

# **Radiosensitivity in Bladder Cancer Cells**

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Medicine at the University of Leicester

By

**Benedict T. Sherwood**

Department of Cancer Studies  
& Molecular Medicine  
University of Leicester

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# Abstract

Potentially curative treatment options for patients with organ-confined transitional cell carcinoma (TCC) of the bladder (T1-4a/N0/M0) are radical cystectomy or radiotherapy (RT)-based 'bladder-preserving' regimens. A substantial number of patients who receive RT fail to respond (approximately 50%). Consequently, a greater understanding of the mechanisms of radioresistance is required, together with predictive information regarding the response of tumours to RT. Hypoxia and intrinsic cellular radiosensitivity (IRS) are examined here, as factors that may influence the outcome of RT.

An immunohistochemical assay using hypoxia-related carbonic anhydrase IX (CA IX) was undertaken to determine the prognostic significance of hypoxia in bladder tumours treated with RT. A modified version of the alkaline comet assay (ACA) was used to examine differences in IRS between cells derived from TCC specimens. Nuclear factors that influence comet formation (and therefore radiosensitivity) were also examined, such as DNA double strand break (DSB) rates and differences in nuclear matrix protein (NMP) composition.

CA IX immunostaining did not provide prognostic information with respect to response to radical RT. ACA analysis indicated a wide range of responses between tumours. In TCC cell lines, DSB rates are not demonstrably different in cells of differential radiosensitivity, however, comparative analysis of nuclear proteins identified differences in their constituent NMPs and repair enzymes.

These results do not provide evidence that hypoxia influences outcome after RT, but support the contention that IRS is important in dictating the response of bladder tumours to RT. Furthermore, in bladder cancer cell lines of differing radiosensitivity, differences in NMP and repair enzymes are identified. Further work is required to determine whether these are of prognostic importance.



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# List of Abbreviations

ACA	Alkaline Comet Assay
AE	Anion exchanger
BCG	Bacille Calmette-Guerin
BER	Base excision repair
CA	Carbonic anhydrase
DSB	Double strand break
EGFR	Epidermal growth factor receptor
FAR	Fraction of activity released
5-FU	5-fluorouracil
GLUT-1	Glucose transporter-1
HEA	Human epithelial antigen
HIF-1	Hypoxia-inducible factor-1
HR	Homologous recombination
IEF	Isoelectric focusing
IPG	Immobilised pH gradient
IRS	Intrinsic radiosensitivity
LMA	Low melting point agarose
MACS	Magnetic cell sorting
MAPK	Mitogen-associated protein kinase
MAR	Matrix associated region
MMC	Mitomycin C
MMR	Mismatch repair
MRI	Magnetic resonance imaging
MTT	Methyltetrazolium
M-VAC	Methotrexate, vinblastine, adriamycin and cisplatin
NBF	Neutral buffered formalin
NER	Nucleotide excision repair
NHE	Sodium-hydrogen exchanger
NHEJ	Non-homologous end-joining
NMA	Normal melting point agarose
NMP	Nuclear matrix protein
PE	Plating efficiency
PFGE	Pulse-field gel electrophoresis
PI3K	Phosphatidylinositol 3-kinase
Rb	Retinoblastoma protein
RT	Radiotherapy
SCGE	Single cell gel electrophoresis
S-PEK	Subcellular proteome extraction kit
SSB	Single strand break
TCC	Transitional cell carcinoma
TNM	Tumour node metastasis
TURBT	Transurethral resection of bladder tumour
VEGF	Vascular endothelial growth factor

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# **CHAPTER 1: INTRODUCTION**

## 1.1 BLADDER CANCER OVERVIEW

### 1.1.1 General Background

Bladder cancer is a common malignancy, with over 11,000 new cases each year in the UK. It is predominantly a disease of elderly men, with 80% of cases occurring in males over the age of 50 (Male:Female ratio = 3:1). Bladder cancer currently ranks as the 4th commonest cancer for men and the 10th commonest cancer for women (excluding non melanoma skin cancer)\*. Approximately 75-85% of patients present with disease confined to the mucosa (stage Ta, Tis) or submucosa (stage T1). Such disease is termed *non-muscle-invasive*. The remaining 15-25% have *muscle-invasive* disease (stages T2-4), often with lymph node involvement or widespread metastasis [1], which is therefore associated with a much worse prognosis.

The tumour node metastasis (TNM) system of classification is widely applied in the pathological staging of bladder cancer (table 1.1, figure 1.1). In addition, tumours are assigned a pathological grade based on the degree of tumour cell differentiation. The World Health Organisation classification (table 1.2 [2]) is applied worldwide.

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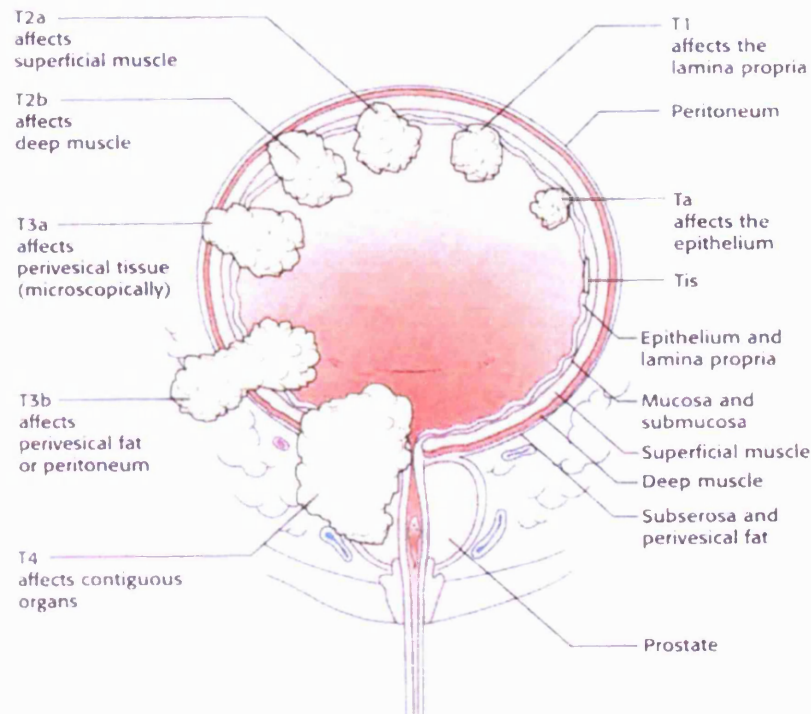
\* Cancer Research UK, Scientific Yearbook 2003/2004 '*Everything points to progress*', chapter 8; pages 8-9, March 2004.

<b>Primary Tumour</b>	<b>Depth of Invasion</b>
Ta	Non-invasive papillary
Tis	<i>In situ</i> : 'flat tumour'
T1	Subepithelial connective tissue
T2	Muscularis propria
T2a	Inner half
T2b	Outer half
T3	Beyond muscularis propria
T3a	Microscopically
T3b	Extravesical mass
T4	Other adjacent structures
T4a	Prostate, uterus, vagina
T4b	Pelvic wall, abdominal wall
<b>Lymph Nodes</b>	<b>Number/Size</b>
N1	Single < 2cm
N2	Single 2-5cm, multiple < 5cm
N3	> 5cm
<b>Distant Metastasis</b>	<b>Presence/Absence</b>
M1	Distant metastasis

**Table 1.1:** The 1997 TNM classification of bladder cancer.

<b>G</b>	<b>Histopathological grading</b>
GX	Differentiation cannot be assessed
G1	Well differentiated
G2	Moderately differentiated
G3	Poorly differentiated

**Table 1.2:** Histological grading of the World Health Organization and International Pathology Consensus Committee, 1988.



**Figure 1.1:** The pathological tumour (T) staging of bladder cancer.

A distinction should be made between non-invasive papillary tumours (Ta-T1) and carcinoma *in situ* of transitional cell epithelium. Macroscopically the latter appears as a flat, red lesion, often with marked cellular atypia, in the absence of invasion. Although non-invasive, molecular biology techniques and clinical experience have demonstrated the highly malignant, invasive potential of such lesions [3, 4].

### **1.1.2 Risk Factors for the Development of TCC**

Transitional cell carcinoma of the bladder (TCC) arises from transitional cell epithelium. There is strong circumstantial evidence that they are caused by environmental agents excreted in high concentration in the urine. TCC is described as a 'field defect', such that the entire urothelium, from kidney to urethra, is at risk of carcinogenesis and multiple tumours are therefore common. Occupational exposure to urothelial carcinogens such as aromatic amines [5] is particularly important, with workers in the analine dye, rubber, textile, printing, iron foundry, aluminium smelting, industrial painting, gas and tar manufacturing industries being at increased risk. Proven bladder carcinogens include  $\beta$ -naphthylamine, 4-aminobiphenyl, 4-nitrobiphenyl and 4,4-diaminobiphenyl. A latent period of 15-20 years from first exposure to carcinogens to tumour diagnosis is not uncommon. The need for accuracy in recording occupational history in patients with bladder TCC is therefore well recognized [6].

Cigarette smoking is the major non-occupational risk factor for the development of bladder TCC. Furthermore, patients with aggressive poorly differentiated disease at presentation are significantly more likely to be smokers than those with well differentiated tumours [7]. Indeed, smoking leads to a higher mortality from bladder cancer during long-term follow up, although in multivariate analysis the prognostic effect is somewhat weaker than other factors such as stage and grade of the tumour [8].

### **1.1.3 Clinical Presentation**

Painless haematuria is the hallmark of bladder cancer. Disease may also present with symptoms of voiding irritability such as urgency, dysuria and increased urinary frequency. Other less common presentations include flank pain resulting from ureteric obstruction and the effects of local invasion beyond the bladder, giving rise to pelvic pain or lower limb oedema.

### **1.1.4 Non-Transitional-Cell Carcinomas of the Bladder**

Non-transitional cell carcinomas of the bladder account for approximately 15% of all bladder neoplasms. They include squamous cell carcinomas, pure adenocarcinomas, mixed tumours (transitional cell and adenocarcinomas), undifferentiated carcinomas and spindle cell carcinomas. Squamous cell carcinomas are derived from metaplastic epithelium, commonly associated with chronic irritation from a calculus. In some geographical areas an important cause of squamous metaplasia and subsequent carcinoma is schistosomiasis. Adenocarcinomas of the bladder are uncommon and are most frequently seen in the dome of the bladder, where they are believed to develop from persistent glandular tissue in urachal remnants. Indeed, rarely, these tumours can spread along a remnant track to the umbilicus. For ease of description in this thesis, 'bladder cancer' pertains to transitional cell carcinoma. Non-transitional-cell carcinomas are not discussed further.

### **1.1.5 Non-muscle-invasive Bladder Cancer**

Non-muscle-invasive (superficial) disease is managed primarily with transurethral resection of the bladder tumour (TURBT). Further treatment is administered on the basis of risk of recurrence and/or progression. The risk of recurrence of superficial bladder cancer is well documented [4, 9], even in adequately treated cases. The risk of progression to muscle-invasion is generally low, but increases dramatically to approximately 50% in high risk T1 G3 disease [3, 4]. Additional therapy may take the form of intravesical instillations of chemotherapeutic agents such as epirubicin or mitomycin C (given over a 6 week course, followed by monthly instillations for tumours with a high risk of recurrence).

