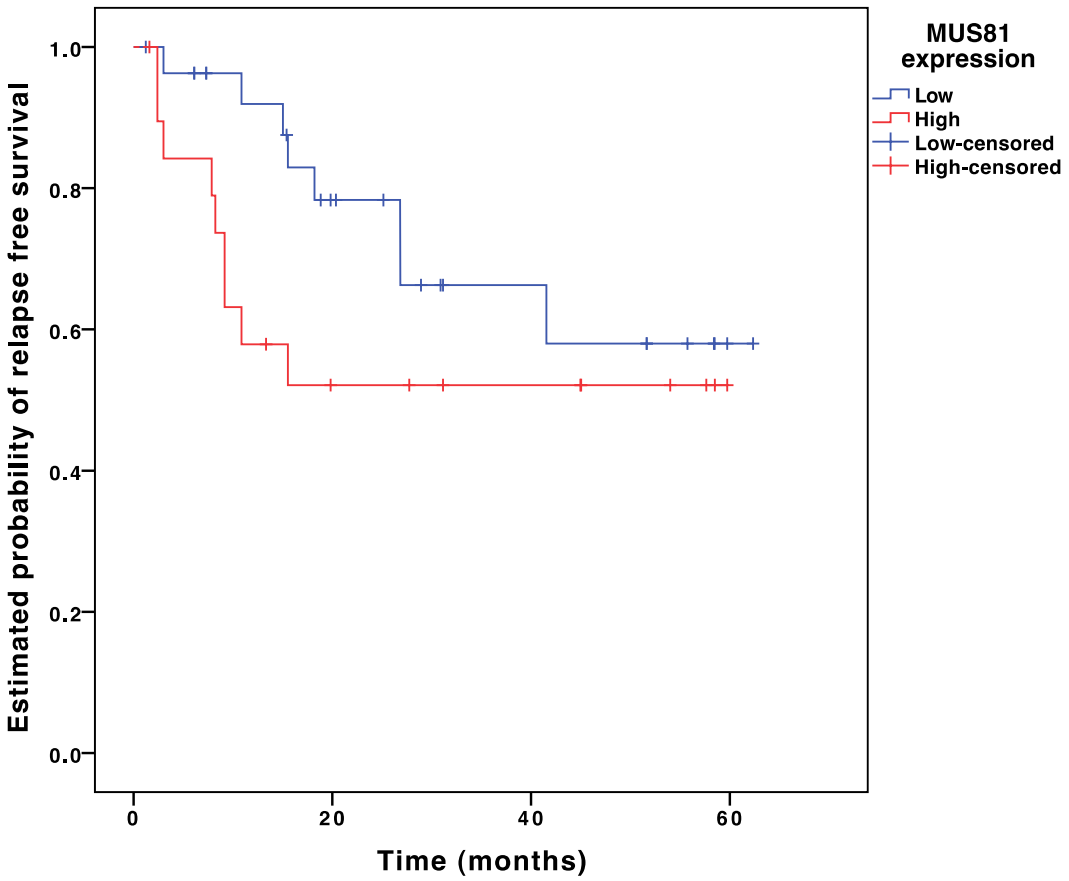


Figure 5.13. Kaplan-Meier analysis of relapse free survival in anal cancer in patients treated with mitomycin C-fluorouracil chemotherapy based on pre-treatment levels of MUS81 protein. There was no significant difference in RFS between those with high MUS81 and those with low MUS81 (median survival time not reached in either group; $\chi^2 = 1.676$; $P = 0.196$; Logrank).

5.8 DISCUSSION

5.8.1 XPF protein levels and response to cross-linking agent-based chemotherapy in other tumour types

In this chapter, to test the hypothesis that high levels of XPF expression were associated with worse outcomes following oxaliplatin chemotherapy, and also assess the confounding effect of concurrent 5-fluorouracil administration, XPF

levels were assayed in pre-treatment biopsies from 209 patients with colorectal adenocarcinoma who participated in the FOCUS2 trial (Seymour et al., 2011). Of these patients, 105 received oxaliplatin and 5-fluorouracil (either as capecitabine or infusional 5-fluorouracil) and 104 received 5-fluorouracil-based drugs alone.

No studies have previously tested for a relationship between XPF levels and response to oxaliplatin in colorectal cancer. It is known that the level of expression of *ERCC1* mRNA (which encodes the functional binding partner of XPF) in colorectal tumours correlates inversely with DFS and OS (Shirota et al., 2001; Uchida et al., 2008). Attempts to correlate levels of ERCC1 protein and outcomes in colorectal cancer have given variable results. Braun *et al*. tested for a correlation between ERCC1 levels and PFS following oxaliplatin-fluorouracil chemotherapy in 1,197 patients from the FOCUS trial and found no significant relationship (Braun et al., 2008). Subsequently, high ERCC1 has been associated with worse outcomes in four small cohorts of patients from Far Eastern countries (containing between 39 and 160 individuals) (Han et al., 2014; Kim et al., 2009b; Li et al., 2013; Noda et al., 2012). All of these results must be treated with caution in view of the concerns about the specificity of commercially available ERCC1 antibodies and their inability to specifically detect the functional ERCC1-202 isoform (Bhagwat et al., 2009; Friboulet et al., 2013a).

In our study, no correlation was found between expression of XPF in biopsies of the primary colorectal tumour and outcomes (radiological response, disease control, PFS and OS) in 105 patients treated with oxaliplatin-fluorouracil

chemotherapy for metastatic colorectal cancer. Likewise, there was no association between XPF expression levels and outcomes in the 104 patients treated with fluorouracil chemotherapy alone. These results fail to support the hypothesis that high levels of XPF expression are associated with worse clinical outcomes following oxaliplatin-based chemotherapy.

One possible explanation for this lack of association is that the link between high XPF expression and lack of pathological response following oxaliplatin-fluorouracil treatment observed in oesophageal cancer may be a tumour-type specific effect and may not occur in colorectal cancer. Another potential source for error in the quantification of XPF levels in these patients is that tumour biopsies for the FOCUS2 trial were taken multiple centres and, as has been discussed at the in Section 4.18.1, differences in sample handling can have an effect on XPF levels, potentially affecting the results (Pierceall et al., 2011).

Another possibility is heterogeneity of composition between a tumour and its metastases – it may be that a biopsy of the primary tumour of a patient with metastatic colorectal cancer does not reflect the biology of the metastases. Gerlinger *et al*. have demonstrated a high degree of heterogeneity between primary renal cell carcinomas and their metastases, calling into doubt the ability of primary tumour biopsies to reflect the composition of metastatic disease (Gerlinger et al., 2012). It has also been shown that levels of *ERCC1* mRNA are approximately two-fold higher in colorectal metastases than they are in primary colorectal tumours, and that there is a lack of significant correlation between *ERCC1* expression in the primary tumour and the metastasis (Uchida et al.,

2008). If this is also the case for expression of XPF, it could explain the failure of XPF protein levels in the primary tumour to correlate with response to oxaliplatin-fluorouracil in a cohort of patients with metastatic disease.

Mitomycin C is a DNA cross-linking agent. It is known to be a potent inducer of ICLs. No studies have looked at the relationship between tumour XPF or ERCC1 levels and response to mitomycin C-based treatment. It is known that, *in vitro*, absence of XPF expression renders cells hypersensitive to mitomycin C (Yagi et al., 1998). In our study, we assayed XPF levels in pre-treatment biopsies from 49 anal squamous cell carcinomas (SCCs) that went on to be treated with mitomycin C-fluorouracil chemoradiotherapy. No significant relationship was found between pre-treatment XPF levels and progression-free survival in this cohort of patients. This may in part be due to the relatively small number of recurrences in these patients. It is also known that anal SCCs are highly sensitive to radiotherapy alone (Byfield et al., 1983), and that, although addition of mitomycin C to 5-fluorouracil chemoradiotherapy improves DFS, it has not been shown to prove OS (Flam et al., 1996). It is therefore possible that tumour response to mitomycin C determines outcome in only a proportion of these patients and that, therefore, XPF levels are not associated with clinical response in the whole cohort.

5.8.2 MUS81 protein levels and response to cross-linking agent-based chemotherapy in other tumour types

In an attempt to test the hypothesis that MUS81 levels in colorectal tumours are associated with clinical outcomes following platinum chemotherapy, MUS81 levels were measured in pre-treatment biopsies from the 209 patients with

colorectal adenocarcinoma who took part in the FOCUS2 trial (Seymour et al., 2011). Of these patients, 105 received oxaliplatin and 5-fluorouracil (either as capecitabine or infusional 5-fluorouracil) and 104 received 5-fluorouracil-based drugs alone. No studies have examined the relationship between MUS81 expression and response to oxaliplatin chemotherapy *in vitro* or *in vivo*. Hiyama *et al*. have demonstrated that HCT116 colorectal adenocarcinoma cell lines in which MUS81 expression has been knocked out show increased sensitivity to cisplatin (Hiyama et al., 2006), and it was hypothesised that a similar relationship might be seen with oxaliplatin-based treatment.

As was the case for XPF, no significant association was found between MUS81 levels and radiological response, disease control and PFS. There was a non-significant trend towards an association between high MUS81 expression and worse OS in the patients receiving oxaliplatin in addition to fluorouracil, and no association was found between MUS81 levels and outcomes in those receiving only fluorouracil.

Possible explanations for the failure replicate the link between MUS81 expression and clinical response to oxaliplatin-based chemotherapy found in oesophageal adenocarcinoma in metastatic rectal cancer are similar to those discussed above for XPF. Firstly, it may be that the processing of oxaliplatin-DNA adducts is different in colorectal cancer and oesophageal adenocarcinomas. Secondly, sample handling differences in a multicentre trial may have affected the assay. Finally, heterogeneity between primary and metastatic tumours may mean that the measurement of MUS81 levels in the primary tumour is not a

reliable assay of MUS81 levesl in metastases. Given that the clinical outcomes evaluated in this group of patients all related to the metastatic rather than the primary disease, this inability to account for MUS81 levels in metastases may be the reason for the failure of pre-treatment MUS81 levels to predict outcomes.

MUS81 null mice are hypersensitive to mitomycin C (McPherson et al., 2004), and knockout of MUS81 expression in HCT116 colorectal adenocarcinoma cells renders them sensitive to mitomycin C (Hiyama et al., 2006). It was therefore hypothesised that MUS81 levels in anal SCC samples taken before treatment would correlate with progression free survival.

When MUS81 expression was measured in 48 patients with anal SCC treated with mitomycin C-fluorouracil chemoradiotherapy, there was a trend towards an association between high MUS81 expression and increase likelihood of early relapse, but this just failed to reach statistical significance ($P = 0.057$). The failure of this result to reach statistical significance may be due to a combination of the small sample size and the fact that, as is discussed above in **Section 5.8.1**, addition of mitomycin C to radiotherapy confers only a small improvement in disease free survival when compared with radiotherapy alone for anal cancer. Further studies in a larger cohort of patients are required to determine whether there is a true relationship between high MUS81 expression and worse clinical outcomes following mitomycin C treatment in anal cancer.

5.9 SUMMARY

These results do not support the hypothesis that high levels of expression of XPF protein and MUS81 protein are associated with lack of response to cross-link generating chemotherapeutic agents. In colorectal cancer, no significant association was found between levels of XPF protein or MUS81 protein before treatment and response to oxaliplatin-fluorouracil chemotherapy. In anal cancer, there was no association between XPF expression and response to mitomycin C-fluorouracil chemoradiotherapy. There was a trend towards an association between high MUS81 expression and early relapse following mitomycin C-fluorouracil chemoradiotherapy in anal cancer, but this failed to reach statistical significance.

6. THE INFLUENCE OF EXPRESSION OF XPF AND MUS81 ON CELLULAR RESPONSE TO OXALIPLATIN CHEMOTHERAPY

6.1 INTRODUCTION

Following the results of the microarray analyses and the association found between pre-treatment levels of both XPF protein and MUS81 protein and clinical outcomes in oesophageal cancer treated with oxaliplatin-based chemotherapy, this chapter aimed to test the hypothesis that levels of expression of the DNA repair proteins XPF and MUS81 determine cellular response to oxaliplatin chemotherapy.

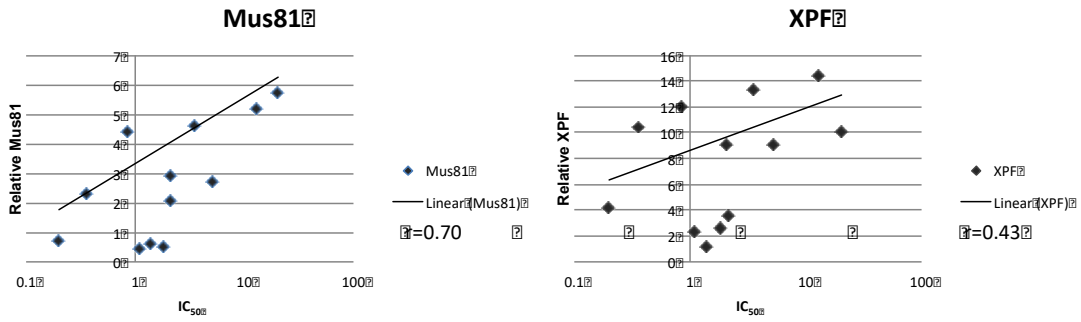
6.2 CORRELATION BETWEEN XPF AND MUS81 EXPRESSION AND SENSITIVITY TO OXALIPLATIN CHEMOTHERAPY

To test the hypothesis that low levels of expression of XPF and MUS81 proteins make tumour cells more sensitive to oxaliplatin, expression of these proteins was tested for correlation with oxaliplatin sensitivity in eleven cell lines derived from gastrointestinal cancers. Six of the cell lines were derived from colorectal cancers (HCT116, DLD1, SW480, LoVo, HT29 and KM12); two were derived from gastric cancers (ST16 and AGS); and four were derived from oesophageal cancers (FLO-1, SKGT-4, OE33 and OE21).

Oxaliplatin IC_{50} values were determined for each of the cell lines using clonogenic survival assays. The cell lines were exposed to graded concentrations

of oxaliplatin for 24 hours. The colonies were then grown on for 7-10 days in the absence of the drugs before the surviving fraction was calculated. Expression of XPF and MUS81 in the individual cell lines was determined by Western blotting. The expression levels of the individual proteins were corrected for tubulin.