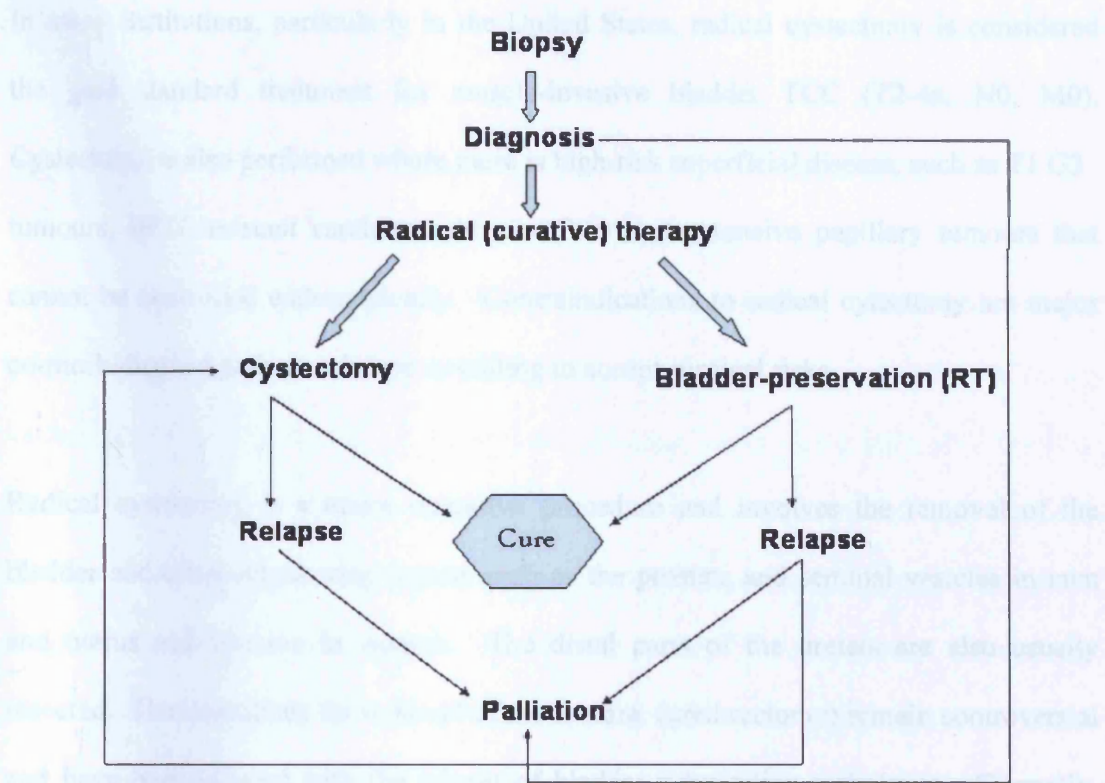
## **1.2 MANAGEMENT OF MUSCLE-INVASIVE BLADDER CANCER**

### **1.2.1 Overview**

The management of muscle-invasive TCC is represented schematically in figure 1.2. Patients are assessed initially with cystoscopy and TURBT or biopsy. Many patients will only be suitable for palliative treatment at the time of diagnosis. Those patients with organ confined (T2-4a/N0/M0) disease may be considered for treatment with curative intent, in the form of radical surgery (cystectomy), or 'bladder preserving' therapy based on radiotherapy (RT).



### 1.3.2 Surgical Management of Muscle-Invasive Bladder Cancer



**Figure 1.2:** The management of muscle-invasive bladder cancer. At presentation, many patients are suitable only for palliative treatment. Patients with organ confined (T2-4a/N0/M0) disease may be considered for treatment with curative intent, in the form of radical surgery (cystectomy), or *bladder-preserving* therapy centered on RT. The overall response rate to radical therapy (surgery or RT) is approximately 50%.

### 1.2.2 Surgical Management of Muscle-invasive Bladder Cancer

In many institutions, particularly in the United States, radical cystectomy is considered the gold standard treatment for muscle-invasive bladder TCC (T2-4a, N0, M0). Cystectomy is also performed where there is high-risk superficial disease, such as T1 G3 tumours, BCG-resistant carcinoma *in situ* (Tis) and extensive papillary tumours that cannot be controlled endoscopically. Contraindications to radical cystectomy are major co-morbidity and patients who are unwilling to accept surgical risks.

Radical cystectomy is a major operative procedure and involves the removal of the bladder and often neighboring organs such as the prostate and seminal vesicles in men and uterus and adnexae in women. The distal parts of the ureters are also usually resected. The indications for removal of the urethra, (urethrectomy) remain controversial and have been reduced with the advent of bladder substitution techniques. Currently, urethrectomy is indicated if the tumour involves the bladder neck in women and the prostatic urethra in men [10]. Extended lymph node dissection is now widely advocated [11].

Unsurprisingly, the operative mortality of radical cystectomy is significant, although the risk has been reduced in the last few decades, to 3.7% in the European Organisation for Research and Treatment of Cancer (EORTC) Medical Research Council (MRC) Trial. In cases where an abdominal wall stoma is created, quality of life may be adversely

affected. In addition, erectile dysfunction occurs almost universally. However, bladder substitution techniques and nerve-sparing cystectomies are now performed increasingly.

### **1.2.3 The Role of RT in the Management of Muscle-invasive Bladder Cancer**

Many patients with bladder cancer would rather keep their bladder. Indeed, preservation of a functional organ without compromising survival is the optimal endpoint in any cancer therapy. The use of RT in invasive bladder cancer dates back to the 1900s. However, as an alternative to cystectomy, it remains a controversial topic amongst urological oncologists. In a recent meta-analysis [12], randomized trials comparing surgery with RT for muscle-invasive bladder cancer were assessed collectively. Although an overall survival benefit for radical surgery was demonstrated, only three trials were included, patient numbers were small and many patients did not receive the treatment to which they were randomised. Furthermore, substantial improvements in RT techniques have occurred since these trials were undertaken.

Radiation treatment as monotherapy has been deemed to be less effective than radical cystectomy because of the apparently poorer survival shown in some published non-randomized clinical trials, where survival rates after RT are 60-80%, 26-59% and 20-38% for T1, T2 and T3 tumours respectively. Patients with T4 tumours fixed to the pelvis rarely survive for five years without tumour progression [13-17]. However, confounding selection bias hinders comparison with outcome in contemporary cystectomy series,

where survival rates are 75-83% for T1 tumours, 63-89% for T2 tumours, 31-62% for T3 tumours and 21-50% for T4 tumours [18, 19]. Patients selected for surgery tend to be younger and fitter than those having RT and consequently, do better than the older, less fit patient sub-population undergoing RT. In addition, cystectomy allows the definitive pathological staging of disease, unlike clinical staging prior to RT. This may result in an increase in the under-staging of some RT patients [20] and further limits comparison of the two treatment modalities.

Since RT has not yet been compared with cystectomy in modern randomized trials of significant statistical power, the decision whether or not to treat a patient with RT is often based on prognostic factors and the patients' wishes, but may be influenced inevitably by the physicians' preference. Advocates of radical cystectomy may regard the extent and duration of RT treatment needed as excessive, or feel concerned that the opportunity for a curative cystectomy may be missed after a failed course of treatment. Cystectomy and continent diversions may certainly be more difficult after RT [21].

Suitable candidates for RT should have adequate bladder capacity, normal bladder function, no recurrent urinary tract infections, no previous inflammation or surgery of the true pelvis with resultant adhesion [22]. Clinical prognostic factors may impact on patient management decisions. Favourable prognostic factors for RT are low stage, a solitary tumour, lack of obstructive uropathy and clinically complete resection of the primary tumour. Other parameters that might positively influence outcome are normal serum haemoglobin, low tumour volume (<5 cm diameter) and lack of concomitant

carcinoma *in situ* [13, 14, 23]. RT is delivered in 30-40 fractions in doses up to 68 Gy. Multiple fractions per day may result in a higher rate of local control, but these strategies are still under evaluation [24].

Brachytherapy is an alternative approach used in a few European centres, for selected patients with small solitary tumours of less than 5 cm diameter. The lesions are exposed by cystotomy, with or without partial resection, and loaded with iridium, tantalum or caesium. This technique provides similar results to external beam RT and indeed may result in higher local control rates for T2-T3 tumours [25].

Many patients undergoing radical RT of pelvic viscera will experience low grade enteritis, proctitis or cystitis that is easily controllable and self-limiting. Late toxic effects of significance (Radiotherapy-Oncology Group 3-5), in the form of radiation cystitis (5%), proctitis (5%) and bowel obstruction are less common in modern series [13, 16, 23]. Erectile dysfunction will occur in over two thirds of male patients [26]. Sexual function is unaffected in females [27].

In summary, RT is administered with curative intent to patients with T2-3/N0/M0 TCC of the bladder. Patients with solitary, completely resected T2 tumours with normal upper tracts have the highest chance of cure and represent the best candidates for treatment using this modality. As is the case after cystectomy, patients require lifelong follow-up with cystoscopy, exfoliative urinary cytology and other investigations to detect disease dissemination.

#### **1.2.4 Concurrent Chemotherapy and RT**

To enhance the therapeutic ratio of RT, the potential radiosensitising effect of concurrent chemotherapy has been investigated. By delivering RT and chemotherapy simultaneously, the opportunity for cross-resistant tumour cells to become established is minimised. In addition, the delivery of potentially curative radiation therapy is not delayed.

Cisplatin is one of the agents most closely investigated with respect to its interactions with ionising radiation. Like several other drugs, it has been shown to inhibit the repair of radiation damage. Specifically, cisplatin exerts its cytotoxic effects by chelating guanine residues, yielding mono-functional adducts and intra-strand or inter-strand cross-links. If a cisplatin adduct and a radiation-induced single strand break (SSB) arise simultaneously within close proximity, the result is mutual inhibition of effective repair [28]. The presence of cisplatin therefore causes an increase in the number of radiation-induced DNA strand breaks. DNA double strand breaks (DSBs, see later) are the lesions most closely associated with radiation induced tumour cell killing. Inhibition of DSB repair or conversion of SSBs to DSBs has the effect of steepening the radiation survival curve, leading to an enhanced response.

The first phase II study of concurrent cisplatin and RT in bladder cancer was reported in 1982 [29] and during the last ten years particularly, a growing number of series have

evaluated the use of concomitant chemotherapy after transurethral resection in the treatment of muscle-invasive bladder cancer at phase II level (table 1.3).

Investigators	Regimen	n	Local Response Rate	Bladder Preservation	Survival
Arias <i>et al</i> 2000[30]	TUR, M-VAC +RT with concomitant Cisplatin	50	47% (5 years)		48% 5 year survival
Danesi <i>et al</i> [31] 1997	TUR, C + 5FU	25	87.5%	71% alive with native bladder	75% actuarial 4-year survival
Rodel <i>et al.</i> 2002[32, 33]	RT +/- C/Carboplatin	289	72%	80% of ten year survivors	45% disease-specific survival
Einstein <i>et al</i> 1996Einstein, Wolf <i>et al.</i> 1996;	TUR, MCV RT	27	55.9%		18% 3 year survival
Fellin <i>et al</i> 1997[34]	TUR, CMV, RT	56	50%	41%	59% disease-specific survival
Hussain <i>et al</i> 2001[35]	MMC, 5FU, RT	31	74%		65% overall 1-year survival
Housset <i>et al</i> 1993[36]	TUR, C + 5FU, RT	54	74%		59% overall 3-year survival
Kaufman <i>et al</i> 1993[37]	TUR, C + 5FU, RT	42	66%	58% at 4 year median	48% actuarial 5-year survival
Kachnic <i>et al</i> 1997[27]	TUR, MCV, RT	106	70%	43% at 5 years	60% 5-year disease specific survival
Orsatti <i>et al</i> 1995[38]	TUR, C + 5FU, RT	76	81%	47% at 4 years	42% actuarial 6 year survival
Shipley <i>et al</i> 2002[39]	TUR, C, RT	190		73%	63% 5-year disease-specific survival 59% 10-year disease-specific survival
Tester <i>et al</i> 1996[40]	TUR, MCV, RT	91	80%	44% 4-year survival with intact bladder	62% 4-year survival
Vikram <i>et al</i> 1994[41]	TUR, MVC, Deoxyrubicin RT	21	89%	84% at median 2 years	60% 3-year survival

**Table 1.3:** selected phase I-II trials of concomitant chemoradiotherapy for bladder cancer, showing the regimen applied, patient numbers, local response and bladder preservation rates and survival (5-FU = 5-fluorouracil; MMC = mitomycin C; M-VAC = methotrexate, vinblastine, adriamycin and cisplatin; TUR = transurethral resection).

A number of published series have shown that complete clinical response, as determined by check cystoscopy, is achievable in 70-80% of patients receiving concomitant chemoradiotherapy, with acceptable toxicity. Overall, survival at 5 years is

approximately 50%, and compares favourably with radical cystectomy for patients with stage T2-3a disease. 5-year survival with an intact bladder is of the order of 40-45%.

### **1.2.5 Neoadjuvant Chemotherapy followed by Radical RT**

Treatment failure in bladder cancer occurs most often from the growth of occult metastases which seeded prior to local therapy, suggesting that if effective adjuvant chemotherapy is given before local treatment, loco-regional relapse and distant metastatic spread may be reduced. Initial cycles of chemotherapy target occult metastasis and may debulk the primary tumour, thereby rendering the reduced number of cells more susceptible to cell death by ionizing radiation. Such a process may also enhance the radiosensitivity of the residual tumour by making it less hypoxic (see 1.4.2.2). It is also suggested that sequential treatment may be less toxic than when given concurrently. Although this approach makes intuitive sense, the results of such strategies are variable in clinical practice. Indeed, in some solid tumours, it has been suggested that initial chemotherapy causes accelerated repopulation of resistant clones, compromising the ability of radiation therapy to achieve control [42] by yielding the so-called 'leaner, meaner' tumours.

In bladder cancer, the Advanced Bladder Cancer (ABC) meta-analysis provided the most comprehensive assessment of neo-adjuvant chemotherapy in this disease to date [43]. The analysis included data from 2,688 patients from ten randomized trials of neo-



adjuvant regimens. Platinum-based combination chemotherapy showed a significant beneficial effect on overall survival, with a 5% improvement in survival at 5 years (from 45 to 50%). This effect was observed when definitive local therapy involved either RT or radical cystectomy. The meta-analysis was insufficiently powered to allow valid evaluation of individual regimens, or to reliably determine the effect of single agent cisplatin on survival. Unfortunately, the implications of this meta-analysis for clinical practice are limited by the fact that few of the included trials allowed meaningful conclusions regarding toxicity and quality of life to be drawn, both of which are of paramount importance when discussing primary treatment options.

#### **1.2.6 Salvage Cystectomy**

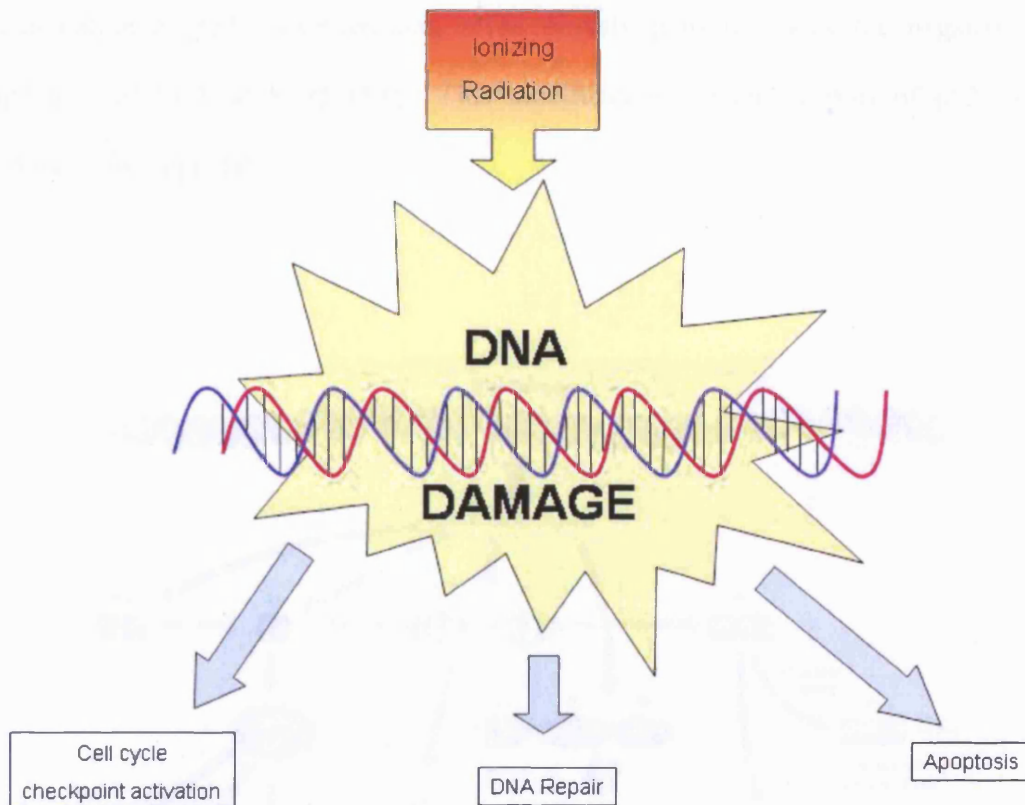
A number of patients who fail bladder preservation programmes are suitable for salvage cystectomy. The proportion of patients entered for bladder preservation that subsequently requires salvage surgery varies considerably between published series, from 24-45% [44]. The long-term outcome in this subgroup is favourable in only 12-40% [16]. The morbidity and mortality rate associated with salvage cystectomy is considerably higher compared with that of primary cystectomy [45]. This may well reflect the technical difficulties arising from operating on previously irradiated tissue, in which healing is impaired. However, a further factor may be the higher co-morbidity in this subgroup, compared with those selected for primary surgery. Nevertheless, these data emphasize the importance of appropriate patient selection for bladder preservation.

### **1.3 CELLULAR RESPONSE TO RADIATION-INDUCED DNA DAMAGE**

Tumour clearance by RT relies on the cytotoxic effect of radiation on tumour cells. DNA is the primary target for the lethal effects of ionizing radiation. DNA-induced lesions include DSBs, SSBs and DNA base damage. Eukaryotic cells exhibit a multifaceted response to the DNA damage induced, involving cell cycle checkpoint activation, DNA repair and apoptosis [46] (figure 1.2).

#### **1.3.1 Checkpoint Activation**

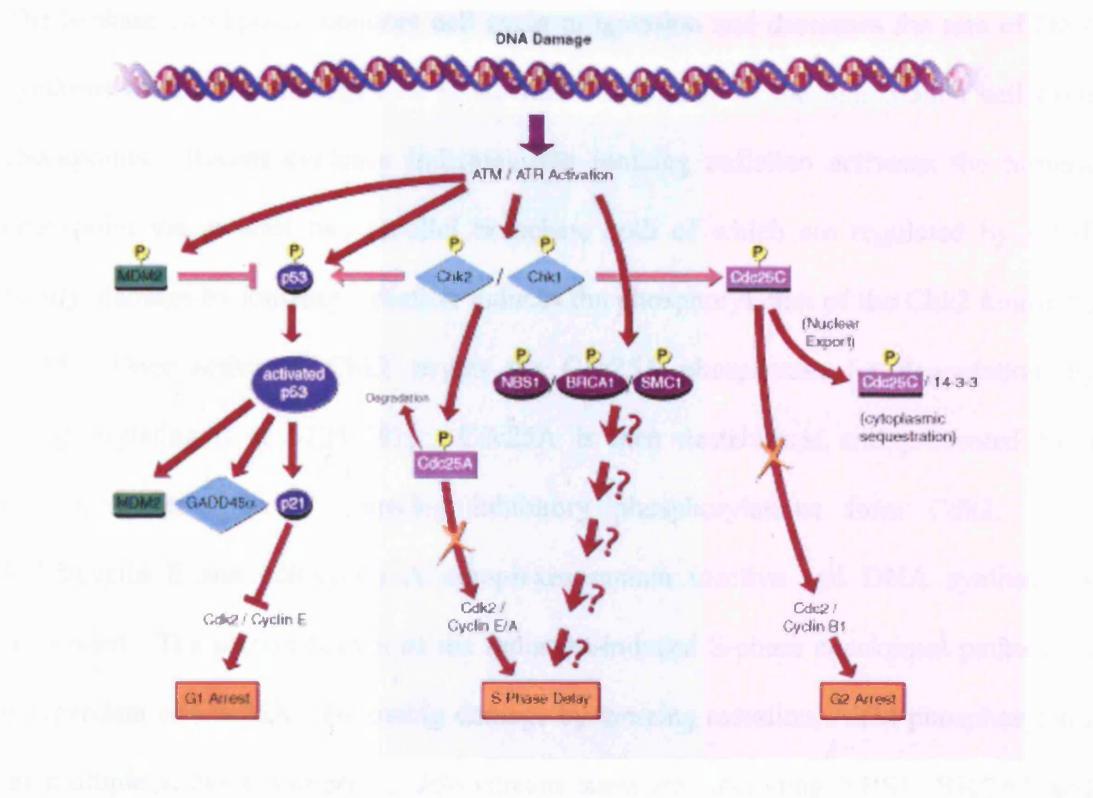
Cell cycle check points govern the speed and order of cell cycle transitions (figure 1.3). In the context of DNA damage or stalls in replication, cell cycle check points arrest cell cycle progression, allowing time for damage repair before the cell replicates. The key regulators of checkpoint pathways in mammalian cells are the ATM (ataxia telangiectasia, mutated) and ATR (ATM and Rad3-related) protein kinases. Although these regulators phosphorylate many of the same substrates [47], they each generally respond to different types of DNA damage. DSBs induced by ionizing radiation are primarily dealt with by an ATM-mediated response. In contrast, ATR plays only a ‘back-up’ role in DSB damage, but is the principal mediator of UV damage response and stalls in DNA replication.



**Figure 1.2:** DNA damage response to ionizing radiation. Eukaryotic cells respond with a combination of cell cycle checkpoint activation, DNA repair mechanisms, or if damage is irreparable, apoptosis.

The G1 checkpoint is the most understood of the cell cycle checkpoints. The accumulation and activation of the p53 protein is pivotal. This in turn is controlled by ATM (in the case of ionizing radiation). Under normal circumstances, p53 levels are low due to interaction with MDM-2, which targets p53 for exportation from the nucleus and degradation by cytoplasmic proteosomes [48]. After ionizing radiation, ATM activates downstream kinase Chk2, by phosphorylation at position T68 [49]. This in turn phosphorylates residue S20 of p53, which blocks p53-MDM-2 interaction, resulting in an

accumulation of p53. Furthermore, ATM directly phosphorylates the negative p53 regulator, MDM-2, at S395 [50]. This modification prevents export of p53 to the cytoplasm for degradation.



**Figure 1.3:** Cell Cycle Checkpoint Pathways. In response to DNA damage, ATM and/or ATR trigger the activation of a checkpoint that leads to cell cycle arrest or delay. Checkpoint pathways are characterized by cascades of protein phosphorylation events (indicated with a 'P') that alter the activity, stability, or localization of the modified proteins. Left, center, and right panels represent the G1, S, and G2 checkpoint pathways respectively (from *R & D Systems, Cytokine Mini-reviews*, 2004).

P53-dependent regulation of the cell cycle proceeds via regulation of expression of *p21*. The p21 protein binds to and inhibits the cyclin/cdk protein kinase complexes. This in turn inhibits cell cycle progression by preventing phosphorylation of the retinoblastoma protein (Rb), which is required for the passage of the cell from G1 to S-phase.

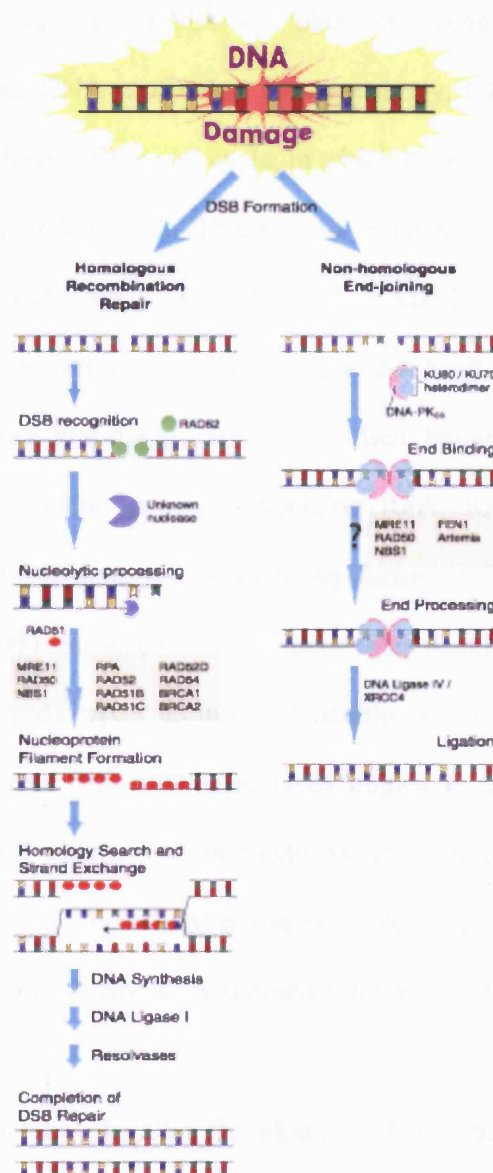
The S-phase checkpoint monitors cell cycle progression and decreases the rate of DNA synthesis after DNA damage and is the least understood of the mammalian cell cycle checkpoints. Recent evidence indicates that ionizing radiation activates the S-phase checkpoint via at least two parallel branches, both of which are regulated by ATM. Firstly, damage by ionizing radiation induces the phosphorylation of the Chk2 kinase by ATM. Once activated, Chk2 targets the Cdc25A phosphatase for degradation, by phosphorylating it at S123 [51]. Cdc25A is then destabilized and prevented from performing its role of removing inhibitory phosphorylations from Cdk2. The Cdk2/cyclin E and cdk/cyclin A complexes remain inactive and DNA synthesis is suspended. The second branch of the radiation-induced S-phase checkpoint pathway is independent of Cdc25A. Following damage by ionizing radiation, ATM phosphorylates (at multiple sites) a number of downstream substrates including NBS1, BRCA1 and SMC1. The precise roles of these proteins in leading to reduced DNA synthesis remains incompletely understood.