There was a positive correlation between expression of XPF expression and oxaliplatin IC_{50} (R = 0.43) and also expression of MUS81 and oxaliplatin IC_{50} (R = 0.70) (**FIGURE 6.1**).



Cell line	IC50	limits (+/-)	Mus81	XPF
HCT116	3.4	0.3	4.6	13.3
DLD1	20.37	0.54	5.7	10
SW480	2.1	0.31	2.1	3.6
Lovo	0.31	0.20	2.3	10.4
HT29	12.73	0.64	5.2	14.4
KM12	6.6	0.05	2.7	9.1
ST16	0.84	0.04	4.4	12.0
AGS	0.2	0.03	0.7	4.2
Flo1	1.8	0.16	0.5	2.6
SKGT4	1.398	0.71	0.6	1.1
OE33	2.075	0.35	2.9	9.1
OE21	1.81	0.19	0.46	2.36

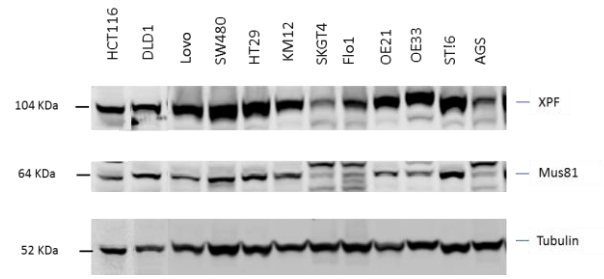


Figure 6.1. The relationship between levels of MUS81 and XPF expression and sensitivity to oxaliplatin in a panel of cell lines derived from gastrointestinal cancers. The two graphs show the positive correlation between MUS81 and XPF levels (measured by Western blotting and corrected relative to tubulin, the results of 3 repeats) and sensitivity to oxaliplatin (IC_{50} values in μM measured by clonogenic survival assays), r = 0.70 and r = 0.43 respectively. (Data courtesy of Alan Westhorpe).

6.3 EXPRESSION OF XPF AND MUS81 IN CELL LINES WITH ACQUIRED OXALIPLATIN RESISTANCE

To further test the hypothesis that cellular expression of XPF and MUS81 determines response to treatment with oxaliplatin, their expression was quantified in five cell lines with acquired oxaliplatin resistance. Levels of expression of the two proteins were tested by Western blotting of cell extracts from paired parental and oxaliplatin-resistant cell lines derived from colorectal cancer (HCT116 and KM12) and gastric adenocarcinoma (AGS, ST16 and MKN).

There was no significant difference between levels of XPF or MUS81 in the parental and oxaliplatin resistant cell lines (**FIGURE 6.2**).

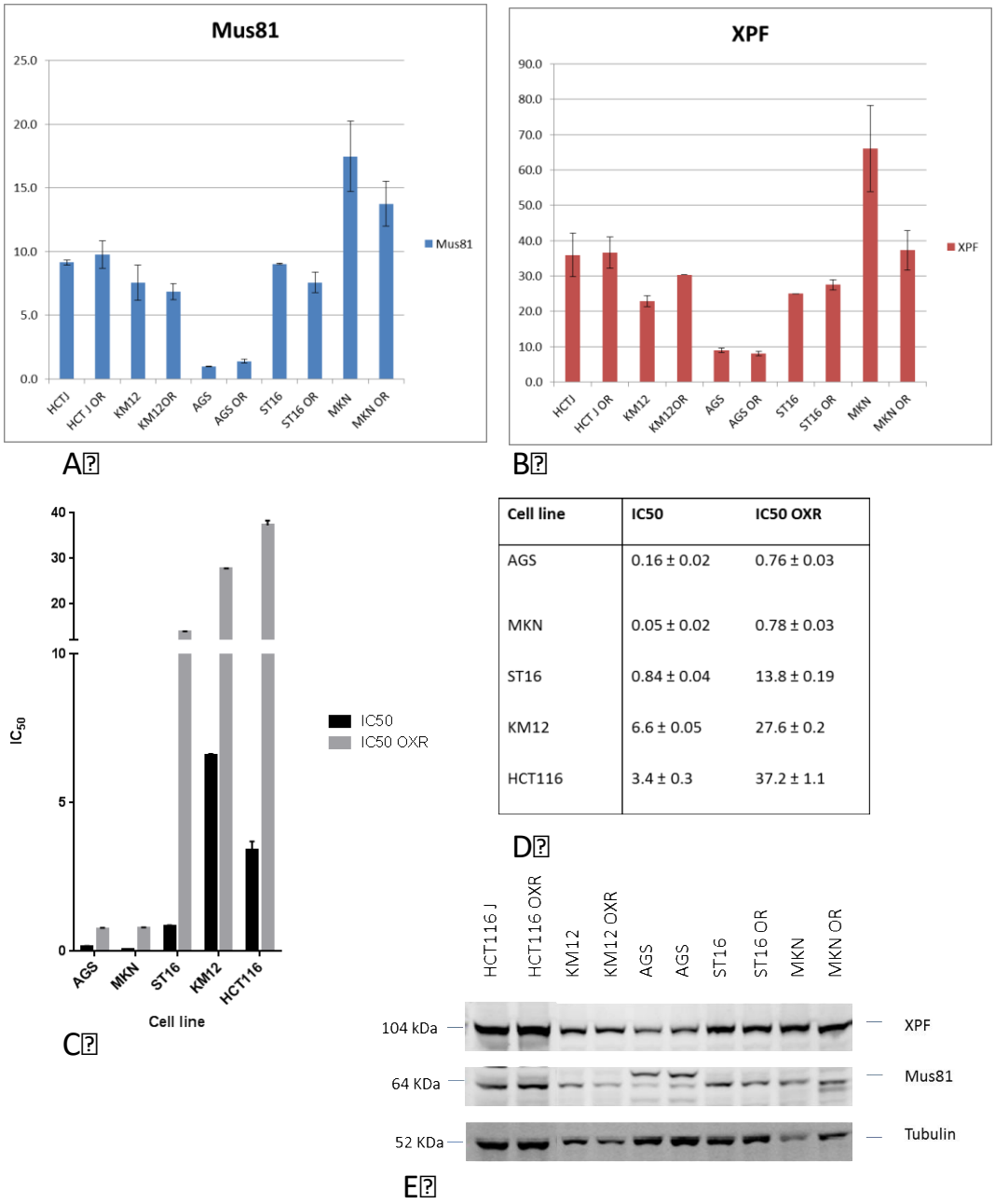


Figure 6.2. Levels of MUS81 and XPF protein levels in parental gastrointestinal cancer cell lines and paired lines with acquired oxaliplatin resistance. A, B & E. Levels of MUS81 and XPF in individual cell lines were determined Western blotting and corrected to tubulin. **C & D.** Acquired resistance was confirmed by resazurin cell viability assay (IC_{50} values are in μM). There was no difference in levels of MUS81 or XPF when parental cell lines were compared with oxaliplatin resistant cell lines.

6.4 INCREASED SENSITIVITY OF MUS81 KNOCKOUT CELL LINES TO OXALIPLATIN

Following the negative results from the comparison of MUS81 expression in wild-type (WT) and oxaliplatin-resistant cell lines, HCT116 cell lines with *MUS81* knockout (*MUS81*-/-) and haplo-insufficiency of MUS81's functional binding partner EME1 (*EME1*+/-) were tested for relative sensitivity to oxaliplatin. Silencing of *MUS81* expression was confirmed by Western blotting (**FIGURE 6.3**).

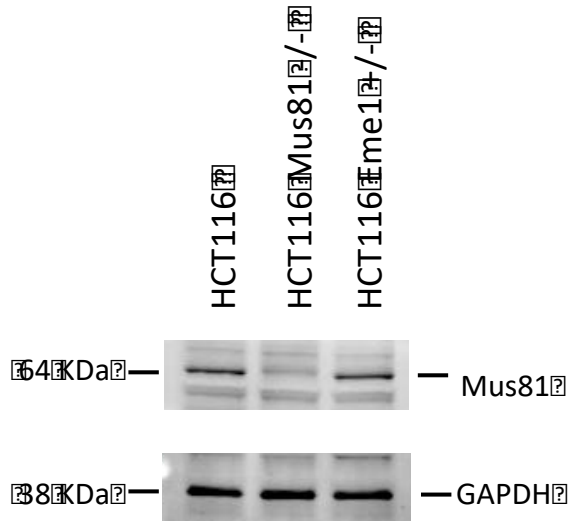
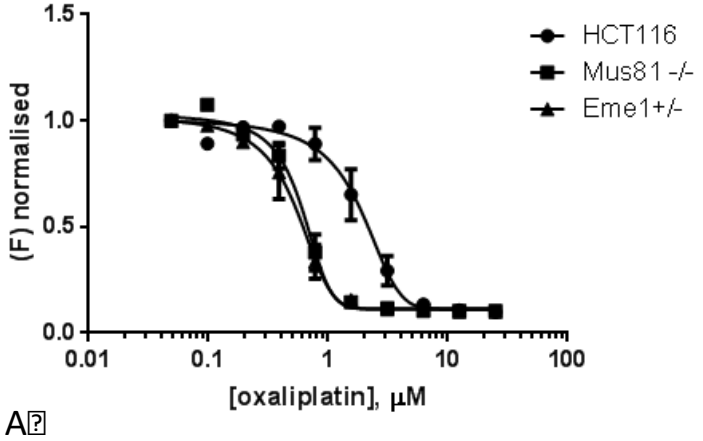


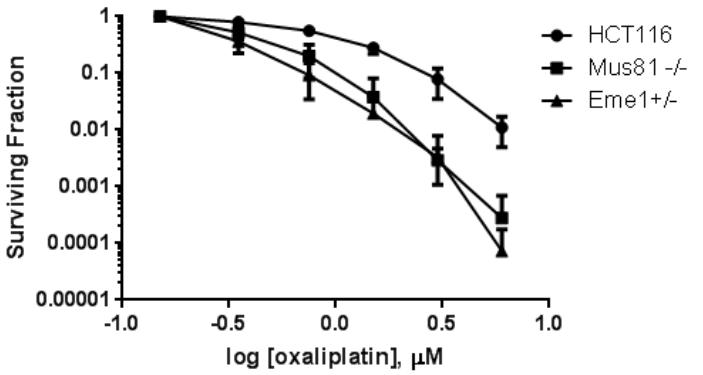
Figure 6.3. Western blot of HCT116 WT, *MUS81 -/-* and *EME1 +/-* cell lines for MUS81.

Clonogenic and resazurin survival assays were performed in the WT, *MUS81 -/-* and *EME1 +/-* cells 10 days after a 24-hour treatment with oxaliplatin. The *MUS81*-/- and *EME1*+/- cells were found to be significantly more sensitive to oxaliplatin than their wild-type counterparts in both the resazurin assay (IC_{50} 2.46 µM – 95% CI 2.31 to 2.61 - for HCT116 WT cells; IC_{50} 0.76 µM – 95% CI 0.71 to 0.81 -for HCT116 *MUS81* -/- cells) (P = 0.035, paired Student's t-test) (IC_{50} 0.66 µM – 95% CI 0.64 to 0.68 - for HCT116 *EME1* +/- cells) (P = 0.017, paired

Student's t-test) and clonogenic survival assay (IC_{50} 0.43 μM – 95% CI 0.37 to 0.48 - for HCT116 WT cells; IC_{50} 0.13 μM - 95% CI 0.09 to 0.17 - for HCT116 *MUS81* -/- cells; $P = 0.049$, at 6 μM, Student's t-test) (IC_{50} 0.05 μM – 95% CI 0.005 to 0.093 - for HCT116 *EME1* +/- cells; $P = 0.049$, at 6 μM, Student's t-test) (**FIGURE 6.4**).



A



B

Figure 6.4. Deficiency of MUS81 and EME1 increases sensitivity to oxaliplatin in HCT116 cell lines.

A. Resazurin assay of cell viability in HCT WT, MUS81-/- and EME1+/- cell lines after treatment with oxaliplatin. Each data point represents five independent experiments, and error bars show the standard error of the mean. Both *MUS81-/-* and *EME1+/-* cells were significantly more sensitive to oxaliplatin than HCT116 WT.

B. Clonogenic survival assay showing increased survival of HCT116 WT compared to *MUS81-/-* and *EME1+/-* following treatment with oxaliplatin. Each data point represents three independent experiments, and error bars show the standard error of the mean. Both *MUS81-/-* and *EME1+/-* cells were significantly more sensitive to oxaliplatin at a concentration of 6 µM than HCT116 WT.

6.5 INCREASED OXALIPLATIN SENSITIVITY IN OESOPHAGEAL ADENOCARCINOMA CELL LINES THROUGH KNOCKDOWN OF MUS81

As shown above, deficiency of MUS81 or its functional binding partner EME1 results in increased sensitivity to oxaliplatin in knockout colorectal cell lines. To test if inhibition of MUS81 in wild-type oesophageal cancer cell lines would also increase sensitivity to oxaliplatin, *MUS81* expression was knocked down in FLO-1 and OE33 oesophageal adenocarcinoma cell lines using an siRNA-based approach.

The cells were exposed to two cycles of 10 nM siRNA against *MUS81* for 48 hours each cycle to obtain knockdown. They were then treated with 72 hours of oxaliplatin before survival was measured with the resazurin assay. MUS81 knockdown was above 85 % when quantified by Western blotting at 96 hours (**FIGURE 6.5**). Knockdown of MUS81 resulted in significantly increased sensitivity to oxaliplatin for both FLO-1 (IC_{50} 0.9 μM for control non-targeting siRNA; IC_{50} 1.4 for mock transfected cells; and IC_{50} 0.4 μM for MUS81 KD cells) (*P* = 0.026, paired Student's t-test) and OE33 (IC_{50} 1.8 μM for non-targeting siRNA; IC_{50} 2.3 for mock transfected cells; IC_{50} 0.7 μM for MUS81 KD cells) (*P* =0.033, paired Student's t-test) (**FIGURE 6.5**).

6.6 INCREASED OXALIPLATIN SENSITIVITY IN OESOPHAGEAL ADENOCARCINOMA CELL LINES THROUGH KNOCKDOWN OF XPF

The findings that levels of XPF expression correlates with sensitivity to oxaliplatin chemotherapy outlined above support the hypothesis that XPF expression levels have a role in determining tumour cell survival following oxaliplatin treatment. To further test the hypothesis that XPF deficiency causes increased oxaliplatin sensitivity in oesophageal adenocarcinoma, *XPF* expression was knocked down in the FLO-1 and OE33 cell lines with siRNA.