The G2 Checkpoint allows cell cycle arrest prior to chromosome segregation. Progression to mitosis is controlled by cyclin-dependent kinase Cdc2 [52]. Maintenance of inhibitory phosphorylations on Cdc2 is essential for G2 checkpoint activation.

Following radiation-induced damage, it is primarily ATR that mediates checkpoint activity by indirectly modulating the phosphorylation status of these sites [53], with ATM playing a ‘back up’ role. Following DNA damage, the downstream kinases Chk1 and Chk2 (activated by ATR and ATM-dependent phosphorylation respectively) are both able to phosphorylate Cdc25C on position S216 [54]. This creates a binding site for 14-3-3 proteins. The 14-3-3/Cdc25C protein complexes are sequestered in the cytoplasm and therefore Cdc25C is prevented from activating Cdc2 by removing the inhibitory phosphorylations. Entry into mitosis is therefore prevented.

### **1.3.2 Repair of Radiation-induced DNA Damage**

DSBs are the radiation-induced lesion directly associated with cell death. They pose problems for transcription, replication and chromosome segregation. In addition to ionizing radiation, other sources may generate DSBs. These include genotoxic chemicals, endogenously liberated reactive oxygen species, replication of SSBs and mechanical stress on the chromosomes. Since DSBs affect opposing strands of the DNA duplex they prevent the use of the complementary strand as a template for repair by alternative strategies. To counteract the detrimental effects of these lesions, two repair mechanisms have evolved [55]. These are termed homologous recombination (HR) and non-homologous end joining (NHEJ) (figure 1.4). The position of the cell in the cell cycle at the time of damage formation appears to be critical in dictating which mechanism is used [56].



**Figure 1.4:** Schematic representation of the repair pathways (homologous recombination (HR) and non-homologous end joining (NHEJ)) that correct radiation-induced DNA DSBs (from *R & D Systems, Cytokine Mini-reviews*, 2004).

HR repair accurately corrects DSBs by obtaining genetic information from a homologous, undamaged DNA molecule. Unsurprisingly, HR occurs most frequently during late S- and G2-phases of the cell cycle, in which an undamaged sister chromatid is available to act as a repair template. This process is mediated by the RAD52 epistasis group of proteins which include RAD50, RAD51, RAD52, RAD54 and MRE11 [57]. The DSB site is initially recognized by RAD52. The damaged ends are then processed to produce 3' single strand overhangs, which are then bound by RAD51 to form a nucleoprotein filament. Other proteins including RPA, RAD52, RAD54, BRCA1, BRCA2 and other related proteins act as accessory factors in filament construction [55]. At this stage, the RAD51 nucleoprotein filament then searches the undamaged sister chromatid for a homologous repair template. Once the relevant DNA has been located, the damaged DNA invades the undamaged DNA duplex in a process known as DNA strand exchange. A DNA polymerase then extends the 3' end of the invading strand and subsequent ligation by DNA Ligase I gives rise to a heteroduplexed DNA structure. This intermediate recombination is resolved and precise correction of the DSB is complete.

Conversely, NHEJ can take place in the absence of a homologous repair template. However this means that the process is more susceptible to errors. The Ku70/Ku80 heterodimeric protein is pivotal to this process [58]. The Ku heterodimer initiates NHEJ by binding to the broken DNA ends and recruiting other factors such as DNA-dependent protein kinase (DNA-PK), XRCC4 and DNA Ligase IV to the site of damage [55]. DNA-PK becomes activated upon DNA binding, and phosphorylates a number of substrates including p53, Ku and the DNA Ligase cofactor, XRCC4. This



phosphorylation is thought to further facilitate the repair process. Where the ends of DSBs are unsuitable for direct rejoining, they are subject to further processing by nucleases and or polymerases before NHEJ proceeds. The final step in the process involves ligation of the DNA ends by Ligase IV, in a complex that includes XRCC4 and Ku.

Base excision repair (BER) targets base damage arising from oxidation, methylation, or deamination [59] and elements of BER contribute to SSB repair. BER can proceed by two distinct pathways. The first, 'short-patch' repair pathway accounts for 80-90% of BER and begins when DNA glycosylase cleaves the N-glycosidic bond between the damaged base and the sugar phosphate backbone of the DNA, generating an apyrimidinic/apurinic (AP) or abasic site. The AP site is then processed by AP Endonuclease 1 (APE1) which cleaves the backbone immediately 5' to the AP site, resulting in a 3' hydroxyl group and a transient 5' abasic deoxyribose phosphate (dRP). Removal of the dRP is accomplished by the action of DNA polymerase  $\beta$ , which adds one nucleotide to the 3' end of the break and removes the dRP moiety [60]. The strand break is ultimately sealed by a DNA ligase, thereby restoring the integrity of the DNA.

The 'long-patch' version of BER involves many of the same factors involved in short patch repair, but is a PCNA-dependent pathway, in which the DNA polymerase adds several nucleotides to the repair defect, displacing the dRP as an oligonucleotide 'flap'. This is excised by flap endonuclease (FEN-1) prior to sealing of the break by DNA ligase.

Nucleotide excision repair (NER) acts upon a range of DNA lesions. The most significant of these are pyrimidine dimers caused by the ultraviolet component of sunlight. Other substrates include bulky chemical adducts, DNA intrastrand crosslinks, and some oxidative damage.

A further pathway, DNA mismatch repair (MMR), is involved in the correction of replication errors, for example base-base mismatches and insertion-deletion loops. In keeping with the other repair pathways described above, MMR involves the recognition of the lesion, patch excision and correction of the defect by DNA repair synthesis and re-ligation. In mammalian cells, MMR is initiated by recognition of the lesion by the MSH2-MSH6 protein heterodimer. Excision and subsequent resynthesis of the affected strand is performed by a number of factors including PCNA, RPA, RFC, exonuclease I, DNA polymerases  $\delta$  and  $\epsilon$ , and FEN-1 [61].

### **1.3.3 Apoptosis**

In addition to its role in causing G1 arrest, p53 acts by directing irreversibly damaged cells to apoptosis (Figure 1.3). During apoptosis, mitochondrial membrane disruption is followed by release of cytochrome C into the cytosol, which in turn leads to activation of caspase cleavage. Antiapoptotic *Bcl-2* genes and proapoptotic *Bax* genes regulate release

of cytochrome C. *Bax* has been shown to contain p53-binding sites in its promoter site and is upregulated in response to DNA damage and increased p53 [62]. P53-mediated cell death can be inhibited by Bcl-2 and Bcl-x. In addition, reactive oxygen species (ROS) are powerful activators of mitochondrial damage and apoptosis. A number of genes that increase production of ROS, and therefore increase oxidative stress, have been found to be induced by p53 [63-65]. Finally, activation and trimerization of Fas (CD95) leads to caspase activation. Over expression of Fas can cause apoptosis and is thought to be p53 dependent, although the mechanism is not yet clear [65].

## **1.4 PREDICTING THE RESPONSE OF BLADDER TUMOURS TO RT**

### **1.4.1 Overview**

Despite the limited response of bladder tumours to radical RT (approximately 50%), it is clear that in some cases, local control and sustained disease remission are achieved, with acceptable toxicity and long term quality of life. Conversely, where tumours are resistant to RT, the decision to treat a patient with RT may in theory be detrimental, as the time elapsing before recognition of treatment failure could allow metastatic spread before salvage surgery is undertaken.

Furthermore, these patients have been exposed to the risks associated with pelvic irradiation, without clinical benefit. Salvage surgery is feasible in only a limited number

of cases and carries a greater risk than primary cystectomy. There is, therefore, a well recognized need for ‘tests’ that provide predictive information regarding bladder tumour radiosensitivity. This would allow the clinician to make informed decisions regarding the most appropriate treatment (i.e. surgery or RT) for individuals; enhancing patient selection for bladder preservation and reducing the need for salvage cystectomy.

These ‘tests’ should be distinguished conceptually from the empirically derived clinico-pathological prognostic factors that are described above. A predictive test of radiosensitivity has long been considered a ‘holy grail’ amongst radiobiologists and cancer therapists alike. A viable predictive assay must prove itself as being sufficiently sensitive, specific, reproducible and practical to be of use. As yet, such a technique is not available to the clinician. Before discussing the relative merits of laboratory techniques in this role, it is important to consider the mechanisms that contribute to radioresistance in bladder tumours.

## **1.4.2 Mechanisms of Radioresistance**

### **1.4.2.1 The Five ‘Rs’ of Radiobiology**

Historically, the mechanisms underlying tumour radiosensitivity were referred to as the four ‘Rs’ of radiobiology; *Repair* (the ability of irradiated cells to repair radiation-induced damage), *Reoxygenation* (hypoxic cells being more radioresistant), *Repopulation* (the rate

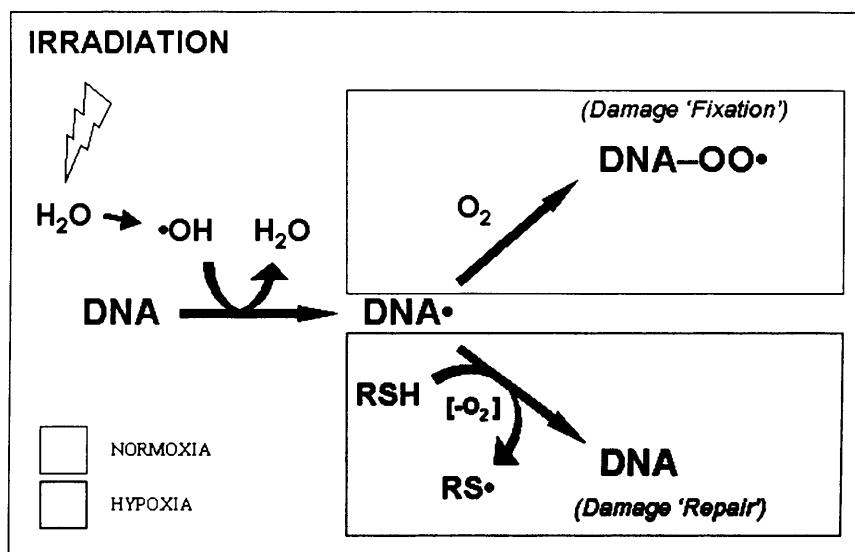
at which tumour cells repopulate after irradiation) and *Reassortment* (the observation that cells vary in sensitivity as they progress through the cell cycle). Latterly, a fifth was added; *Radioresistance*, which acknowledges the observation that tumour cells are intrinsically different in their susceptibility to death from the effects of ionizing radiation.

#### **1.4.2.2 Hypoxia**

The radioprotective effect of hypoxia has long been implicated as a cause of radioresistance; indeed, hypoxic cells *in vitro* are up to three times more resistant to the lethal effects of ionising radiation than well-oxygenated cells [66]. This has led to a substantial number of treatment strategies aimed at countering the presence of hypoxic but viable cells *in vivo*.

Hypoxia in tumours can occur by two distinct mechanisms. Acute hypoxia is the result of a transient reduction in flow through a tumour blood vessel. Conversely, chronic hypoxia results from the limited diffusion distance of oxygen through tumour tissue. In aggressive lesions this is compounded by the fact that tumour cells grow faster than the endothelial cells that make up the blood vessels and because the newly formed vascular supply is disorganised [67]. This results in areas of acidosis and nutrient deprivation as well as regions of hypoxia.

The radiosensitising effect of oxygen arises from the fact that it is required for the 'fixation' of radiogenic DNA damage; an irreversible radiation-induced change in the chemical composition of the DNA (figure 1.5). Ionising radiation generates hydroxyl free radicals that lead to the formation of DNA-centred radicals. In the presence of oxygen, this damage becomes fixed via the formation of peroxy radicals (DNA-OO•). However, under hypoxic conditions, endogenous thiols (RSH) are able to repair this damage (by donating hydrogen), thereby restoring the structure of the DNA and leading to radioresistance. A reduction in the available oxygen level at the time of irradiation therefore increases radioresistance in the target cells.