Knockdown was obtained by exposure of the cells two cycles of 10 nM siRNA against *XPF* for 48 hours. XPF knockdown was above 85 % when quantified by Western blotting at 96 hours (**FIGURE 6.5**). Cellular survival was measured at 7 days using the resazurin assay. Knockdown of XPF resulted in significantly increased sensitivity to oxaliplatin for both both FLO-1 (IC_{50} 0.9 μM for control non-targeting siRNA cells; IC_{50} 1.4 for mock transfected cells; and IC_{50} 0.4 μM for MUS81 KD cells) (P = 0.026, paired Student's t-test) and OE33 (IC_{50} 1.8 μM for control non-targeting siRNA cells; IC_{50} 2.3 for mock transfected cells; IC_{50} 0.7 μM for MUS81 KD cells) (P =0.32, paired Student's t-test) (**FIGURE 6.5**).

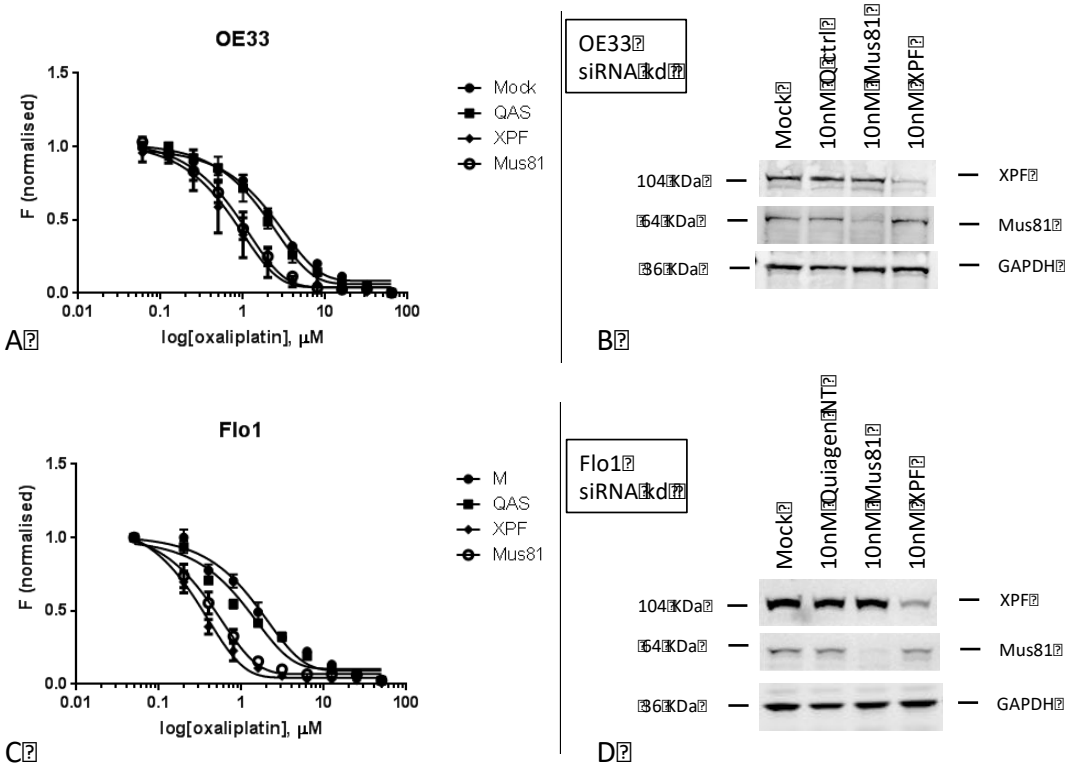


Figure 6.5. Deficiency of MUS81 and XPF increase sensitivity of oesophageal adenocarcinoma cell lines to oxaliplatin. Resazurin assay of cell viability in OE33 (**A**) and Flo1 (**B**) oesophageal adenocarcinoma cell lines after knockdown of XPF or MUS81 and treatment with oxaliplatin. Each data point represents three independent experiments, and error bars show the standard error of the mean. **B, D.** Western blot analysis showing efficiency of knockdown of XPF and MUS81 in the two cell lines at 72 hours. Knockdown efficiency was above 85% in each case.

6.7 DISCUSSION

This chapter used a variety of adenocarcinoma cell line models to test the hypothesis that levels of expression of the DNA repair proteins XPF and MUS81 determine cellular response to oxaliplatin chemotherapy.

As has been discussed previously, XPF-ERCC1 is a key nuclease of the NER pathway, where it makes incisions 5' to the lesion. It has been shown that ERCC1

and XPF provide reciprocal stability with XPF containing the nuclease domain (Biggerstaff et al., 1993) and that the stability of XPF depends on the presence of the ERCC1-202 isoform (Friboulet et al., 2013a). Mutations in ERCC1 that disrupt its interaction with XPA, a key NER factor, stop NER but not ICL repair. This supports a hypothesis that the XPF-ERCC1 heterodimer plays a role in ICL repair separate to its function in NER (Orelli et al., 2010).

The role of XPF-ERCC1 in the repair of platinum-induced ICLs has previously been studied *in vitro*, with high levels of expression of both XPF and ERCC1 demonstrated to correlate with resistance to oxaliplatin in a variety of cell lines (Hatch et al., 2014). Gastric cancer cell lines with acquired resistance to cisplatin have increased ERCC1 expression, although, interestingly, these cells do not exhibit cross-resistance to oxaliplatin (Tozawa et al., 2008).

In keeping with the literature, this study demonstrated that XPF levels in gastrointestinal cell lines showed a positive correlation with IC_{50} to oxaliplatin. The finding in clinical samples that high XPF protein levels in oesophageal adenocarcinomas were associated with lack of pathological response following oxaliplatin-fluorouracil chemotherapy (**Section 4.9**) was lent functional support by the result of the experiments in which *XPF* was knocked down by RNA interference in the oesophageal adenocarcinoma cell lines FLO-1 and OE33. These showed that, in keeping with these clinical findings and data from other cell types (Hatch et al., 2014), knock down of *XPF* increased the sensitivity of oesophageal adenocarcinoma cell lines to oxaliplatin.

MUS81 is a member of the XPF-ERCC1 nuclease family and contains the nuclease ERCC4 domain (Ciccia et al., 2008). MUS81-EME1 has the ability to cleave a variety of complex branched DNA structures including replication forks, 3' DNA flaps and Holliday Junctions (Ciccia et al., 2003; Ogrunc and Sancar, 2003). Depletion of XPF-ERCC1 leads to an accumulation of DSBs following induction of ICLs, which is suppressed by depletion of MUS81 (Wang et al., 2011). The formation of these DSBs occurs in late S/G2 phase, supporting a hypothesis that MUS81-EME1 represents an alternative pathway if XPF-ERCC1 has failed (Sengerova et al., 2011; Wang et al., 2011). In these circumstances, forks may converge more frequently on ICLs, leading to more complex structures, better suited to the endonuclease function of MUS81-EME1.

MUS81-EME1 is critical for replication-dependent ICL repair (Hanada et al., 2006), with deficiency of expression of MUS81 or EME1 rendering cells hypersensitive to the cross-linking agents mitomycin C and cisplatin (Abraham et al., 2003; Hiyama et al., 2006; Tomoda et al., 2009). High levels of both EME1 and MUS81 have been shown to correlate with resistance to cisplatin in a variety of human cancer cell lines and knock-out of MUS81 or haploinsufficiency of EME1 render colorectal cancer cells sensitive to cisplatin and mitomycin C (Hiyama et al., 2006; Tomoda et al., 2009).

No previous studies have examined levels of MUS81 or EME1 as markers of sensitivity to oxaliplatin-based chemotherapy, either *in vitro* or in patients. In this study, it was shown that MUS81 levels correlated with IC_{50} for oxaliplatin in a panel of gastrointestinal cell lines. In a colorectal cell line, knockout of *MUS81*

or haploinsufficiency of its binding partner *EME1* increased sensitivity to oxaliplatin. The findings in patients that levels low of MUS81 in tumours correlated with DFS and OS following oxaliplatin-based chemotherapy in oesophageal adenocarcinomas described in **Section 4.14**, were supported by the *in vitro* findings that knockdown of *MUS81* in FLO-1 and OE33 oesophageal adenocarcinoma cell lines increase sensitivity to oxaliplatin. Taken together, these findings support a role for levels of MUS81 in determining sensitivity to oxaliplatin chemotherapy. This novel finding has not been published previously.

Since decreasing levels of XPF or MUS81 in cell line models resulted in increased sensitivity to oxaliplatin, we wished to test a separate hypothesis relating acquired oxaliplatin resistance to levels of DNA repair proteins. In these experiments, no difference was seen in levels of either protein when paired oxaliplatin resistant and sensitive gastrointestinal adenocarcinoma cell lines were compared. To explain this finding, it is known that acquired resistance to cytotoxic chemotherapy is multifactorial. The factors that can cause it include changes in intracellular transport proteins; increased extracellular detoxification by glutathiones (GSH), alterations in levels of metallothioneins and antioxidants; increased removal of platinum-DNA adducts; altered expression of oncogenes; and activation of anti-apoptotic pathways (Rabik and Dolan, 2007).

6.8 SUMMARY

In this chapter the hypothesis that levels of expression of the DNA repair proteins XPF and MUS81 determine cellular response to oxaliplatin chemotherapy was tested. In support of the hypothesis, there was a positive correlation between levels of both MUS81 and XPF and IC_{50} for oxaliplatin in a panel of gastrointestinal adenocarcinoma cell lines. Further evidence in support of the hypothesis was provided by the findings of increased sensitivity to oxaliplatin in colorectal cancer cell lines with knockdown of *MUS81* expression and also haplo-insufficiency of its functional binding partner *EME1*. The finding from the clinical series of patients that low levels of both XPF and MUS81 were associated with better clinical outcomes following oxaliplatin-based treatment of oesophageal adenocarcinoma were supported by the experiments that showed that decreasing levels of XPF or MUS81 by RNA interference resulted in increased sensitivity to oxaliplatin in two oesophageal adenocarcinoma cell lines.

Going against the hypothesis, there was no significant difference in levels of XPF or MUS81 when parental cell lines and cell lines with acquired oxaliplatin resistance were compared. This may, however, be a reflection of the multifactorial nature of acquired chemotherapy resistance in cell line models.

7. CELL CYCLE PROFILE AND RESPONSE TO OXALIPLATIN-FLUOROURACIL CHEMOTHERAPY IN PATIENTS WITH OESOPHAGEAL ADENOCARCINOMA

7.1 INTRODUCTION

In Chapter 3, the results of functional pathway analysis of gene expression in oesophageal adenocarcinoma before and after treatment with neoadjuvant oxaliplatin-fluorouracil chemotherapy were described. Alterations in tumour expression of genes involved of a number of pathways were associated with pathological response to treatment. One of these was down-regulation of genes associated with cell cycling.

There are two possible explanations for the finding that down-regulation of cell cycling was seen in pathological responders, but not pathological non-responders. It may be that the down-regulation of cell cycle pathways seen in the responders is simply a reflection of oxaliplatin-induced cell cycle arrest, and is therefore a marker of response to treatment. It may, however, be that pathological non-responders have a different tumour cell cycle profile, and that cell cycle stage dictates the strategy used by a cell to repair platinum-DNA adducts. For example, it is known that repair of platinum-induced interstrand cross-links (ICLs) occurs in both a replication-dependent and replication-independent fashion (see **Section 1.1.7**). It is thought that replication-dependent ICL repair is more efficient, as futile cycles of incision and ligation can occur in replication-independent ICL repair (Mu et al., 2000). It was therefore

hypothesised that tumours with a high proportion of cells in replicative phases of the cell cycle would have worse outcomes following oxaliplatin-fluorouracil chemotherapy.

7.2 PRE-TREATMENT CELL CYCLE PROTEIN LEVELS AND RESPONSE TO NEOADJUVANT OXALIPLATIN-FLUOROURACIL CHEMOTHERAPY

To investigate the relationship between tumour cell cycle profile and outcomes following oxaliplatin-fluorouracil chemotherapy, pre-treatment tumour samples were stained for Cyclins A, B1, D1 and E and for Ki-67. Levels of expression of these proteins vary during the cell cycle, indicating the proportion of cells likely to be in a given phase. Cyclin A is a marker of S/G2-phase; Cyclin B1 is a marker of G2/M-phase; Cyclin D1 is a marker of G1-phase; and Cyclin E is a marker of G1/S-phase (**FIGURE 7.1**). Ki-67 is a marker of actively cycling cells (*i.e.* it is expressed in the nuclei of cells in all phases except G0), and is therefore often regarded as a surrogate marker for the S-phase fraction. The whole section was examined, and slides were scored on the basis of a representative X20 magnification field. The percentage of cell expressing the marker was counted by two observers using a double headed microscope and, in cases of disagreement, a consensus score was reached.

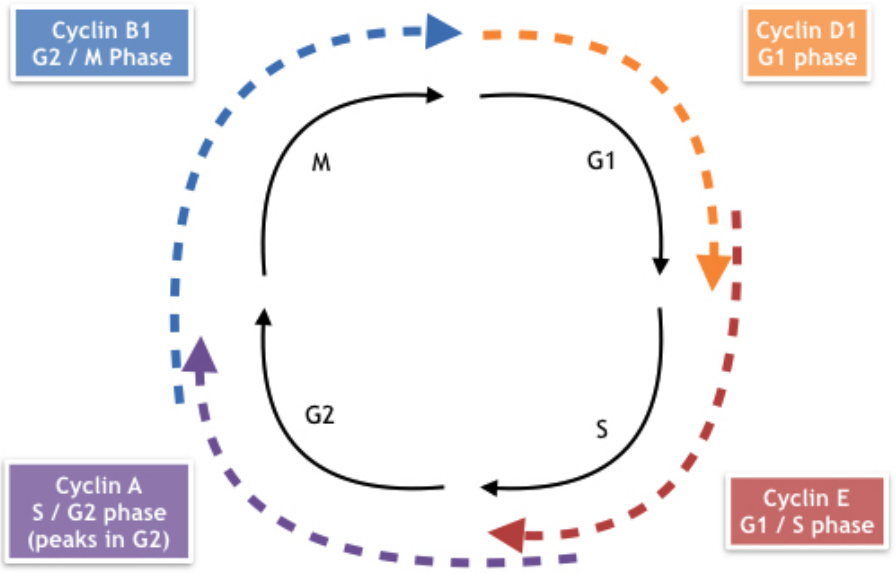


Figure 7.1. Relationship between expression of Cyclins A, B1, D1 and E and cell cycle phase.

For categorical analyses, a greater than median cut-off was used to define high expression of an individual marker. The distribution of expression of the cell cycle markers and cut-offs used to define high expression are shown in **TABLE 7.1**. To assess for a link between cell cycle profile and prognostic factors, the independent samples t-test was used to test for association between cell cycle marker expressions and stage (I/II *vs* III/IV) (**TABLE 7.2**), and age (**TABLE 7.3**). No association was found between expression of any of these proteins and tumour stage or patient age.

Table 7.1. Distribution of percentage of cells expressing individual cell cycle markers.

Cell cycle marker	Range (%)	Median (%)
Cyclin A	0–50	10
Cyclin B1	5–50	10
Cyclin D1	10–90	70
Cyclin E	5–100	60
Ki-67	10–100	60

Table 7.2. Relationship between pre-treatment tumour expression of cell cycle markers and tumour stage.

	Tumour Stage	*N*	Mean	Std. Deviation	Std. Error of Mean	t-statistic	Degrees of freedom	*P*-value
Cyclin A	I or II	23	13.09	15.95	3.33	0.32	27	0.75
	III or IV	6	10.83	12.01	4.90			
Cyclin B1	I or II	24	21.04	19.61	4.00	1.14	32	0.26
	III or IV	10	13.50	10.55	3.34			
Cyclin D1	I or II	21	46.90	30.27	6.61	-0.45	27	0.65
	III or IV	8	52.50	27.65	9.77			
Cyclin E	I or II	20	3.00	1.30	0.29	1.44	26	0.16
	III or IV	8	2.13	1.81	0.64			
Ki-67	I or II	22	58.41	31.22	6.66	-0.64	25	0.53
	III or IV	5	68.00	24.90	11.14			

Table 7.3. Relationship between pre-treatment tumour expression of cell cycle markers and patient age.

	Marker Expression	*N*	Mean age	Std. Deviation	Std. Error of Mean	t-statistic	Degrees of freedom	*P*-value
Cyclin A	Low	24	67.58	8.22	1.68	0.68	27.00	0.50
	High	5	64.80	8.98	4.02			
Cyclin B1	Low	19	66.68	8.04	1.84	-0.19	32	0.85
	High	15	67.20	7.59	1.96			
Cyclin D1	Low	18	65.67	8.08	1.90	-0.75	27	0.46
	High	11	67.91	7.27	2.19			
Cyclin E	Low	16	65.88	7.52	1.88	0.24	26	0.82
	High	12	65.17	8.34	2.41			
Ki-67	Low	15	66.13	7.73	1.99	-0.29	25	0.77
	High	12	67.08	9.29	2.68			

To test for an association between cell cycle marker expression and clinical outcomes, expression of the individual proteins were tested for association with pathological response and 1 year DFS (chi-square), and DFS and OS (logrank statistic). No association was found between expression of any of the cell cycle markers and pathological response (**TABLE 7.4**). High expression of Cyclin B1 correlated with an increased likelihood of death or recurrence within a year (χ^2 = 7.174, P = 0.007), no other significant associations between cell cycle marker expression and 1 year DFS were found (**TABLE 7.5**).

Table 7.4. The relationship between tumour cell cycle marker expression and pathological response following neoadjuvant oxaliplatin-fluorouracil chemotherapy.

Cell cycle protein	Chi-square statistic	*P*-value
Cyclin A	1.77	0.184
Cyclin B1	1.27	0.260
Cyclin D1	0.06	0.812
Cyclin E	1.45	0.229
Ki-67	0.36	0.547

Table 7.5. The relationship between tumour cell cycle marker expression and one-year disease free survival following neoadjuvant oxaliplatin-fluorouracil chemotherapy.

Cell cycle protein	Chi-square statistic	*P*-value
Cyclin A	1.72	0.189
Cyclin B1	7.17	0.007
Cyclin D1	0.86	0.355
Cyclin E	0.05	0.820
Ki-67	1.43	0.247

High expression of Cyclin B1 was associated with worse DFS (high *vs* low expression, median 238 days *vs* 1062 days; $\chi^2 = 4.175$; $P = 0.041$) (**FIGURE 7.2**). This did not, however, translate into a statistically significant association with OS (high *vs* low expression, median 639 days *vs* not reached; $\chi^2 = 2.479$; $P = 0.115$) (**FIGURE 7.3**).

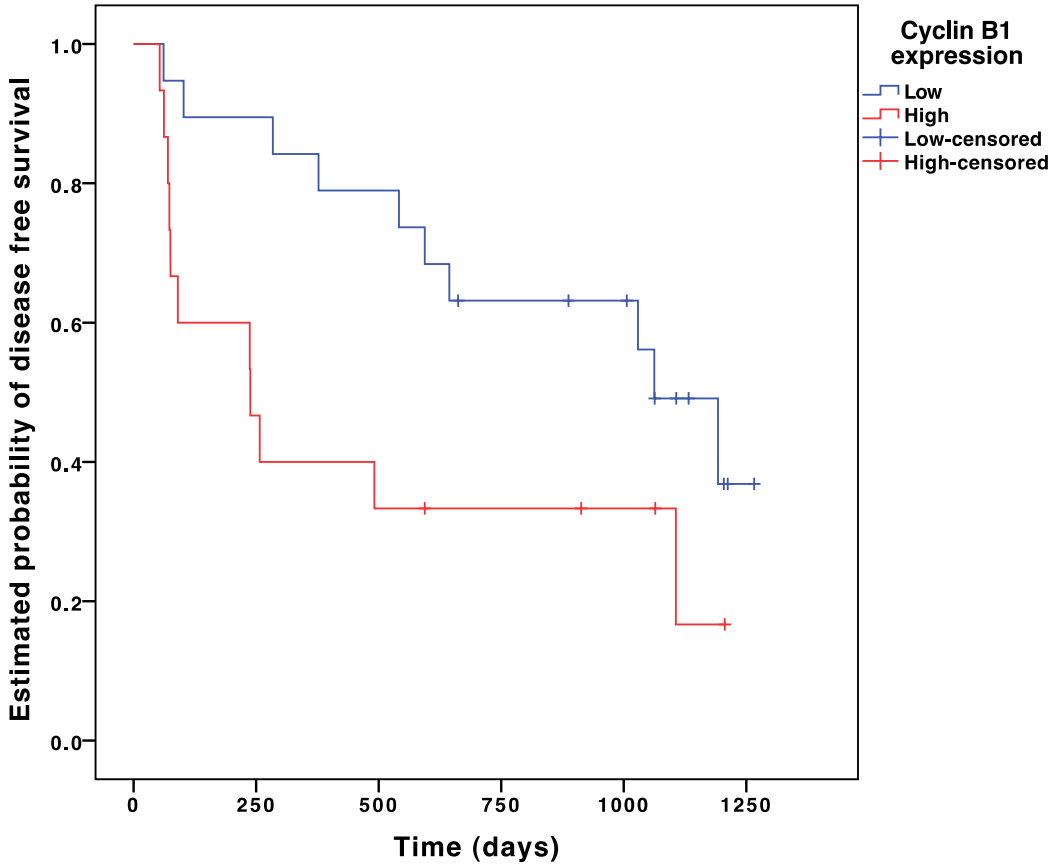


Figure 7.2. Kaplan-Meier analysis of disease free survival of oesophageal adenocarcinoma patients treated with neoadjuvant oxaliplatin-fluorouracil chemotherapy based on pre-treatment level of Cyclin B1. High levels of Cyclin B1 expression were associated with worse DFS (high *vs* low expression, median 238 days *vs* 1062 days; $\chi^2 = 4.175$; $P = 0.041$).

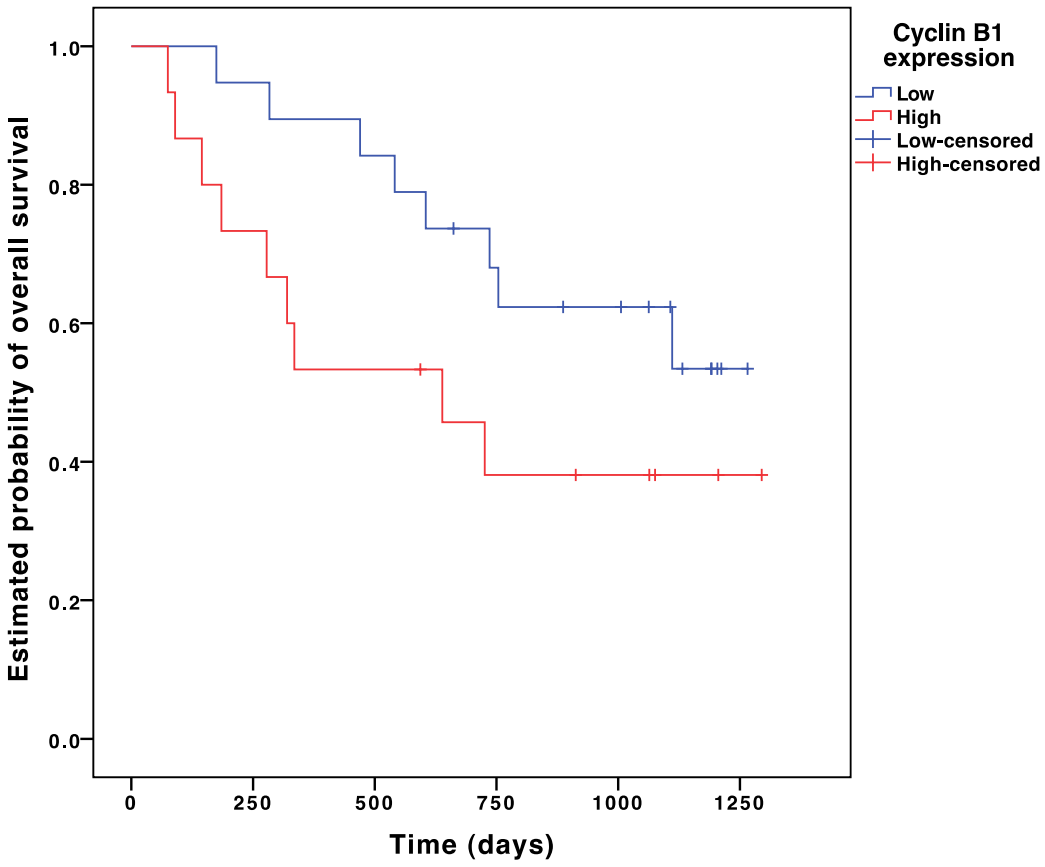


Figure 7.3. Kaplan-Meier analysis of overall survival of oesophageal adenocarcinoma patients treated with neoadjuvant oxaliplatin-fluorouracil chemotherapy based on pre-treatment level of Cyclin B1. There was no significant association between pre-treatment Cyclin B1 expression level and OS in these patients (high *vs* low expression, median 639 days *vs* not reached; $\chi^2 = 2.479$; $P = 0.115$).

7.3 ASSOCIATIONS BETWEEN TUMOUR DNA REPAIR PROTEIN EXPRESSION AND CELL CYCLE MARKERS

It is known that ICLs show their greatest toxicity in S-phase, when they cause stalling of the advancing replication fork. There are thought to be two pathways for ICL repair – a replication-dependent pathway, that involves both XPF and MUS81, and a less well-defined replication-independent pathway that is known

to involve XPF but in which MUS81 is not known to have a role. In order to examine whether there was any relationship between tumour cell cycle profile and expression of XPF and MUS81, patients expressing high and low levels of the two proteins were compared for variance in distribution of expression of Cyclins A, B1, D1 and E and Ki-67 by the independent sample t-test. No significant relationship was found between XPF expression and any of the cell cycle markers (**TABLE 7.6**). High expression of MUS81 was associated with a significantly increased proportion of the tumour cells expressing the G2/M-phase marker Cyclin B1 (P = 0.02) (**FIGURE 7.4**). No other cell cycle markers were found to correlate with MUS81 expression (**TABLE 7.7**).

Table 7.6. Relationship between level of XPF expression and cell cycle markers in oesophageal adenocarcinomas.

Cell cycle protein	XPF expression	*N*	Mean	Std. Deviation	Std. Error of mean	t-statistic	Degrees of freedom	*P*-value (2-tailed)
Cyclin A	Low	16	12.06	12.32	3.08	-0.22	27	0.83
	High	13	13.31	18.39	5.10			
Cyclin B1	Low	19	13.95	15.15	3.47	-1.88	32	0.07
	High	15	25.00	19.09	4.93			
Cyclin D1	Low	16	52.19	26.64	6.66	0.76	27	0.45
	High	13	43.85	32.54	9.03			
Cyclin E	Low	15	2.60	1.50	0.39	-0.57	26	0.57
	High	13	2.92	1.50	0.42			
Ki-67	Low	14	57.14	33.15	8.86	-0.54	25	0.59
	High	13	63.46	27.03	7.50			

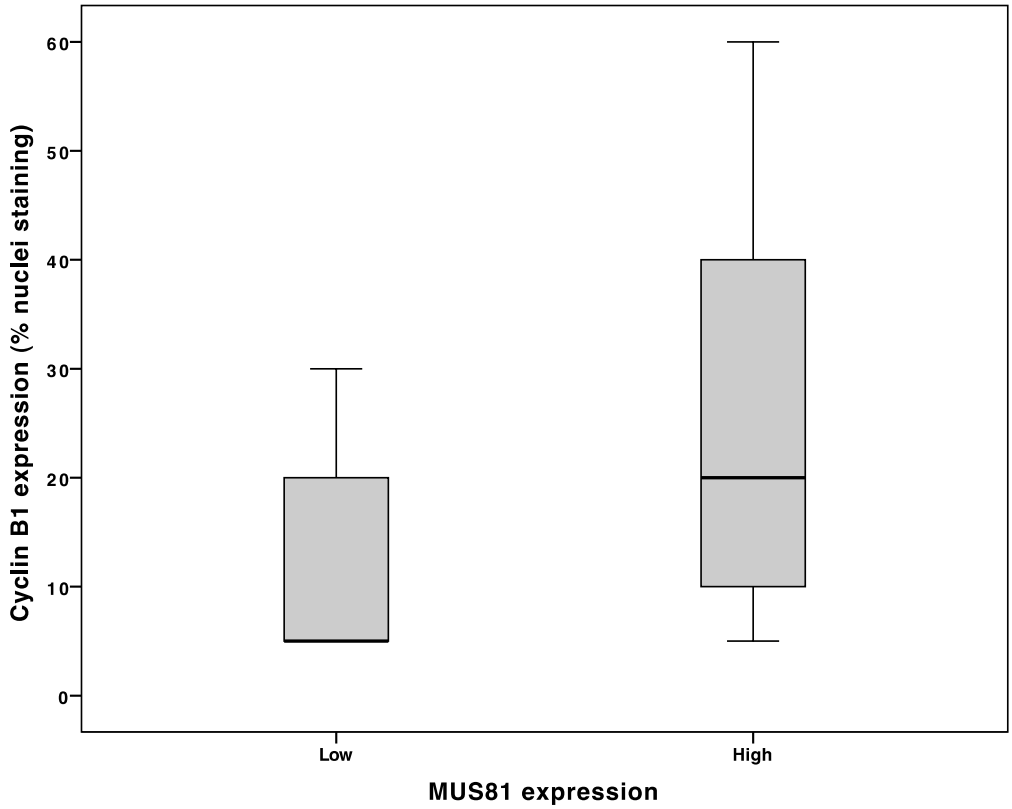


Figure 7.4. A box and whisker plot comparing Cyclin B1 expression in tumours with high and low levels of MUS81 protein. Median Cyclin B1 positivity in low MUS81 tumours 5% (IQR 5-20%); median Cyclin B1 positivity in high MUS81 tumours 20% (IQR 10-40%) (*P* = 0.02).