**Figure 1.5:** The pivotal role of oxygen in the fixation of radiation induced DNA damage. Ionising radiation generates hydroxyl free radicals ( $\bullet OH$ ) that lead to the formation of DNA-centred radicals ( $DNA\bullet$ ). In the presence of oxygen, this damage becomes fixed. However, under hypoxic conditions, endogenous thiols (RSH) are able to chemically repair this damage (by donating hydrogen), effectively contributing to radioresistance.

In addition, it is now clear that hypoxia triggers mechanisms which may give tumours additional survival advantages. The transcriptional complex hypoxia-inducible factor-1 (HIF-1) is recognised as a key mediator of gene expression in hypoxic tumours, although the range of target genes has yet to be completely defined. HIF-1 comprises two subunits from the basic helix-loop-helix PAS domain family, HIF-1 $\alpha$  and HIF-1 $\beta$ /ARNT. The HIF-1 $\alpha$  subunit is thought to be the specific regulator of the hypoxia response [68].

Angiogenesis is essential to meet the requirements of a rapidly proliferating tumour [69]. The vascular endothelial growth factor (VEGF) gene is under the control of HIF-1 $\alpha$  [70]. Activation of angiogenic pathways confers a cancer cell proliferation advantage and triggers regeneration of tumour vasculature during fractionated RT. VEGF expression is elevated and is of prognostic importance in bladder carcinoma and also breast, gastric and other malignancies [71]. Hypoxia has also been shown to cause HIF-1 dependent expression of the tumour associated carbonic anhydrase genes, CA9 and CA12 [72], indicating a further biochemical pathway regulated by this mechanism. Carbonic anhydrases are important for regulation of pH, therefore expression of extracellular carbonic anhydrases is likely to affect microenvironmental pH. There is evidence that acidic pH may promote invasion in other human tumours [73]. CA9 and VEGF expression are strongest on the luminal surface of bladder tumours and adjacent areas of necrosis [72]. The role of intratumoural hypoxia as a cause of radioresistance and tumour progression is discussed further in chapter 3.

#### 1.4.2.3 Repopulation and Reassortment

Tumour control by RT requires sterilization of all clonogenic cells that are present at any time up to the end of treatment. Therefore, repopulation of cells that survive the early part of a course of treatment may result in failure to achieve local control. In some tumours, after several days the rate of cell division exceeds that prior to irradiation, such that over time, tumour cell numbers are more rapidly repopulated [74]. This concept is known as accelerated repopulation and theoretically could confer greater radioresistance. In bladder cancer cell lines, tumour clonogen repopulation accelerates after a lag period of approximately 5-6 weeks [75]. Ki67 immunostaining, bromodeoxyuridine and flow cytometry have all been used to demonstrate high proliferative rates in large subsets of muscle-invasive bladder tumours [76].

If rapid proliferation of tumour cells is a common cause of RT failure in muscle-invasive bladder cancer, maintaining the dose whilst reducing the overall treatment time (within the limits of toxicity) might be a reasonable strategy to reduce the local relapse rate. The relationship between treatment duration and outcome has been intensively studied in head and neck malignancies, with randomized prospective trials suggesting a therapeutic gain when accelerated radiation regimes are employed [77]. However, there is little evidence to support this approach in muscle-invasive bladder cancer. In a retrospective analysis of 77 patients, Maciejewski *et al.* [75] found evidence for a large decrease in bladder control rate when treatment duration was prolonged, while De Neve *et al.* [78], in a study of 147 patients, could not establish an influence of treatment time on outcome after continuous



courses of radiation. In the largest series (379 patients) to examine the influence of overall treatment time, [79] no effect on response rates could be identified when comparing continuous treatment, treatment interruptions or alterations in treatment duration, implying that tumour cell repopulation was not a significant determinant of treatment outcome.

The radiosensitivity of tumour cells depends on their position in the cell cycle. Cells are most sensitive in G2 and mitosis, slightly less so in G1 and become progressively more resistant through S phase. After a single dose of ionising radiation, more resistant S phase cells will survive as the majority of the residual tumour cell population. The result achieves increased synchronisation of surviving cells with respect to cell cycle position. Although this population of cells will subsequently move into G2 phase and become more sensitive, this reassortment has not been exploited for therapeutic gain.

#### **1.4.2.4 Repair and Intrinsic Cellular Radiosensitivity**

Intuitively, tumours whose clonogenic cells are more inherently radioresistant should be more difficult to treat with RT. Indeed, differences in the intrinsic radiosensitivity of human tumour cells (IRS) are now acknowledged, and these differences relate to clinical radiocurability [80, 81]. One mechanism proposed to explain variation in IRS is that the amount and distribution of damage induced in critical targets may vary between tumour cells of different radiosensitivity. The view that initial DNA damage formation is a major

determinant of cell IRS is supported by previous work with human bladder and breast cancer cell lines [82].

Cells may also vary in their ability to repair the damage induced by radiation. Following damage induction, numerous processes remove and repair the damage in an attempt to restore the genetic sequence (see section 1.3.2). DNA damage may be correctly repaired, incorrectly repaired or completely unrepaired. The DNA DSB rejoining process follows biphasic kinetics with a rapid initial rate followed by a much slower second component [83]. The speed of repair of DNA DSBs is a critical factor underlying survival in irradiated human tumour cell lines [84] and the importance of DNA repair enzymes in recovery from radiation-induced DNA damage is well known [84, 85]. Radiosensitive rodent cell lines have been identified that are defective in the rejoining of DSBs [86, 87]. Repair of DNA damage requires not only functional repair enzymes, but also adequate access to damaged sites. Deficiencies in DNA repair have been linked with alterations in the spatial organisation of DNA [88-90]. Variations in the conformation and organisation of chromatin between cell lines could, therefore, affect both the extent of DNA damage formation and repair of these lesions. Using a restriction endonuclease as a model for radiation-induced DNA DSBs, repair fidelity has been shown to correlate closely with radiosensitivity. It has been suggested that repair fidelity can be a measure of correct repair relative to misrepair, resulting from the processing of DSBs [91].

#### **1.4.2.5 Tumour Heterogeneity**

Although differences in IRS may explain at least in part why tumours exhibit different responses to RT, it should be appreciated that cellular radiosensitivity may not be uniform within a given tumour. Like many other cancers, human bladder cancer is often heterogeneous, containing numerous biologically different populations within a single neoplasm. This can give rise to tumour heterogeneity with respect to cellular morphology, karyotype, ploidy, cell cycle and cell proliferation kinetics, clonogenic potential, receptor expression and metastatic and tumorigenic properties [92, 93]. Biological heterogeneity has been shown to cause differing radiosensitivity in human bladder cancer cell lines *in vitro* [94] and should therefore be considered as a cause of differing clinical response to radiation treatment for invasive bladder cancer.

#### **1.4.3 Predictive Assays**

##### **1.4.3.1 Overview**

To date, predictive assays for tumour control have had limited success and none has entered clinical practice. In light of the mechanisms of radioresistance outlined above, this is perhaps not too surprising, since tumour response is almost certainly multifactorial. Further problems in bringing tumour predictive assays to the clinic include the relative inaccessibility of the majority of tumour types for sampling, patient tolerance of invasive

sampling procedures, precise definition of the clonogenic population and the accuracy and time required for most assays. Nevertheless, over the last decade, much work has been undertaken to develop techniques that measure IRS and might therefore serve as a predictive assay of RT outcome.

#### **1.4.3.2 Measuring Tumour Cell Hypoxia**

As discussed earlier, the radioprotective effect of hypoxia has long been implicated in RT treatment failure. Intuitively, quantitation of hypoxia might therefore be expected to serve as a predictive assay. Only in recent years, however, has direct measurement of intratumoural oxygen levels become feasible, with the introduction of polarographic probe or microelectrode. Although limited in size, a number of studies applying this technique to solid tumours have consistently shown an adverse effect on both local tumour control and subsequent survival in uterine cervical, head and neck carcinomas, and soft tissue sarcomas, where oxygenation levels are low [95, 96]. Interestingly, in cervical cancers treated surgically, hypoxia was again an adverse prognostic factor, suggesting that hypoxia may play a more general role in the pathogenesis of malignant disease. The clinical application of microelectrode measurements is hampered by the fact that polarographic measurements of tumour oxygen tensions are invasive by their very nature; demanding direct access to tumours *in vivo* which is technically difficult in bladder cancer.

An alternative approach involves the systemic administration of pimonidazole, which binds to thiol-containing proteins specifically in hypoxic cells. This technique has been successfully applied to demonstrate hypoxia in bladder tumours, by pre-biopsy administration of pimonidazole and immunohistochemical staining for bound pimonidazole fragments after nitroreductase-induced breakdown in hypoxic areas [72]. The disadvantage of this approach is the need for systemic administration of the agent several hours before biopsy and the associated risk of toxicity. An intrinsic immunohistochemical marker of hypoxia would therefore have considerable advantages over this extrinsic approach.

Several putative intrinsic markers of hypoxia are under investigation. They share a common feature in their regulation by HIF-1 $\alpha$  [70]. Of these markers, CA IX and glucose transporter-1 (GLUT-1) protein have received much recent attention. The potential of CA IX immunostaining as a surrogate marker of hypoxia in invasive bladder cancer is discussed further in chapter 2.

#### **1.4.3.3 Tumour Cell Repopulation Assays**

Although studies of tumour kinetics are potentially of great clinical value since they reflect a tumour's biological activity and may define its potential aggressiveness, their present value to the clinician has been found to be limited. In a multicentre analysis in head and neck cancers, Begg *et al.* [97] found that pre-treatment measurements of

proliferation rate provided only a weak prediction of RT outcome. In bladder cancer, the monoclonal antibody Ki67 has been used to recognize an antigen present in actively dividing cells. Immunohistochemical staining therefore provides an easy method of determining tumour cell turnover [98]. Using Ki67 immunostaining, Lara *et al.* [99] observed that patients with very low proliferating tumours seemed to achieve better local control after fractionated RT compared with other patients. However, as was discussed in 1. 4. 2.3, the relative importance of tumour cell repopulation as a cause of RT failure in bladder cancer remains questionable.

#### **1.4.3.4 Predicting Intrinsic Cellular Radiosensitivity**

In measuring IRS in other tumour models, one of the most significant developments has been the clonogenic SF2 assay for cervical cancer, developed by West *et al.* [100]. This assay has been shown to be highly predictive of the outcome of RT in this disease. Unfortunately, application of this assay to bladder cancer is hindered by the fact that it fails to provide information on a timescale appropriate for treatment planning (typically 4-5 weeks). Additionally, tumour cells from different organs may vary considerably in their ability to form colonies. For example, in a study of primary bladder tumours, only 8% of biopsies gave rise to colony formation\*.

Other means of predicting IRS have also been evaluated. The methyltetrazolium (MTT) assay, which measures metabolically viable cells, provides reproducible measurements

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\* McKeown, McKelvey-Martin and Ho, personal communication.

that correlate well with SF2 clonogenic cell survival values [101]. Unfortunately, this technique is relatively insensitive at low doses and requires optimization of the assay conditions for each cell line under investigation, thus rendering it inappropriate for a comparative assessment of cells from tumour preparations [102].

An alternative approach, in bladder cancer, has been to measure the disruption of DNA organization using nucleoid light scattering [103]. One factor that may influence initial damage formation is the arrangement of DNA in the nucleoid, as demonstrated by the ability of DNA to maintain its 'supercoiled' state following irradiation. The ability of tumour cell DNA to adopt and maintain positive supercoiling in the presence of radiation-induced DNA damage may be related to a stronger association between individual DNA loops and their attachment to the nuclear matrix (explored further in chapter 4). Two predominant forms of supercoiling behaviour have been demonstrated *in vitro*, as indicated by the extent of nucleoid light scattering [103]. In clinical studies, patients who achieved local control after RT were associated with a greater nucleoid light scatter in the tumour cell nucleoid bodies than those with residual disease at 6 months. A similar correlation between nucleoid light scattering and radiosensitivity has been reported previously in human squamous cell carcinoma cell lines, ataxia telangectasia fibroblasts and variants of the LY5178 murine leukaemia cell line [104-106]. Unfortunately, clinical application of this technique is hindered by poor specificity, but such results do indicate that nuclear DNA configuration influences the response of tumour cells to irradiation.