Table 7.7. Relationship between level of MUS81 expression and cell cycle markers in oesophageal adenocarcinomas.

Cell cycle protein	MUS81 expression	*N*	Mean	Std. Deviation	Std. Error of mean	t-statistic	Degrees of freedom	*P*-value (2-tailed)
Cyclin A	Low	10	10.20	16.21	5.12	-0.86	25	0.40
	High	17	15.47	14.77	3.58			
Cyclin B1	Low	15	12.00	9.41	2.43	-2.56	30	0.02
	High	17	26.76	20.46	4.96			
Cyclin D1	Low	12	46.25	28.85	8.33	-0.75	25	0.46
	High	15	54.67	29.00	7.49			
Cyclin E	Low	13	2.54	1.33	0.37	-0.70	26	0.49
	High	15	2.93	1.62	0.42			
Ki-67	Low	9	75.56	26.51	8.84	1.61	23	0.12
	High	16	57.81	26.39	6.60			

7.4 THE PROGNOSTIC VALUE OF CELL CYCLE PROTEIN EXPRESSION IN OESOPHAGEAL ADENOCARCINOMA TREATED WITH SURGERY ALONE

To test the prognostic role of cell cycle protein expression in the absence of chemotherapy, expression of Cyclins A, B1, D1 and E and Ki-67 were assayed in the cohort of patients treated with surgery alone. No association was found between expression of Cyclins A, B1, D1 and E and OS. The shape of the Kaplan-Meier curve for OS in Ki-67 high vs. low patients (**FIGURE 7.5**) indicated a possible early survival advantage for tumours with low Ki-67 levels. This was confirmed by the finding that high levels of Ki-67 expression were associated with worse early OS when tested by the Generalised Wilcoxon test (high *vs* low expression, median 517 days *vs* 1338 days; $\chi^2 = 4.506$; $P = 0.034$), although this failed to reach significance when the logrank statistic was used ($\chi^2 = 3.637$; $P = 0.056$) (**FIGURE 7.5**).

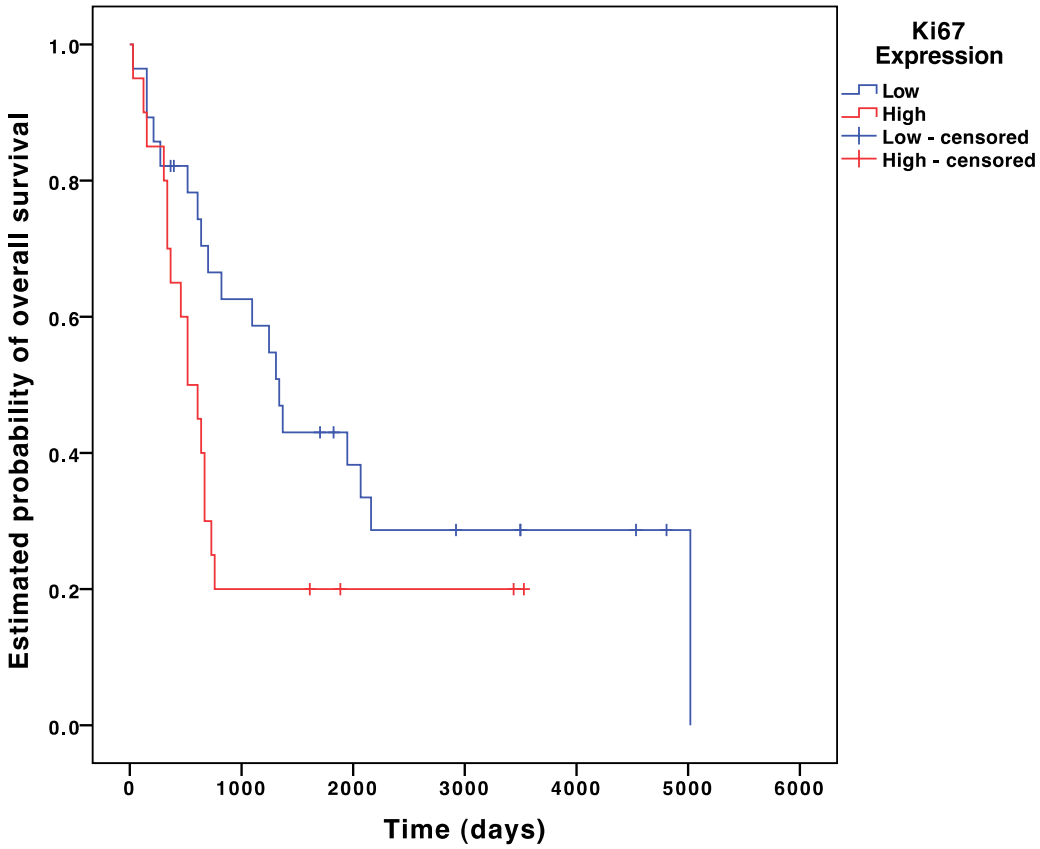


Figure 7.5. Kaplan-Meier analysis of overall survival of oesophageal adenocarcinoma patients treated with surgery alone based on tumour Ki-67 expression. High levels of tumour Ki-67 expression were associated with worse overall survival in patients with oesophageal adenocarcinoma treated with surgery alone (high *vs* low expression, median 517 days *vs* 1338 days; $\chi^2 = 4.506$; $P = 0.034$; Generalized Wilcoxon).

7.5 LACK OF ALTERATION IN EXPRESSION OF KI-67 AND CYCLIN B1 PROTEIN LEVELS FOLLOWING TWO CYCLES OF OXALIPLATIN-FLUOROURACIL CHEMOTHERAPY

As pre-treatment levels of Cyclin B1 and Ki-67 were associated with clinical outcomes in the cohort of patients treated with neoadjuvant chemotherapy and the cohort of patients treated with surgery alone, respectively, post-treatment levels of these cell cycle markers were measured in the cohort of patients treated

with neoadjuvant oxaliplatin-fluorouracil. If high levels of either of these proteins confers a survival advantage following oxaliplatin-fluorouracil chemotherapy, then the proportion of cells expressing them may increase following treatment.

The percentage of cells positive for Cyclin B1 and Ki-67 was measured by IHC in tumour samples before and after two cycles of oxaliplatin-fluorouracil chemotherapy. After two cycles of oxaliplatin-fluorouracil chemotherapy there was no significant alteration in Cyclin B1 expression (median pre-treatment 10%, IQR 5-30%; median post-treatment 10%, IQR 5-20%; $P = 0.570$, Wilcoxon's sign rank test) (**FIGURE 7.6**). Nor was there any alteration in Ki-67 expression (median pre-treatment 60%, IQR 30-90%; median post-treatment 60%, IQR 40-90%; $P = 0.400$, Wilcoxon's sign rank test) (**FIGURE 7.7**).

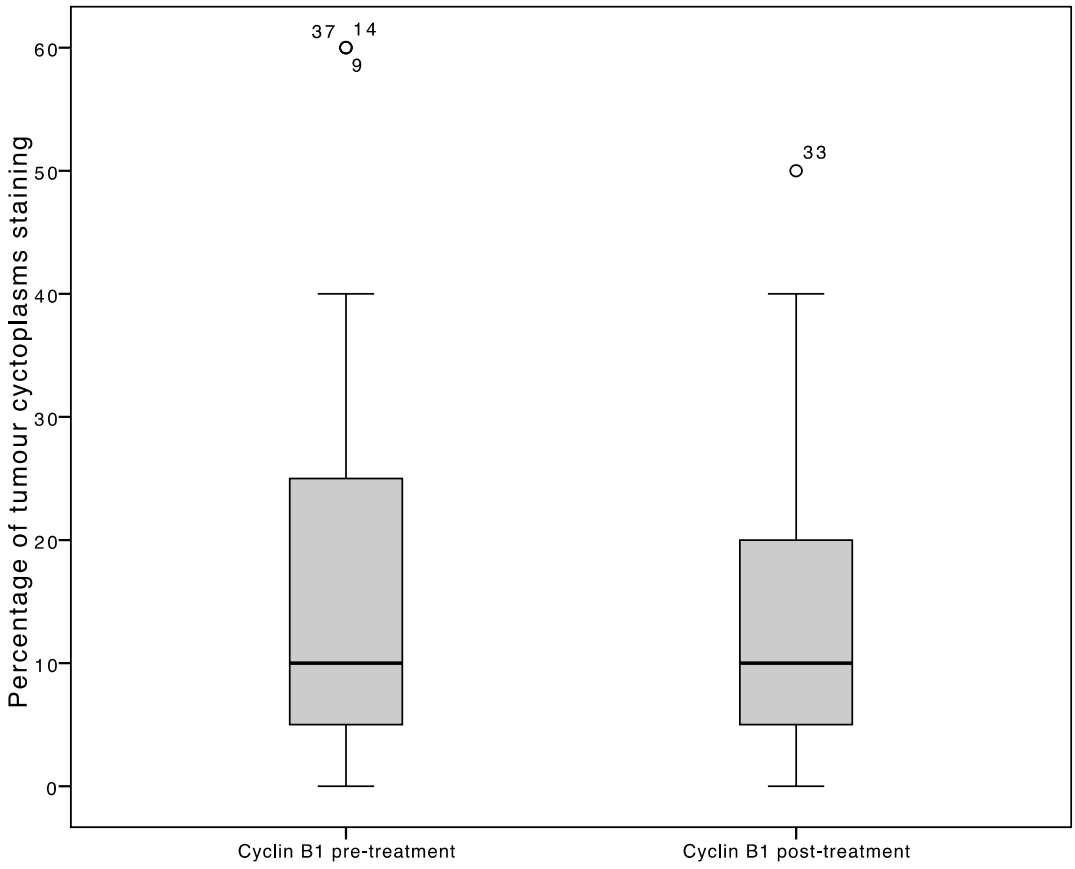


Figure 7.6. Comparison of proportion of oesophageal adenocarcinoma cells positive for Cyclin B1 before and after oxaliplatin-fluorouracil chemotherapy. There was no significant alteration in Cyclin B1 expression.

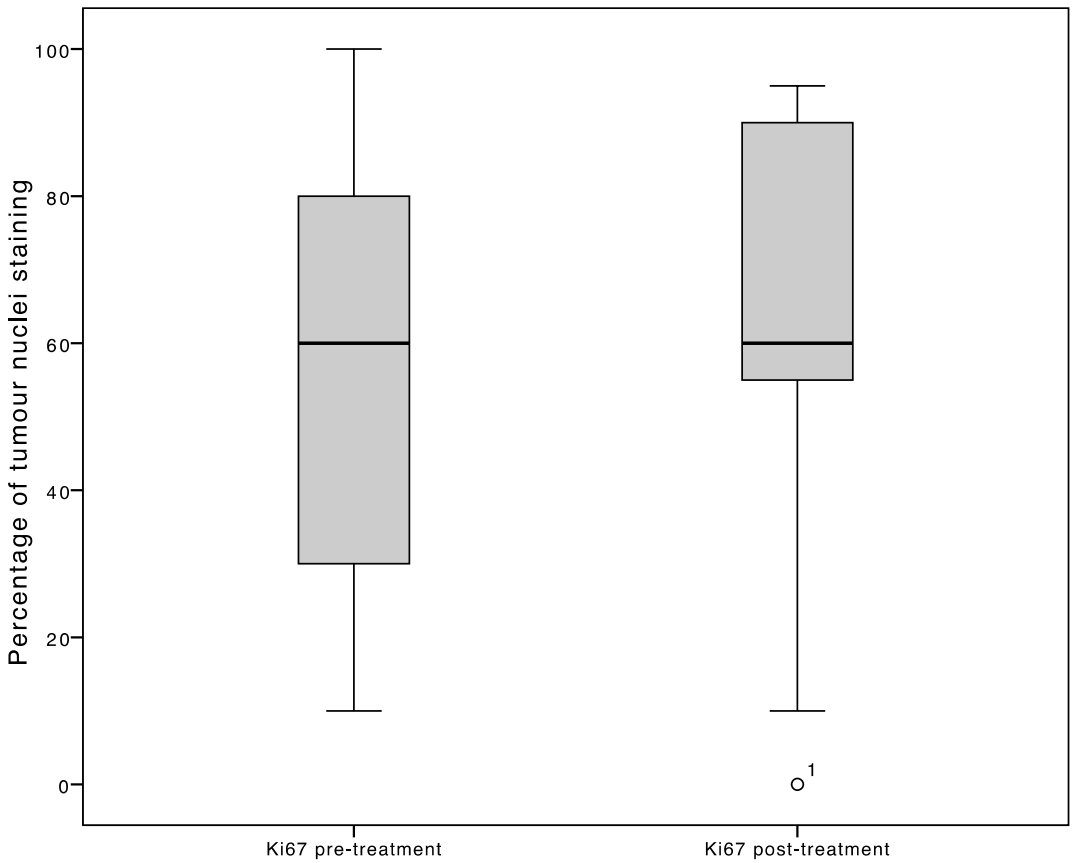


Figure 7.7. Comparison of proportion of oesophageal adenocarcinoma cells positive for Ki-67 before and after oxaliplatin-fluorouracil chemotherapy. There was no significant alteration in Ki-67 expression.