Telomere abnormalities have also been implicated as a cause of differing IRS and have been assessed in different tumour cell lines. Some investigators have observed a relationship between telomere shortening and radiosensitivity whilst others have demonstrated an association with telomere functioning [107, 108]

#### **1.4.3.5 The Comet Assay**

The single cell gel electrophoresis (also known as the comet assay) has evolved as a means of evaluating the induction and repair of DNA strand breakage. The comet assay (discussed in more detail in chapter 3) provides a measurement of DNA damage, whereby damaged DNA migrates from the lysed remnants of the nucleus (the nucleoid body), under the influence of electrophoresis, forming the comet 'tail'. Intact DNA remains in the nucleoid body, forming the comet 'head'. The relative extent of DNA migration into the tail indicates the level of induced damage (or the level of residual damage following repair) and is readily analysed using fluorescent microscopy coupled to purpose-designed software. The comet assay is rapid, sensitive and is relatively straightforward to perform. Since it operates at the level of single cells, it does not depend on the formation of viable colonies. It therefore has considerable advantages over the techniques described above, as a potential means of measuring IRS in a clinical setting.



Using the neutral version of the assay, Price *et al.* [109] reported an inverse correlation between comet formation and clonogenic cell survival at 10 Gy (SF10), in a panel of bladder tumour cell lines. However, no such correlation was observed at 2 Gy. The sensitivity of the assay at lower (clinical) doses of radiation can be significantly improved by using alkaline operating conditions (the alkaline comet assay (ACA)). Using this approach, Mckelvey-Martin *et al.* [110] observed a correlation between clonogenic survival and ACA measurements in a panel of 3 bladder cancer cell lines. More recently, we have demonstrated that ACA measurements of DNA damage correlate closely with clonogenic cell survival in six bladder cancer cell lines, over a dose range of 2-6 Gy [111]. This is consistent with the independent findings of McKeown *et al.*, [112] in a similar series of bladder cancer cell lines. Similar correlations have been recently demonstrated in colorectal cell lines [113].

Overall, these studies indicate that ACA provides an accurate prediction of clonogenic cell survival *in vitro*, providing a solid foundation for further studies using cells derived from tumour biopsies (chapter 3).

#### **1.4.3.6 The Genetics of Radiosensitivity**

The belief that the underlying differences in IRS may be genetically determined has prompted the search for genotypes that might be associated with radiosensitivity or resistance. For example, the various stages of cellular response to ionizing radiation

(damage induction, repair, cell cycle control and control of apoptosis (1.3)) are genetically controlled and the activity of the relevant genes or proteins may be expected to provide prognostic information.

Several studies have attempted to link mutated candidate genes with a radiosensitive phenotype. Mutations in the *p53* gene are associated with human bladder cancer, occurring in 30-60% of cases [114]. P53 plays a role both in causing G1 arrest in the cell cycle and allowing apoptosis to proceed (discussed in 1.3.1 and 1.3.3). In bladder cancers, mutations in the *p53* gene lead to conformational changes resulting in stabilization and hence, accumulation of the p53 protein, which is detectable by immunohistochemical methods. In one study, concordance between p53 nuclear reactivity and mutations analysed by single strand conformational polymorphism was 77% [115]. However, although there is an overall association between p53 accumulation and *p53* gene mutation assessed by this and other techniques, significant accumulation of *p53* can arise in the absence of detectable mutations [116].

There appear to be inconsistencies regarding the relationship between p53 positivity and outcome following RT for bladder cancer. Jahnson *et al.* [117] found that p53 and Rb immunostaining did not correlate with radiation response or patient survival in patients undergoing radical RT for locally invasive disease. In contrast, Osen *et al.* [118] found that in 83 patients irradiated for bladder cancer, low apoptotic index and p53 positivity were associated with worse local control. In another series, the prognostic significance of p53 and p21 expression in a cohort of patients with non-metastatic muscle-invasive

bladder cancer treated by RT was investigated [119]. Stratification for p21 and p53 status identified distinct prognostic groups, with p21+ and p53+ associated with the best survival advantage and p21- p53+ tumours the worst. The authors therefore advocated offering either adjuvant treatment or radical cystectomy to patients with phenotypes other than p21+ p53+.

The retinoblastoma protein (Rb) is also a key regulator of the G1 cell cycle checkpoint and has been implicated as having a role in G1 arrest and apoptosis induced by radiation damage (see section 1.3.1). Loss of Rb function as measured by immunohistochemical staining is a strong correlate of radiation response in bladder cancer, [15] making this a potential marker for selecting patients for bladder preservation with RT.

In bladder cancer, the prognostic value of proteins downstream of p53 in the regulation of apoptosis including Bcl-2 and Bax, have been investigated, together with p21 involved in cell cycle control [120]. Of these, Bcl-2 positivity was shown to be an independent adverse prognostic indicator. Its predictive value was enhanced when combined with p53 positivity. In a separate study investigating a cohort of patients undergoing definitive RT [118], p21 immunopositivity, Stage T2/3 and grade 2 tumours were independent prognostic indicators of improved survival.

Given the fact that a multitude of genes are likely to be involved, it is unsurprising that such studies have been largely contradictory. However, it remains plausible that subgroups of radiosensitive or radioresistant patients might carry mutations in specific

ionizing radiation damage-processing genes. Should this be the case, screening of the RT population for a particular gene defect before therapy may allow treatment to be tailored to the individual.

The advent of microarray technology has facilitated an alternative approach, whereby large-scale genetic ‘fingerprinting’ of individuals may be undertaken. In one such procedure, DNA corresponding to part of thousands of gene sequences is coupled to a solid support and exposed to a mixture of two different labeled RNA pools corresponding to the two different states of interest (in this case radiosensitive versus radioresistant). The relative hybridization of each sample to each DNA spot gives a measure of activity (expression) of each individual gene in the two samples. Thus a profile of gene activity for the two states of interest can be generated. This approach has been employed in bladder cancer to achieve tumour subtype classification of cell lines *in vitro* with respect to pathological stage, grade and squamous differentiation in the tumours from which the cell were derived [121]. Studies using clinical specimens have involved the development of a predictive classifier of Ta, T1 and T2+ tumour subtypes. Smad6 and cyclin G2 were identified as Ta/T1 classifier genes and their immunostaining patterns were validated on tissue microarrays by immunohistochemistry [122]. A more recent study compared expression profiles of early stage and advanced bladder tumours using cDNA microarrays. In this case, Gene profiling successfully classified tumours according to their progression and clinical outcome [121]. The application of microarray technology to radiation sensitivity clearly has promise, but as yet no such studies in bladder cancer have been undertaken. Furthermore, combining this technology with histological

microdissection now enables the derivation of cell type-specific gene expression profiles [123].

Similarly, the evolution of proteomic technology may pave the way for the identification of novel proteins associated with tumour cell response to RT. Proteomics encompasses platform technologies for protein separation and identification, delineation of their biomolecular interactions, function, and regulation, and for annotating, storing, and distributing protein information. The proteome is far more complex and dynamic than the genome. The prospect of deciphering even a single cell type is daunting, as many thousands of proteins, including post-translational modifications, and cleavage products, may be present in a cell at any given time. To date, proteomic studies have revealed several protein markers of TCC progression and have led to the development of novel strategies for the identification of tumor heterogeneity among low-grade papillary TCCs [124, 125]. Furthermore, proteomic studies have highlighted proteins that may be associated with radiation response; for example, in Chinese hamster ovary cells *in vitro* [126] and in cells derived directly from colorectal tumours treated with radiotherapy [127]. The identification of proteins associated with radiation response in bladder cancer is the focus of chapter 4.

## **1.5 SUMMARY AND AIMS**

The development of a technique that predicts the response of a tumour to radiation has long been considered a ‘holy grail’ amongst radiobiologists. In muscle-invasive bladder cancer, where in many cases there is a choice of primary intervention (surgery or RT), the need for predictive information is particularly great. A review of current evidence in this and other disease models has highlighted both hypoxia and IRS as potentially important factors in dictating the response of bladder tumours to RT. There is little convincing evidence at present to suggest that differences in repopulation rates account for the variable response to RT.

This thesis will therefore focus on hypoxia and IRS as factors that may potentially dictate the response of human bladder tumours to RT. The specific aims of this study are:

1. To investigate the prognostic significance of hypoxia within bladder tumours, with regard to outcome after RT, using the novel hypoxia-related marker, carbonic anhydrase IX.
2. To examine differences in IRS between tumour cell preparations derived from TURBT specimens using the ACA.

3. To examine the nuclear factors that influence comet formation (and therefore radiosensitivity) such as DSB rates and nucleoid proteins (by using proteomic analysis).

## **CHAPTER 2: CA IX EXPRESSION IN MUSCLE-INVASIVE BLADDER CANCER**



## **2.1 INTRODUCTION**

### **2.1.1 General Background**

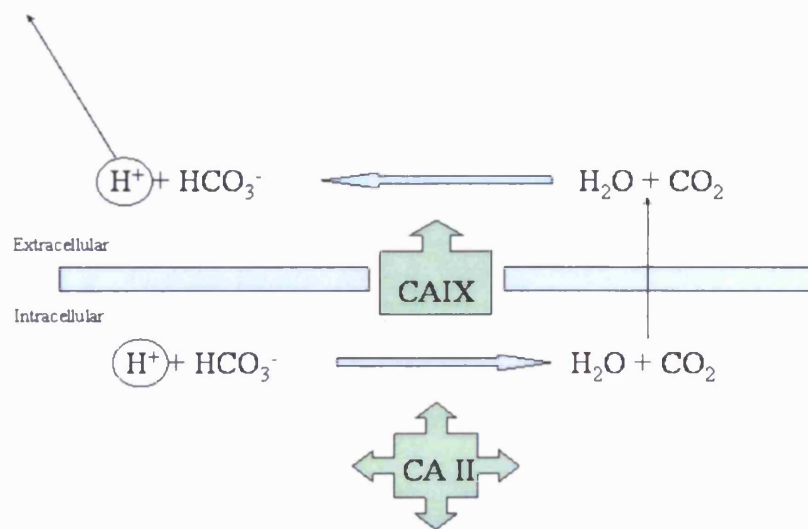
Since tumour hypoxia is widely regarded to enhance radioresistance, an intrinsic marker of hypoxia (as discussed in 1.4.3.2) in bladder tumours could be expected to provide predictive information with respect to tumour response to ionising radiation. One potential candidate marker is Carbonic anhydrase (CA) IX; part of a family of phylogenetically ancient enzymes found ubiquitously in nature.

The transcriptional complex hypoxia-inducible factor-1 (HIF-1) has been identified as a key mediator of hypoxic gene expression in human tumours (see figure 2.3). Hypoxic induction of the carbonic anhydrase genes *CA 9*, *CA 12* and corresponding proteins (CA IX, CA XII), has been shown to be HIF-1-dependent [71]. In tumour cells, these enzymes are pivotal in maintaining the intracellular pH at physiological levels. Their activity is therefore up-regulated in tissue undergoing anaerobic respiration, where there is an accumulation of intracellular hydrogen ions ( $H^+$ /protons).

### **2.1.2 Carbonic Anhydrases and pH Homeostasis**

CAs achieve pH homeostasis in two ways. Firstly, they assist with passive diffusion of  $CO_2$  and  $H_2O$  out of the cell. Membrane associated CA IX maintains the diffusion gradient by catalysing the conversion of  $H_2O$  and  $CO_2$  to  $H^+$  and  $HCO_3^-$  in the

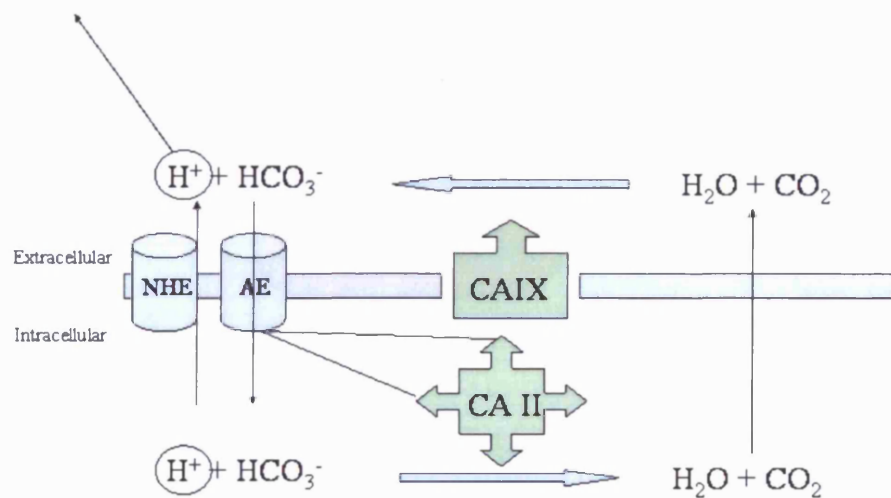
extracellular space, whilst intracellular cytosolic CA II generates  $\text{CO}_2$  and  $\text{H}_2\text{O}$  (figure 2.1) from  $\text{H}^+$  and  $\text{HCO}_3^-$ .



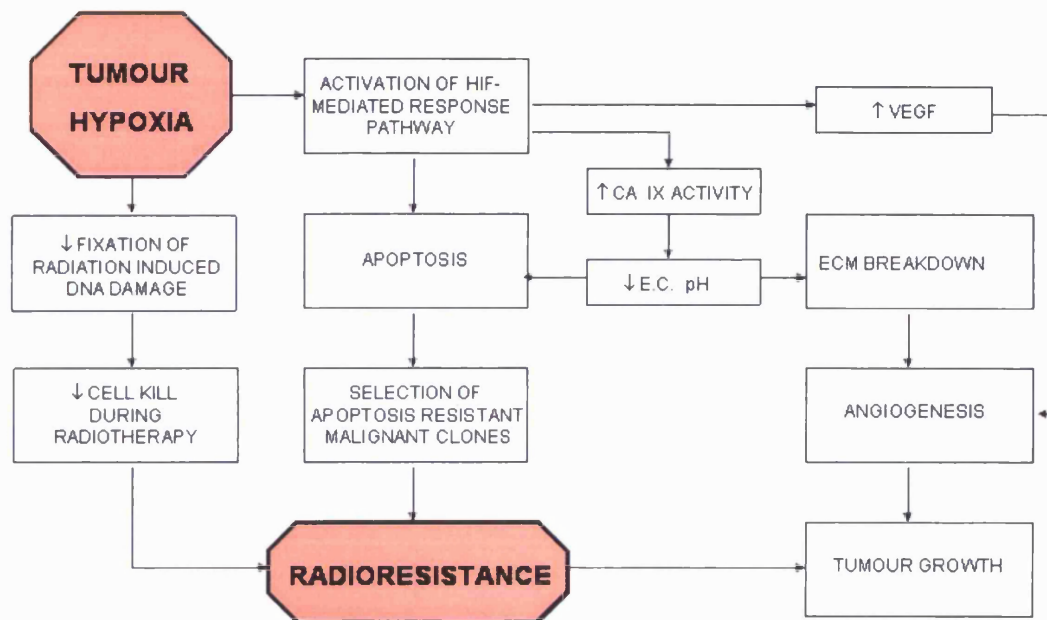
**Figure 2.1:** The role of CA IX in assisting passive diffusion of  $\text{H}^+$  from the cell.  $\text{H}^+$  ions produced through anaerobic metabolism within the cell must first be converted to  $\text{CO}_2$  to allow diffusion across the lipid bilayer. This reaction with bicarbonate is catalysed by cytoplasmic CA II. Once  $\text{CO}_2$  has diffused out to the extracellular space, the opposite reaction occurs catalysed by membrane associated CA IX. Protons are regenerated, and the diffusion gradient is maintained (adapted from Potter & Harris, British Journal of Cancer, 2003[128]).

Alternatively, CAs may act in conjunction with membrane associated ion transport systems such as the sodium-hydrogen exchanger (NHE) and chloride-bicarbonate anion exchanger (AE, figure 2.2), to remove protons from the cell. An additional model is proposed; whereby membrane associated CAs regenerate bicarbonate ions which are

shuttled back into the cell and recombined with  $H^+$ . This in turn is converted to  $H_2O$  and  $CO_2$  which diffuses out of the cell. Indeed CA II has been shown to bind to the cytoplasmic tail of the chloride-bicarbonate anion exchanger (AE). The recycling of bicarbonate ions with net extrusion of protons is known as a Jacobs-Stewart cycle.



**Figure 2.2:** CA IX and active ion transport. Acting in concert with active ion transport mechanisms, membrane associated CAs regenerate bicarbonate ions which are shuttled back in to the cell and recombined with  $H^+$ . This in turn is converted to  $H_2O$  and  $CO_2$  which diffuses out of the cell. CA II has been shown to bind to the cytoplasmic tail of the chloride-bicarbonate anion exchanger (AE) (adapted from Potter & Harris, British Journal of Cancer, 2003[128]).



**Figure 2.3:** Schematic diagram showing the role of hypoxia in causing radioresistance. In addition to its physical effects on the ‘fixation’ of radiogenic DNA damage (discussed in 1.4.2.2), hypoxia triggers specific patterns of gene expression, mediated by HIF-1 $\alpha$ . CA IX is pivotal in this response, causing acidification of the extracellular space, promoting extracellular matrix breakdown and apoptosis, which in turn may provide a selective pressure favouring apoptosis-resistant subclones.

The overall effect of CA activity is the relative acidification of the extracellular space. This has important ramifications in promoting further tumour growth and invasion. An acidic extracellular environment promotes tumour invasion; in part by the induction of endopeptidases that break down the extracellular matrix, allowing further tumour spread [73]. In addition, low extracellular pH suppresses the anti-tumour effects of the cell

mediated immune system. Importantly, hypoxia can induce apoptosis by a mechanism dependent upon a drop in extracellular pH [129]. Although intuitively this process would be expected to reduce the tumour cell population, it may in fact provide a selective pressure for the emergence of apoptotic-resistant sub-clones (figure 2.3). In this way, hypoxia may select for cells with p53 mutations, since cells expressing wild-type p53 tend to undergo apoptosis more readily [130]. In keeping with this, p53 is one of the most commonly mutated gene in bladder cancer [72, 114].

### **2.1.3 CA IX as a Marker of Hypoxia**

CA IX expression is reported to be a possible endogenous surrogate marker of hypoxia in solid neoplasms. In cervical carcinoma, CA IX expression correlates well with polarographic measurements of tumour oxygen tension [131]. More recently, CA IX expression has been associated with poor prognosis in non-small cell lung cancer [132] and has been associated with poor response to chemoradiotherapy in head and neck cancer [133].

In bladder cancer, CA IX immunostaining is attractive as an endogenous marker of hypoxia, since it is non-invasive and does not require systemic administration, compared with polarographic needle measurements and pimonidazole, respectively. Significant correlations have been observed between CA IX expression and pimonidazole levels in bladder cancer [72]; however, there is no clear consensus as to the prognostic value of

CA IX immunostaining in this disease. Turner *et al.* [134] studied the distribution of vascular endothelial growth factor mRNA (by in situ hybridisation) and CA IX expression in 22 bladder cancers of varied pathological stages. Co-localisation of VEGF and CA IX expression was observed; both being expressed predominantly on luminal surfaces and in perinecrotic areas. Expression of both factors was greater in superficial compared with invasive disease. The authors went further, to study the relationship between expression of CA IX and clinical outcome in 49 patients with superficial bladder cancer. CA IX expression was not predictive of clinical outcome. Hoskin *et al.* [135] investigated GLUT1 and CA IX as endogenous markers of hypoxia and their relationship with outcome in a retrospective series of 64 patients treated with radical RT with carbogen and nicotinamide (ARCON). GLUT1 and CA IX staining were found to be independent predictors for overall and disease-specific survival, but not for local control or metastasis-free survival. A prospective study was also reported in which pimonidazole, GLUT1 and CA IX staining was compared in 21 patients with bladder cancer. A good correlation was reported between CA IX/GLUT1 expression and pimonidazole staining. More recently, CA IX expression has been studied in 57 patients with superficial or invasive disease [136]. Again, significantly more superficial bladder cancers expressed CA IX strongly. No significant association between CA IX staining and survival was established in either superficial or invasive disease.

#### **2.1.4 Summary and Aims**

Although intuitively quantitation of tumour hypoxia might be expected to provide predictive information regarding the response of muscle-invasive bladder tumours to RT, relatively few studies to date have explored this hypothesis. Measurement of hypoxia in bladder tumours using well established techniques such as polarographic needle studies and pimonidazole is hampered by the need for direct tumour access and pre-biopsy systemic administration of a chemical agent, respectively. CA IX expression has been associated with hypoxia in other tumour models and potentially overcomes these limitations. In the present study, we aim to evaluate the prognostic significance of CA IX expression in patients treated with RT as monotherapy. We aim to determine whether CA IX expression, as determined by standard immunohistochemistry, correlates with local control and patient survival following radical RT in muscle-invasive bladder cancer.

## **2.2 MATERIALS AND METHODS**

### **2.2.1 Study Population**

Ethical approval was obtained for the study of archival paraffin-embedded tissue sections from 110 patients with pathological stage T2-T3 bladder cancer. All patients were treated with radical RT (mean total dose = 57.9 Gy) with curative intent. Of the stained

specimens, 91 (82.7%) were from male and 19 (17.3%) were from female patients. Staging was based on biopsy reports from the initial transurethral resection of the tumour, scan reports and the findings of examination under anaesthesia. Clinico-pathological data is summarised in table 2.1. Hospital notes were reviewed to determine clinical outcomes. Response to RT was determined by check-cystoscopy after completing treatment. A positive response was defined as disease regression or remission. A negative response was defined as persistence or progression.

	No.	%
<b>No of patients</b>	110	100
<b>Age at diagnosis (years)</b>		
<b>50-59</b>	8	7.3
<b>60-69</b>	25	22.7
<b>70-79</b>	53	48.2
<b>80-89</b>	24	21.8
<b>Sex</b>		
<b>Male</b>	91	82.7
<b>Female</b>	19	17.3
<b>Tumour Grade</b>		
<b>2</b>	16	14.5
<b>3</b>	93	84.5
<b>Unknown</b>	1	0.9
<b>Tumour Stage</b>		
<b>T2/3</b>	109	99.1
<b>Unknown</b>	1	0.9
<b>Radiation Therapy</b>		
<b>Mean total dose</b>	57.9Gy	
<b>Modal fraction size</b>	2Gy	
<b>Modal fraction number</b>	30	
<b>Mean treatment duration</b>	45.1Days	

**Table 2.1:** Summary of clinico-pathological data.



### 2.2.2 Immunohistochemistry

The tumour specimens evaluated were routinely processed, formalin-fixed and paraffin-embedded. Tissue sections of 4  $\mu\text{m}$  thickness were cut onto glass slides that were previously treated with 2% 3-aminopropylethoxysilane (in methanol, APES) and dried overnight at 37°C to promote section-to-slide adhesion.

The murine monoclonal antibody M75, recognising the *N*-terminal domain of the CA IX protein, has been previously reported by Pastorekova *et al.* [137]. The specificity of the monoclonal antibody M75 for CA IX has been previously reported using Western blots and immunostaining of COS-7 cells transfected with CA IX cDNA [138]. The secondary antibody was polymer-conjugated rabbit anti-mouse from the Envision kit (Dako, Ely, UK).

No antigen retrieval was required (optimization studies performed previously\*). Slides were dewaxed in xylene before rehydration by passage through graded alcohols. Endogenous peroxidase was blocked by applying 0.03% hydrogen peroxide containing sodium azide from the Envision kit (Dako) for 10 minutes. Non-specific staining was prevented by the application of 100  $\mu\text{l}$  of 10% human serum for 15 minutes. M75 working solution (stock solution diluted 1:50 (v/v) in 5% human serum) was added for 30

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\* Swinson DL, *Hypoxic Markers in Non-Small Cell Lung Cancer*. DM thesis, September 2003.

minutes. Polymer-conjugated rabbit anti-mouse secondary antibody from the Envision kit (Dako) was then added for 30 minutes. Diaminobenzidine substrate (DAB, applied for 8 minutes) was used to visualise CA IX. Sections were washed in tris-buffered saline for 5 minutes between incubations. After DAB staining, slides were immersed in running tap water for 5 minutes and counterstained with haematoxylin. Slides were dehydrated by reverse passage through graded alcohols and mounted.