Post-treatment Cyclin B1 expression was tested for association with pathological response and 1 year DFS (chi-square), and DFS and OS (logrank statistic). There was no significant association between post-treatment Cyclin B expression and pathological response (χ^2 = 1.660; *P* = 0.198), 1 year DFS (χ^2 = 1.981; *P* = 0.159), or OS (high *vs* low expression, 754 days *vs* median not reached; χ^2 = 1.462; *P* = 0.227). There was a trend towards high post-treatment Cyclin B1 expression being associated with worse DFS (high *vs* low expression, 541 days *vs* 1192 days;

χ^2 = 3.420; P = 0.064) (**FIGURE 7.8**), but this did not reach statistical significance.

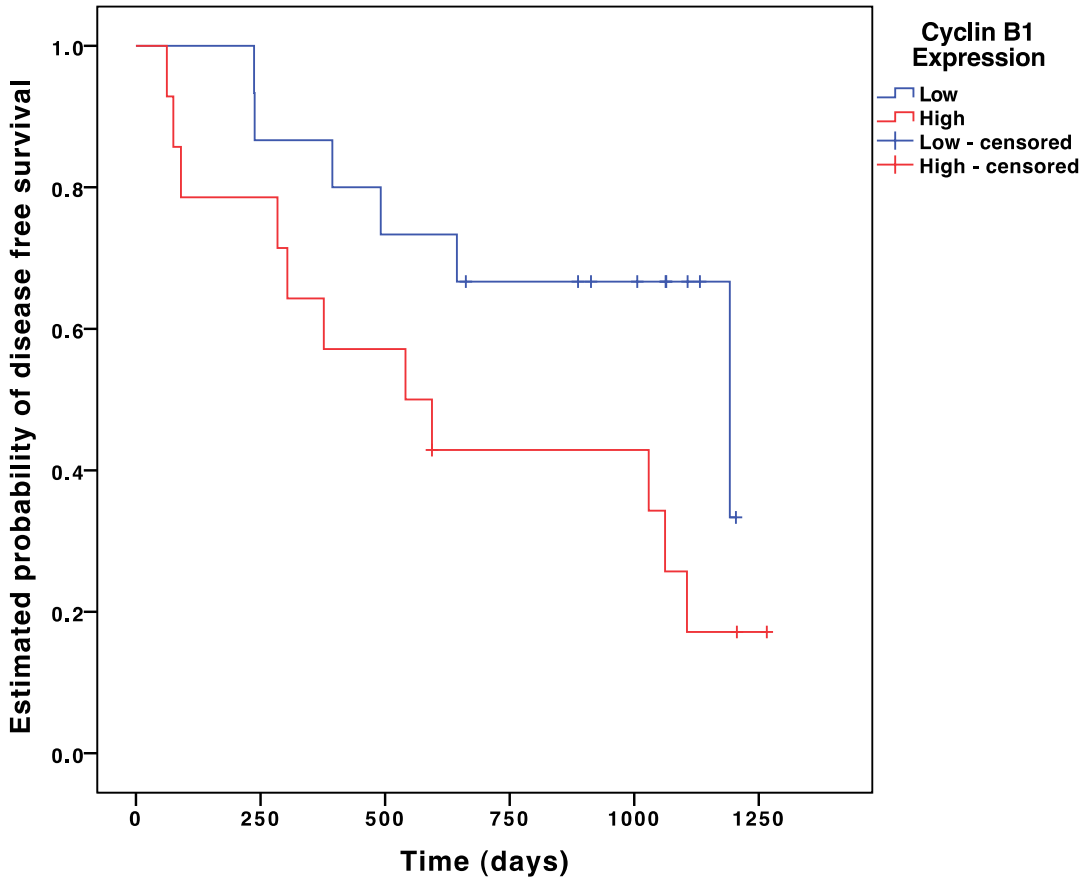


Figure 7.8. Kaplan-Meier analysis of disease free survival in oesophageal adenocarcinoma patients receiving neoadjuvant oxaliplatin-fluorouracil chemotherapy based on post-treatment tumour Cyclin B1 expression. There was no significant association between post-treatment Cyclin B1 expression and DFS in these patients (high *vs* low expression, 541 days *vs* 1192 days; χ^2 = 3.420; P = 0.064).

The association between post-treatment Ki-67 expression and pathological response and one-year DFS (chi-square), and DFS and OS (logrank statistic) was also tested. No relationship was found between Ki-67 expression and pathological response (χ^2 = 0.017; P = 0.897), one-year DFS (χ^2 = 1.395; P =

0.238), DFS (high *vs* low expression, 644 days *vs* 1029 days; χ^2 = 0.022; *P* = 0.883), or OS (high *vs* low expression, median not reached *vs* 1111 days; χ^2 = 0.020; *P* = 0.887).

7.6 DISCUSSION

The study presented above followed the findings of the functional pathway analysis presented in **Section 3.12**. The functional pathway analysis showed that tumours exhibiting pathological response to oxaliplatin-fluorouracil treatment had down-regulation of expression of cell cycle pathway genes following treatment. This finding posed the question, is a different cell cycle profile seen in patients that respond to treatment, and, if so, is there a correlation between cell cycle profile and expression of DNA repair proteins?

It was found that high pre-treatment levels of the G2/M phase marker Cyclin B1 were associated with worse one year and overall DFS. The finding that Cyclin B1 level was not associated with clinical outcomes in the cohort of patients treated with surgery alone goes against an argument that Cyclin B1 is simply a prognostic marker.

One explanation for the finding that tumours with a high proportion of cells in G2/M phase have worse DFS following oxaliplatin-fluorouracil chemotherapy is that these tumours have a higher proportion of cells that have completed DNA strand synthesis. It is known that oxaliplatin induces G2/M phase arrest in HCT116 colorectal cancer cell lines (Arango et al., 2004). It may be that in these

tumours, a high proportion of cells have already completed DNA replication, thus they are less vulnerable to oxaliplatin-induced ICLs as they have the entirety of G0 and G1 to repair these lesions before they cause replication fork stalling in S-phase.

Another explanation is that cells in G2/M phase have the ability to perform replication-dependent ICL repair, which is thought to be more efficient that replication-independent ICL repair (Mu et al., 2000), thus enabling them to remove oxaliplatin-DNA adducts more efficiently. In keeping with this hypothesis, it was found that tumours with a high proportion of cells expressing Cyclin B1 also tended to have high levels of MUS81 protein. MUS81-EME1 is a key component of replication-dependent ICL repair, and is thought to have a particular role in the processing of ICLs that persist into late G2/M phase (Sengerova et al., 2011; Wang et al., 2011). It is also known that MUS81-EME1 has a crucial role in the maintenance of genome stability by allowing controlled cleavage of common fragile sites (CFS) in the early stages of mitosis (Ying et al., 2013). However, it thought that activation of MUS81-EME1 in G2/M phase occurs via phosphorylation of EME1, and that levels of MUS81-EME1 do not vary significantly during the active phases of the cell cycle (Gallo-Fernandez et al., 2012; Minocherhomji and Hickson, 2014). If time had allowed, further investigation by double staining would have been performed to identify whether MUS81 levels are higher in individual tumour cells expressing Cyclin B1, or if the finding is simply coincidental.

In the patients treated with surgery alone, a high proportion of cells expressing the marker cellular proliferation Ki-67 was associated with worse OS. This is in keeping with two published studies in oesophageal adenocarcinoma - increased Ki67 levels were associated with worse OS in 35 patients treated with surgery alone and in another cohort study of 121 patients, 74 of whom received no neoadjuvant chemotherapy before tumour resection (Dutta et al., 2012; Evangelou et al., 2008). One other study exists of oesophageal adenocarcinoma managed with surgery alone, in which low levels of Ki-67 expression were associated with worse OS (Falkenback et al., 2008); however, the authors acknowledge that the results of this study must be treated with some caution as only a small proportion of the patients in the study were classified as low Ki67 expressers.

7.7 SUMMARY

In this study, it has been shown that high pre-treatment expression of the G2/M-phase marker Cyclin B1 is associated with worse 1 year and overall DFS following oxaliplatin-fluorouracil chemotherapy. There is no association between pre-treatment expression of any other cell cycle protein and outcomes following oxaliplatin-fluorouracil chemotherapy.

When the prognostic value of the cyclins (including Cyclin B1) was tested in a cohort of patients treated with surgery alone, there was no association with OS. In keeping with other tumour types, high levels of Ki-67 expression were associated with worse OS in this group of patients.

High levels of pre-treatment Cyclin B1 expression correlated with high levels of expression of the DNAR protein MUS81. There was no correlation between any other markers of cell cycling and expression of the DNAR proteins MUS81 and XPF.

When tumour samples taken after two cycles of oxaliplatin-fluorouracil were compared with pre-treatment samples, there was no significant difference in their G2/M-phase population or overall proportion of cells cycling.

8. DISCUSSION

8.1 SUMMARY OF FINDINGS

This research project aimed to test the hypothesis that high levels of the DNA repair proteins XPF and MUS81 in tumours before treatment are associated with lack of response to platinum chemotherapy. These two proteins are endonucleases that are known to play a role in the interstrand cross-link (ICL) repair pathway. It is thought that ICLs are the most toxic lesion induced by platinum chemotherapeutic agents, and the ability of cells to repair these lesions may, therefore, be a key determinant of cellular sensitivity to platinum-based chemotherapy.

The first part of this research project used microarray analysis of gene expression in pre-treatment samples of oesophageal adenocarcinomas to discover if expression of genes known to be involved in ICL repair was associated with clinical responses to oxaliplatin-fluorouracil chemotherapy. These results are summarised in **Table 8.1**. High levels of expression of *ERCC1* and *EME1* were associated with worse one-year disease free survival (DFS). High levels of *ERCC1* expression were also associated with worse DFS and overall survival (OS). In addition, it was found that mean levels of expression of *ERCC1* and *MUS81* increased following two cycles of oxaliplatin-fluorouracil chemotherapy.

Table 8.1. Summary of associations between outcomes and level of tumour expression of *XPF* and *MUS81* in patients with oesophageal adenocarcinoma treated with neoadjuvant oxaliplatin-fluorouracil chemotherapy. □ = no significant association; □ = $P < 0.05$; □□ = $P < 0.05$ adjusted for multiple testing.

	XPF	*ERCC1*	*MUS81*	*EME1*
Pathological response	×	×	×	×
One year disease free survival	×	✓	×	✓
Disease free survival	×	✓	×	×
Overall survival	×	✓	×	×
Increase in level post treatment	×	✓✓	✓✓	×

Functional pathway analysis was also performed on the microarray data, looking for other potential pathways involved in determining clinical response to oxaliplatin-fluorouracil chemotherapy in oesophageal adenocarcinoma. This analysis showed that patients who exhibited pathological response had down-regulation of cell cycling pathways and down-regulation of the anti-apoptotic HSP27 pathway in their tumours following treatment and increased expression of a histone deacetylase pathway after treatment.

These findings led to further studies of the relevance of protein levels of XPF-ERCC1 and MUS81-EME1 in determining clinical responses to oxaliplatin-based chemotherapy in patients with oesophageal cancer. Immunohistochemical analysis of XPF and MUS81 protein levels was performed to test their potential use as biomarkers for response to ICL-generating chemotherapy in gastrointestinal cancers. The results of these analyses are summarised in **Table 8.2**. High levels of XPF expression were associated with lack of pathological response in oesophageal adenocarcinoma treated with oxaliplatin-fluorouracil chemotherapy. High levels of MUS81 expression were associated with worse DFS

and OS in the same patient cohort. XPF expression was also found to be at higher levels in post-treatment resection specimens when compared with pre-treatment biopsies from the same tumours. There was no relationship between XPF or MUS81 expression and OS in a matched historic cohort of patients with oesophageal adenocarcinoma treated with surgery alone. These two sets of results support the hypothesis that XPF and MUS81 have the potential to be used as biomarkers for response to oxaliplatin-based chemotherapy in patients with oesophageal adenocarcinoma.

Despite these findings that XPF and MUS81 protein expression are associated with clinical outcomes following oxaliplatin-fluorouracil chemotherapy in patients with oesophageal adenocarcinoma, no significant association was found between protein levels of these two biomarkers and clinical outcomes in a historic cohort of patients who received neoadjuvant epirubicin-cisplatin-fluorouracil chemotherapy for oesophageal adenocarcinoma. Likewise, no significant association was found between protein levels and outcomes in colorectal cancer treated with oxaliplatin-based chemotherapy and anal cancer treated with the ICL-generating agent mitomycin C.

Table 8.2. Summary of the results of analyses of levels of MUS81 and XPF proteins in gastrointestinal tumour samples. □ = no significant association; ■ = $P < 0.05$; ★ = finding that failed to reach statistical significance, but $P < 0.06$

Tumour type/treatment	Outcome measured	XPF	MUS81
Oesophageal adenocarcinoma	Pathological response	✓	×
Oxaliplatin-fluorouracil chemotherapy	One year disease free survival	×	✓
	Disease free survival	×	✓
	Overall survival	×	✓
	Increase in level post treatment	✓	★
Oesophageal adenocarcinoma	Pathological response	×	×
Epirubicin-cisplatin-fluorouracil chemotherapy	Disease free survival	×	×
	Overall survival	×	×
Metastatic colorectal cancer	Radiological response	×	×
Oxaliplatin-fluorouracil chemotherapy	Disease control	×	×
	Progression free survival	×	×
	Overall survival	×	×
Anal squamous cell carcinoma	Recurrence free survival	×	★
Mitomycin-cisplatin-fluorouracil chemoradiotherapy			

In the third part of this research project, immunohistochemical detection of cell cycle proteins was used to test for a relationship between tumour cell cycle profile and response to oxaliplatin-fluorouracil chemotherapy in patients with oesophageal adenocarcinoma, testing the hypothesis that cell cycle phase affects the ability of tumour cells to remove oxaliplatin-DNA adducts. In support of this hypothesis, high levels of expression of Cyclin B1, a marker of G2/M-phase, was associated with worse disease free survival in these patients. There was no association between other cell cycle markers (Cyclin A, Cyclin D1, Cyclin E and Ki67) and clinical outcomes in this cohort. There was no association between cell cycle markers and XPF expression, but there was an association between high levels of tumour MUS81 expression and high Cyclin B1 expression. These markers were also assayed in the cohort of patients with oesophageal adenocarcinoma treated with surgery alone and, consistent with the hypothesis, no association was found between expression of any of the Cyclins (including

Cyclin B1) and OS in this group of patients. In keeping with the published literature, high levels of tumour Ki67 expression was associated with worse OS in patients treated with surgery alone.

In the final part of the project, tumour cell line models were used to test the hypothesis that MUS81 and XPF levels determine sensitivity of tumour cells to oxaliplatin. In support of the hypothesis, there was a positive correlation between XPF protein levels and oxaliplatin IC_{50} values in a panel of gastrointestinal adenocarcinoma cell lines; likewise MUS81 protein levels positively correlated with the oxaliplatin IC50 value. Adenocarcinoma cells with acquired resistance to oxaliplatin had higher levels of XPF expression than their paired wild-type cell lines, although they did not have significantly higher MUS81 expression. Colorectal cell lines with MUS81 knockout and haploinsufficiency for EME1 showed increased sensitivity to oxaliplatin treatment. The clinical finding that high levels of MUS81 expression were associated with worse DFS and OS following oxaliplatin-based chemotherapy in oesophageal adenocarcinoma was supported by the novel finding *in vitro* that knockdown of MUS81 expression in the oesophageal adenocarcinoma cell lines FLO-1 and OE33 by siRNA increased sensitivity to oxaliplatin treatment.

8.2 DNA DAMAGE REPAIR PROTEINS AS BIOMARKERS FOR RESPONSE TO PLATINUM CHEMOTHERAPY

The findings presented in Chapters 3, 4 and 5 indicate that measurement of levels of the XPF-ERCC1 and MUS81-EME1 heterodimers in pre-treatment tumour biopsies may have the potential to be developed as predictive

biomarkers for response to oxaliplatin chemotherapy in patients with oesophageal adenocarcinoma.

There is considerable controversy in the literature about the use of DNA repair proteins as predictive biomarkers for response to platinum chemotherapy. In published clinical trials, ERCC1 levels have been successfully used to predict response to a range of platinum-based chemotherapies in patients with different cancer types. High pre-treatment tumour levels of *ERCC1* mRNA and protein have been correlated with worse outcomes following platinum-based therapy in a range of tumour types, including gastric, colorectal and lung cancer (Cobo et al., 2007; Metzger et al., 1998; Shirota et al., 2001; Wei et al., 2014).

Although immunohistochemistry has been used extensively in studies for the quantification of ERCC1 protein levels in tumour samples, concerns have been expressed about the specificity of the antibodies used for immunodetection of ERCC1 since Bhagwat *et al*. demonstrated that the most commonly used antibody for ERCC1, the 8F1 clone, was not specific for ERCC1 and stained another unidentified protein (Bhagwat et al., 2009). Subsequently, it has been found that the 8F1 antibody also binds to choline phosphate cytidylyltransferase-α (CCTα), a phospholipid synthesis enzyme regulated by RAS (Vaezi et al., 2014). Bhagwat et al. demonstrated that the rabbit polyclonal antibody FL-297 was the only antibody then available that was specific for ERCC1 detection by immunohistochemistry (Bhagwat et al., 2009).

In this project, attempts were made to develop a protocol for the use of the FL-297 antibody for detection of ERCC1 in different human cancers by immunohistochemistry. Considerable cytoplasmic staining occurred with the FL-297 antibody, and it was decided that this called into question its suitability for detection of the functional XPF-ERCC1 heterodimer (as its role in DNA damage repair requires a nuclear location). Subsequently, Friboulet *et al*. have shown that four isoforms of ERCC1 exist (201 – 204) and that one of these, ERCC1-203, is inactive in DNA repair and has a cytoplasmic location, explaining the high level of cytoplasmic staining seen with the FL-297 antibody in this study (Friboulet et al., 2013b). The same group examined sixteen commercially available ERCC1 antibodies and found that none of them were specific for the ERCC1-202 antibody, which is the only isoform that is active in DNA damage repair (Friboulet et al., 2013a). To date, no commercial antibody has been produced that has proven specificity for ERCC1-202 alone.

Assay of levels of XPF protein represents an alternative to ERCC1 for measurement of levels the XPF-ERCC1 heterodimer. It has been shown that XPF binds only to the functional ERCC1-202 isoform, and that it is dependent on this interaction for its stability (Friboulet et al., 2013b); therefore, measurement of XPF levels also gives a measurement of the levels of the XPF-ERCC1 heterodimer. Previous work by our group has demonstrated the specificity of the SPM228 mouse monoclonal antibody for immunodetection of XPF (Hatch et al., 2014). Immunohistochemistry for MUS81 was developed, and specificity of the antibody was demonstrated by knockdown experiments.

Academic consortia have defined pathways for the development of predictive biomarkers. These include the Cancer Research UK (CR-UK) Biomarker Roadmap and the US FDA's Biomarker Qualification Program ((CDER), 2014; Lioumi and Newell, 2010). The CR-UK Biomarker roadmap (**FIGURE 8.1**) sets out a series of stages required to bring a biomarker into routine clinical use. The first step is "Biomarker Discovery and Assay Development". This is divided into three stages.

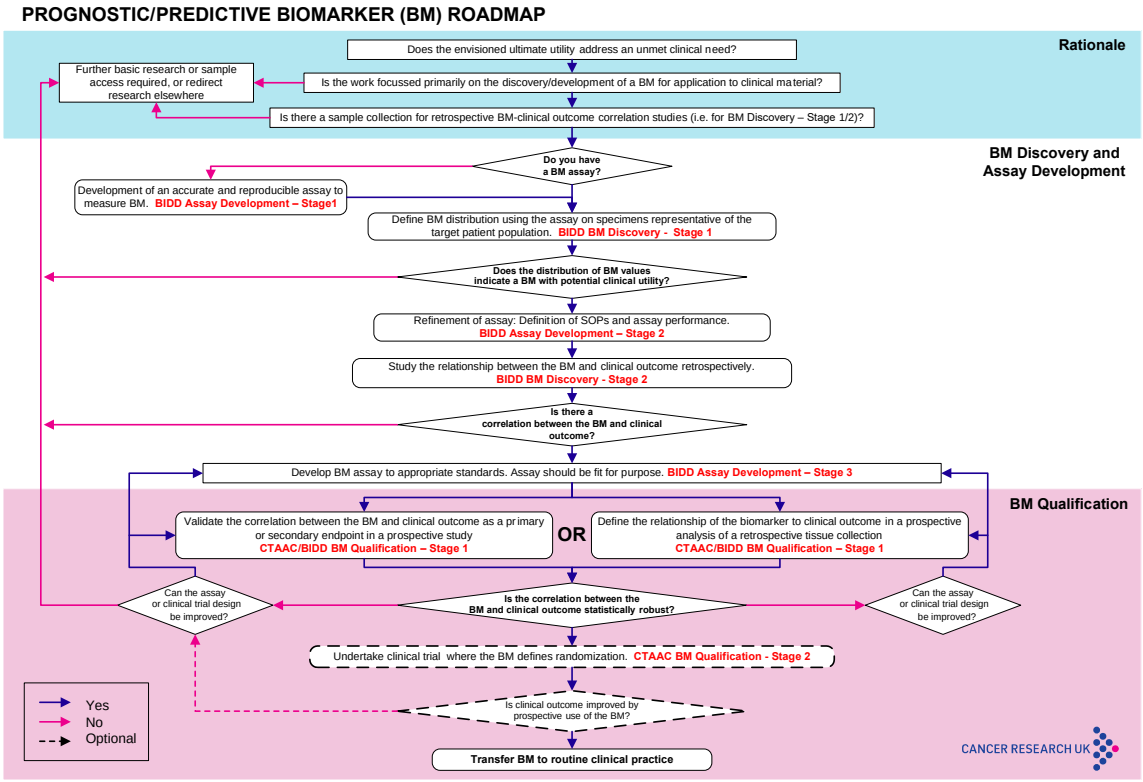


Figure 8.1. The Cancer Research UK Prognostic/Predictive Biomarker Roadmap. This summarises the steps required for the development of a validated biomarker for use in clinical practice.
(http://www.cancerresearchuk.org/prod_consump/groups/cr_common/@fre/@fun/documents/generalcontent/cr_027486.pdf)

The first stage is the development of an accurate and reproducible biomarker and definition of the distribution of that biomarker within the target patient population. The second stage is refinement of the assay and retrospective

studying of the relationship between the assay and clinical outcome. In the third and final stage of Biomarker Discovery and Assay Development, the assay should be developed to appropriate standards and be fit for purpose. The next series of stages is "Biomarker Qualification". The first stage of this is either validation of the biomarker as a primary or secondary endpoint in a prospective study or test the relationship of biomarker expression prospective analysis of a retrospective tissue collection. If the biomarker passes all of these hurdles, then the final stage is for the biomarker to be tested in a clinical trial in which the biomarker can be used for randomisation. If this is successful, then the biomarker is suitable for generalised use in clinical practice.

The findings that XPF and MUS81 may be predictive biomarkers in oesophageal adenocarcinoma treated with oxaliplatin-fluorouracil chemotherapy were made in a group of 38 patients receiving neoadjuvant chemotherapy. As this sample size was relatively small, it was decided that biomarker qualification should be performed in a prospective analysis of a retrospective tissue collection, before its use in a clinical trial could be considered. Unfortunately, no equivalent series of oxaliplatin treated oesophageal cancer specimens were available for analysis.

No relationship between XPF or MUS81 expression and outcomes were found in a cohort of 48 patients who received neoadjuvant epirubicin-cisplatin-fluorouracil chemotherapy. There are a number of possible explanations for this including the inclusion of an intercalating agent in the chemotherapy regimen, differences in sample handling and the small sample size. XPF and MUS81 levels were also assayed in a cohort of 48 patients with anal cancer treated with

mitomycin C-fluorouracil chemoradiotherapy. This showed an interesting, but non-statistically significant, trend towards correlation between high levels of MUS81 expression and worse relapse free survival. These findings highlight the difficulties of biomarker development in small cohorts of patients.

In an attempt to overcome the limitations of small sample sizes in the other cohorts, pre-treatment XPF and MUS81 levels were measured in patients from the FOCUS2 trial, in which patients with metastatic colorectal cancer received either oxaliplatin-fluorouracil based treatment. Pre-treatment primary tumour biopsies were analysed in 209 patients, 105 of whom received oxaliplatin in addition to fluorouracil. No significant association between levels of either of the biomarkers and response to oxaliplatin was demonstrated in these patients.

The failure of levels of XPF or MUS81 in pre-treatment biopsies to correlate with clinical outcomes in oxaliplatin-treated metastatic colorectal cancer demonstrates a major potential criticism of biomarkers based on single markers assessed in single pre-treatment biopsies. It has been shown by Gerlinger *et al*. in renal cell carcinoma that considerable heterogeneity occurs both within individual tumours and between primary tumours and metastases (Gerlinger et al., 2012). Thus, a single biopsy from a tumour may fail to represent the heterogeneity of tumour cells occurring within that tumour and may also not take into account the composition of metastases originating from that tumour. Uchida *et al*. have shown that *ERCC1* mRNA levels in colorectal metastases are overall approximately double those in primary tumours, and that there is no correlation between *ERCC1* expression in the primary tumour and the metastasis

(Uchida et al., 2008). It is therefore possible that the failure to replicate the oesophageal results in the colorectal patient cohort was due to the inability of primary tumour biopsies to represent the heterogenous biology of metastatic disease

Another potential criticism of the use of MUS81 and XPF as biomarkers for response to oxaliplatin chemotherapy is that they only represent a relatively narrow point in cellular response to DNA damaging chemotherapy. An alternative strategy is to develop assays that take into account multiple potential biomarkers such as the DNA damage response deficiency assay (DDRD). This mRNA expression assay was developed by identifying genes associated with FA/BRCA pathway dysfunction and has been shown to be associated with response and relapse free survival after anthracycline/cyclophosphamide–based chemotherapy in breast cancer (Mulligan et al., 2014).

It was found that, after two cycles of oxaliplatin-fluorouracil chemotherapy in oesophageal cancer, there are significant increases in expression of fifteen DNA damage repair genes, including ERCC1 and MUS81, and that XPF protein levels also rise. This raises another concern about the use of pre-treatment biopsies for determination of treatment – it does not take into account on treatment development of resistance to treatment. In a subcohort of 24 of the patients in the trial of oxaliplatin-fluorouracil chemotherapy for oesophageal adenocarcinoma, it has been shown that, even over the short time period of the two cycles of neoadjuvant chemotherapy followed by a 4-6 weeks of pre-operative recovery, considerable clonal evolution of the tumours occurred

(Findlay et al., 2016). It may be that, even in localised disease, the biology of a primary tumour rapidly evolves during initial treatment, meaning that information from a pre-treatment biopsy may quickly become redundant with regard to further management.

Another problem with development and use of predictive biomarkers is the handling and storage of the tumour biopsies. It has been shown that tissue fixation times have a significant impact on detection of XPF in tumour samples, with IHC-based measures of protein levels increasing with time spent in formalin before tissue processing (Pierceall et al., 2011). There is evidence that cut sections stored at room temperature undergo significant deterioration in detectable levels of a variety of proteins, but that sections stored at 4 °C show little deterioration in protein levels for up to one year (DiVito et al., 2004; Karlsson and Karlsson, 2011). In the oxaliplatin-fluorouracil treated cohort of oesophageal patients, a strict sampling handling protocol was imposed, with uniform fixation times, tissue processing and storage conditions. It may be that the positive results found in this cohort of patients are partially accounted for by this strict sample handling and the lack of distant metastases.

8.2.1 Future perspectives

In this study, levels of MUS81 and XPF were correlated with clinical response to oxaliplatin-fluorouracil chemotherapy in oesophageal adenocarcinoma. The role of XPF and MUS81 in determining sensitivity to oxaliplatin was confirmed by in vitro work in oesophageal adenocarcinoma cell line models. Furthermore, there was a possible link between high levels of MUS81 protein and lack of response to

mitomycin C-fluorouracil chemoradiotherapy in anal cancer, but this failed to reach significance in a small group of patients with this rare cancer.

On the basis of these findings, MUS81 and XPF warrant further investigation as potential biomarkers of response to oxaliplatin and other cross-linking chemotherapeutics. As has been discussed above, however, concerns regarding inter- and intra-tumour heterogeneity and tumour evolution on treatment need to be addressed. It is also possible that measurement of levels of the two proteins, or mRNA for them, could be incorporated into a multi-gene/protein signature as in the DDRD assay described above.

There is much current interest in the use of "liquid biopsies", that is the analysis of peripheral blood samples for either circulating tumour cells (CTCs) or circulating tumour DNA (ctDNA)(Crowley et al., 2013). Liquid biopsies offer the opportunity to examine the genetic landscape of a patients cancer, both primary tumour and metastases, and evolution of genetic changes during treatment. They therefore may allow alterations in the genetic profile of a tumour with time to be assessed by serial blood samples, rather than the conventional, and often impractical, alternative of multiple tumour biopsies.

ctDNA analysis has the potential to be useful for predicting and monitoring disease response patients with oesophageal adenocarcinoma. A recent meta-analysis has shown an association between two gene polymorphisms and major pathological response to platinum-based chemo/radiotherapy in Caucasians: wild-type XRCC1 rs25487, and variant ERCC1 rs11615 (Findlay et al., 2015).

Whole exome and deep sequencing of 24 of the individual patients with oesophageal adenocarcinoma receiving neoadjuvant oxaliplatin-fluorouracil chemotherapy failed to yield any association between specific mutations and response to treatment (Findlay et al., 2016). Interestingly, the only aberration seen for *XPF*, *ERCC1*, *MUS81* and *EME1* in these patients was an increase in copy number for *ERCC1* in a single patient. This demonstrates the importance of studying copy number in the potential use of ctDNA for DNA repair proteins as biomarkers for response to oxaliplatin.

It is now technically possible to isolate mRNA from CTCs (Sperger et al., 2016). This offers the tantalizing possibility that it may, in the future, be possible to monitor alteration in DNA repair gene expression, especially *XPF*, *ERCC1*, *MUS81* and *EME1*, in patients during treatment. This technology, however, remains to be proven, and the relationship between gene expression in CTCs and cells within tumours themselves has not yet been fully established.

For MUS81 and XPF to be further developed as potential biomarkers for response to platinum-based chemotherapy in oesophageal cancer, a number of steps must be performed. Firstly, the relationship between level of expression of the two proteins in primary tumours and metastases must be established. It is critical that the question of inter-tumour variability between primary and metastatic disease, and between metastatic lesions, is answered before the precise area of use for the biomarkers can be established. More detailed imaging of tumour heterogeneity with a view to developing new clinical trials of image-

directed biopsies may be of benefit, but there is a clinical limit to the number of biopsies that can be taken in one patient at a single timepoint.

Secondly, in accordance with Stage I of Biomarker Qualification as per the CRUK Biomarker Roadmap (**Figure 8.1**), the biomarkers need validation in a separate cohort of patients. No clinical trials of oxaliplatin-based chemotherapy in oesophageal cancer have human tissue biobanked for such a purpose. It is hoped that such a resource may be available in future years. The Neo-AEGIS trial (ICORG 10-14) aims to test whether there is any difference in response when patients with operable oesophageal cancer are treated with pre- and post-operative chemotherapy as per the MAGIC regimen [Etoposide, Cisplatin, Fluorouracil (Capecitabine)] or neoadjuvant chemoradiation as per the CROSS protocol (Carboplatin and Paclitaxel with concurrent radiotherapy, 41.4Gy/23Fr, over 5 weeks) (Keagan et al., 2014). Patients in this study are also to be recruited for samples of tumour and normal tissue, both before treatment and from resection specimens, to be taken for biomarker studies. Measurement of levels of XPF and MUS81 in pre-treatment biopsies from the patients in the arm receiving MAGIC protocol chemotherapy would allow a definitive result to be gained as to whether levels of the two proteins are associated with response to platinum-based chemotherapy in locally advanced oesophageal cancer.

This study found that high levels of tumour XPF and MUS81 are associated with lack of response to oxaliplatin-fluorouracil chemotherapy in oesophageal adenocarcinoma. It was also demonstrated, *in vitro*, that *MUS81* knock-out cells showed increased sensitivity to oxaliplatin; levels of MUS81 and XPF correlated

with sensitivity to oxaliplatin in a panel of gastro-intestinal adenocarcinoma cell lines; and knock-down by RNA interference of both *MUS81* and *XPF* sensitised oesophageal adenocarcinoma cell lines to oxaliplatin. These findings suggest that inhibition of XPF or MUS81 protein levels may be a mechanism by which oxaliplatin's anticancer efficacy may be increased.

Small molecule inhibitors of the XPF-ERCC1 interaction (NSC 130813 and E-X PPI2) and the endonuclease function of XPF (E-X AS5-4) have been developed that sensitise cell lines to cisplatin (Jordheim et al., 2013; McNeil et al., 2015). It is possible that future development of these, or similar compounds, could be used to overcome platinum resistance. Pre-treatment measurement of XPF level using the methods outlined in this thesis could help select patients for treatment with small molecule XPF inhibitors to potentiate the action of oxaliplatin-based chemotherapy.

A number of other compounds that are already in widespread use as anticancer agents have been shown to interact with XPF-ERCC1. The monoclonal antibody cetuximab, an EGFR pathway inhibitor, has been shown to reduce expression of XPF and ERCC1, resulting in an increase in the number of platinum adducts on DNA following oxaliplatin chemotherapy *in vitro* (Prewett et al., 2007). The purine analogue, fludarabine, which is incorporated into DNA at stalled replication forks incised by XPF following oxaliplatin treatment, may represent a potential method of overcoming ICL repair-induced resistance to oxaliplatin (Zecevic et al., 2011). It is therefore possible that pre-treatment testing of XPF

levels could be used to stratify patients for combination treatments with oxaliplatin chemotherapy.

This is the first study to examine the role of MUS81 in tumour response to oxaliplatin-based chemotherapy. It demonstrated both *in vitro* and in clinical samples, that MUS81 determines sensitivity to oxaliplatin-based chemotherapy. As yet, no pharmacological inhibitors of MUS81 have been developed, but it is a potential target for treatment to increase sensitivity of tumours to oxaliplatin-based chemotherapy.

8.3 FUNCTIONAL PATHWAY ANALYSIS OF GENE EXPRESSION IN OESOPHAGEAL ADENOCARCINOMA TREATED WITH OXALIPLATIN-FLUOROURACIL CHEMOTHERAPY

Functional pathway analysis showed that patients who exhibited pathological response had down-regulation of cell cycling pathways and down-regulation of the anti-apoptotic HSP27 pathway in their tumours following oxaliplatin-fluorouracil chemotherapy. Tumours that showed pathological response also had increased expression of a histone deacetylase (HDAC) pathway after treatment. Following treatment, normal oesophageal squamous epithelium exhibited up-regulation of the NF-κB pathway and the IL1R pathway and decreased expression of the EIF4 pathway.

The results from the analysis of gene expression in normal oesophageal mucosa (squamous mucosa biopsied 10 cm proximal to the tumour) raise interesting questions about the interplay between the tumour and the microenvironment. In particular, up-regulation of the NF-κB pathway and IL1R pathways after oxaliplatin-based chemotherapy may promote both the generation and function of cancer associated fibroblasts (CAFs) (Erez et al., 2010; Maleszewska et al., 2013; Nie et al., 2014). It is therefore possible that alterations in gene expression in response to oxaliplatin-based chemotherapy have the effect of promoting development of the tumour microenvironment. To further investigate this relationship, gene expression analysis could be undertaken in post-treatment resection specimens in which microdissection is performed to separate tumour from surrounding stroma. A three way comparison in gene expression between tumour, surrounding stroma and adjacent "normal" mucosa may provide further information on how the interaction between gene expression in these tissue types affects response to oxaliplatin.

The finding that down-regulation of cell cycle genes was associated with pathological response indicated that cell cycle profile may have a role in determining tumour sensitivity to oxaliplatin-based chemotherapy. This hypothesis was supported by the finding that high levels of the G2/M-phase marker Cyclin B1 were associated with worse DFS following treatment with oxaliplatin-fluorouracil chemotherapy.

Interestingly, there was also a correlation between high levels of tumoural Cyclin B1 and high levels of MUS81. This is out of keeping with evidence from the

literature that levels of MUS81-EME1 do not vary significantly during the active phases of the cell cycle, with function of the heterodimer instead being controlled by phosphorylation of EME1 (Gallo-Fernandez et al., 2012; Minocherhomji and Hickson, 2014).

It is possible that the finding that high levels of Cyclin B1 are associated with worse DFS is explained by the different repair processes for interstrand cross-links. ICL repair may occur in both a replication-dependent and replication-independent fashion. Replication-dependent ICL, repair occurs with higher fidelity, due to the presence of the sister chromatid for use as a template. It is therefore conceivable that cells in G2/M-phase are better able to repair oxaliplatin-induced ICLs, thus enabling them to avoid cell cycle arrest and cell death, and ultimately conferring decreased sensitivity to oxaliplatin. To investigate these findings further, experiments could be performed in synchronised cell lines *in vitro* examining alterations in levels of MUS81-EME1 with the cell cycle and studying whether there is any differential sensitivity to oxaliplatin during particular stages of the cell cycle.

8.4 CONCLUSIONS

In conclusion, this thesis aimed to test the hypothesis that high levels of tumour expression of the DNA repair proteins XPF and MUS81 are associated with lack of response to platinum chemotherapy. In support of the hypothesis, microarray analysis of gene expression in samples from patients with oesophageal adenocarcinomas treated with neoadjuvant oxaliplatin-fluorouracil

demonstrated an association between high levels of genes encoding ICL repair proteins (including *ERCC1*, *MUS81* and *EME1*) and poorer clinical outcomes; high levels of XPF and MUS81 proteins in pre-treatment biopsies were correlated with worse outcomes following neoadjuvant oxaliplatin-fluorouracil chemotherapy for oesophageal adenocarcinoma; and it was demonstrated *in vitro* that reducing levels of XPF and MUS81 proteins increased sensitivity of colorectal and oesophageal cancer cells to oxaliplatin treatment. Collectively, these findings suggest that XPF and MUS81 have the potential to be developed as biomarkers for clinical response to oxaliplatin-based chemotherapy for patients with oesophageal cancer.

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APPENDIX 1: PUBLISHED ABSTRACTS ARISING FROM THIS WORK

Association of a DNA damage response deficiency (DDRD) assay and prognosis in early-stage oesophageal adenocarcinoma

RC Turkington, LA Hill, D McManus, S McQuaid, K Arthur, J James, M Salto-Tellez, TS Davison, C Harrison, C Purcell, RH Wilson, TP MacGregor, RA Sharma, RC Fitzgerald, P Johnston, PD Harkin, M McKinlay Eatock, RD Kennedy

J Clin Oncol 32:5s, 2014 (suppl; abstr 4015)

Discovery of prognostic and predictive tissue biomarkers in patients with resectable esophageal cancer

TP MacGregor, RS Gillies, N Sahgal, R Chetty, LM Wang, RC Turkington, ND Maynard, PJ McHugh, RD Kennedy, MR Middleton, RA Sharma

J Clin Oncol 32, 2014 (suppl 3; abstr 45)

DNA repair proteins XPF and MUS81 are predictive biomarkers for neoadjuvant oxaliplatin-fluorouracil chemotherapy in patients with operable oesophageal adenocarcinoma.

TP MacGregor, RS Gillies, N Sahgal, R Carter, R Chetty, LM Wang, ND Maynard, PJ McHugh, MR Middleton, RA Sharma

Br J Surg 2013; 100 (Suppl 8): 56.

Use of DNA repair proteins translated from biomarker screening to predict overall survival in patients with esophageal cancer treated with oxaliplatin chemotherapy

RA Sharma, T MacGregor, R Gillies, S Hatch, L Swift, T Humphrey, R Chetty, PJ McHugh, MR. Middleton

J Clin Oncol 2012; 30 (suppl; abstr e13587)

Metabolic response at repeat PET/CT examination predicts survival and recurrence after neoadjuvant chemotherapy and surgery for oesophageal cancer.

RJ Gillies, TP MacGregor, C Blesing, N Warner, N Maynard, R Marshall, KM Bradley, FV Gleeson, M Middleton

Br J Surg 2011; 98 (Suppl 7): 8.

APPENDIX 2: PAPERS ARISING FROM THIS WORK

SUBMITTED PAPERS

Identification and validation of XPF and MUS81 as predictive biomarkers for oxaliplatin-fluorouracil chemotherapy in patients with esophageal adenocarcinoma

TP MacGregor, RS Gillies, R Carter, JM Findlay, C Kartsonaki, N Sahgal, F Castro, L-M Wang, RJ Chetty, ND Maynard, J-B Cazier, F Buffa, PJ McHugh, I Tomlinson, MR Middleton, RA Sharma

Submitted to Annals of Oncology March 2016

PAPERS ACCEPTED FOR PUBLICATION

Differential clonal evolution in oesophageal cancers in response to neo-adjuvant chemotherapy

JM Findlay, F Castro-Giner, S Makino, E Rayner, C Kartsonaki, W Cross, M Kovac, D Ulahannan, C Palles, RS Gillies, TP MacGregor, D Church, ND Maynard, F Buffa, J-B Cazier, TA Graham, L-M Wang, RA Sharma, MD Middleton, I Tomlinson.

Accepted for publication in Nature Communications 3rd February 2016

PUBLISHED PAPERS

XPF protein levels determine sensitivity of malignant melanoma cells to oxaliplatin chemotherapy: Suitability as a biomarker for patient selection

SB Hatch, LP Swift, S Caporali, R Carter, EJ Hill, TP MacGregor, S D'Atri, MR Middleton, PJ McHugh, RA Sharma

Int J Cancer 2013; 134(6):1495-503.

Pathological grading of regression following neoadjuvant chemoradiation therapy: the clinical need is now.

TP MacGregor, TS Maughan, RS Sharma

J Clin Pathol 2012; 65(10):867-71.