### **2.2.3 Interpretation of CA IX staining**

Tissue sections from a Non-Small Cell Lung tumour were used as positive controls. Negative controls consisted of bladder tumour sections processed without the use of the primary antibody. Tissue sections were evaluated blind using light microscopy by two independent observers. Consensus was then reached using a conference microscope, with adjudication by a third observer. Sections were initially classified as positive or negative for CA IX expression. In addition, the type of staining (membranous, cytoplasmic or nuclear) and the presence or absence of necrosis was documented. For further analysis, slides showing CA IX positivity were sub-classified according to the percentage of tumour cell population stained ( $0 \leq 5\%$ ,  $1 = 6-25\%$ ,  $2 = 26-50\%$ ,  $3 = 51-75\%$ ,  $4 \geq 76\%$ ).

### **2.2.4 Statistics**

The SPSS software system (SPSS for Windows, version 9.0; SPSS Inc., Chicago, IL) was used to perform statistical analysis. Survival curves were plotted using the Kaplan-Meier method and statistical significance was assessed using the log-rank test. Multivariate analysis was performed using Cox's regression to determine any potential independent prognostic factors for diminished bladder-cancer specific survival. In the first step of the model, univariate analysis was used to identify potential prognostic factors. Factors which predicted diminished survival at a significance level of  $<0.10$  were retained for further stepwise conditional log rank analysis.

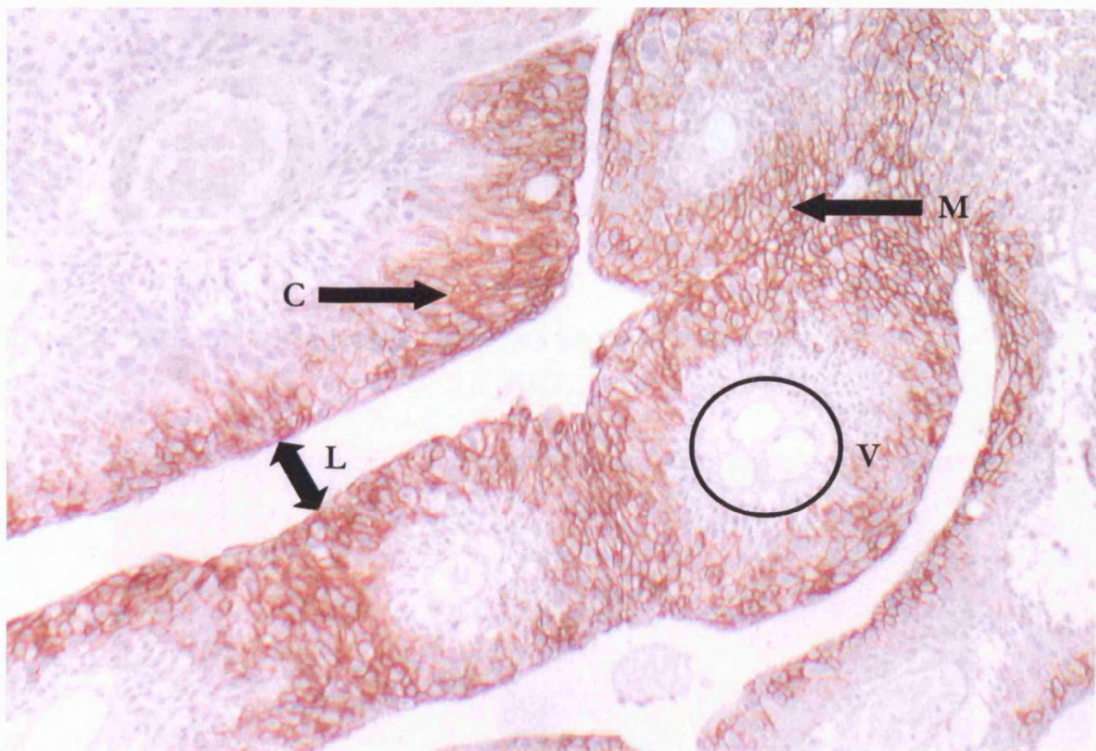
## **2.3 RESULTS**

### **2.3.1 CA IX Tumour Cell Expression**

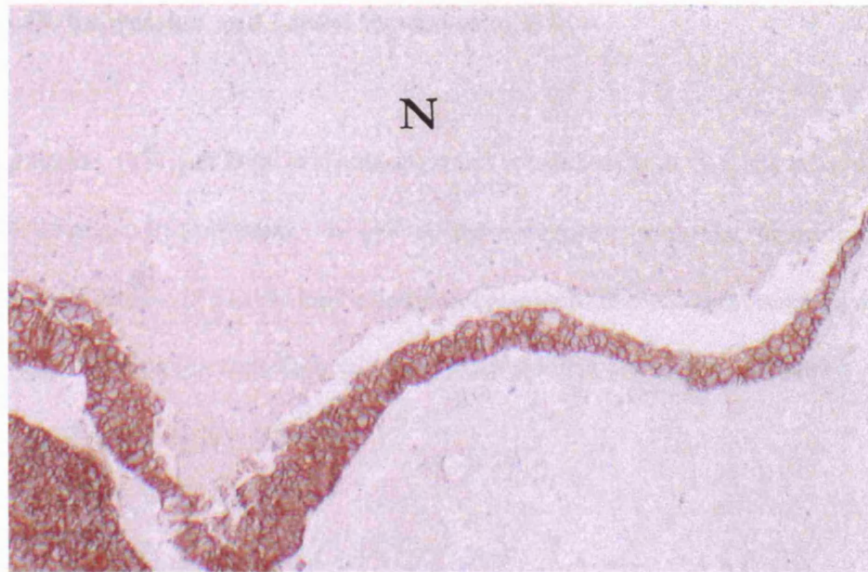
Concordance between the two observers with respect to positivity of immunostaining in this series was 97%. For subclassification by percentage of tumour cells staining positive, concordance was 91%. Consensus was reached on disputed cases using the conference microscope.

Tumour cell CA IX immunostaining was detected in 80.9% (89) of patients. Staining was predominantly membranous, with areas of concurrent cytoplasmic staining ( $n = 42$ ) (figure 2.4). CA IX expression was abundant in luminal and perinecrotic areas (figures 2.4 and 2.5). In 25 cases, areas of nuclear staining (figure 2.6) were observed along with

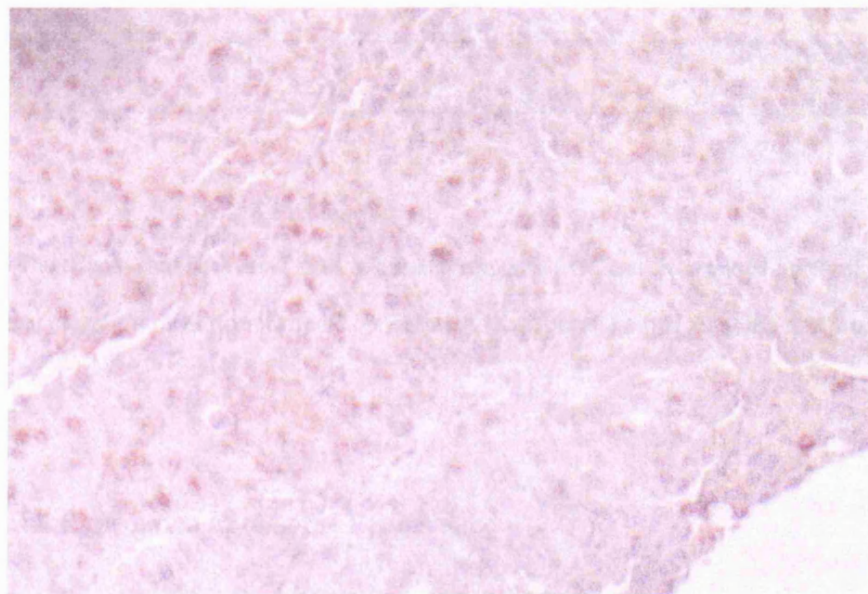
membranous/cytoplasmic patterns. Where staining was sub-classified by category, the majority of slides (56.4%) were assigned to category 1 (5-25%). Only 1 slide (0.9%) was assigned to category 4 ( $\geq 76\%$ ).



**Figure 2.4:** Typical CA IX staining distribution. CA IX expression is predominantly membranous (M) with areas of concurrent cytoplasmic staining (C). Expression was strongest in tumour cells near the luminal surface (L) and increased with distance from the tumour microvasculature (V).



**Figure 2.5:** Prominent CA IX expression adjacent to a large area of necrosis (N).



**Figure 2.6:** Nuclear CA IX staining (n = 25)

### 2.3.2 CA IX Expression and Initial Response to RT

79% of patients (87) patients underwent check cystoscopy at 3-6 months post RT, to determine response to treatment. 60 (69%) had a negative response (disease progression or persistence), whilst 27 (31%) had a positive response (disease remission or regression). No significant correlation was demonstrated between CA IX status and initial response to RT (Fisher's Exact Test,  $p = 0.561$ ).

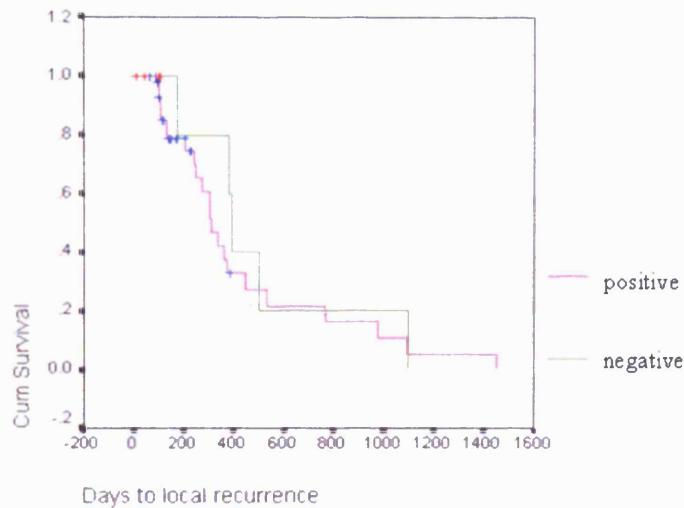
CA IX Status	Positive Response	Negative Response	Total
Negative	5	12	17
Positive	22	48	70
Total	27	60	87

**Table 2.2:** The relationship between CA IX status and response to RT, as determined by check cystoscopy.

No significant correlation was demonstrated between CA IX status and initial response to RT ( $p = 0.561$ ).

### 2.3.3 CA IX Expression and Local Recurrence

55 (50%) patients suffered local recurrence of their bladder cancer. No significant association between CA IX status and time (days) to local recurrence was demonstrated ( $p = 0.4679$ , figure 2.7).

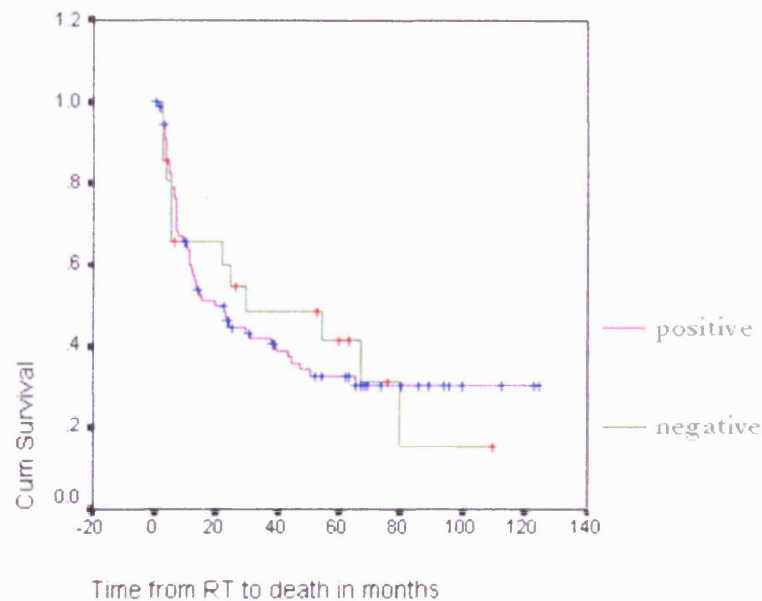


**Figure 2.7:** Kaplan-Meier curve for CA IX status and time to local recurrence (days) following completion of treatment. No significant association was demonstrated ( $p = 0.4679$  by log rank).

#### 2.3.4 CA IX Expression and Survival Following RT

Overall 5-year survival was 23.6% in this series. 5-year bladder cancer-specific survival was 40.9%. No significant association was observed between CA IX status and disease-specific survival following RT treatment ( $p = 0.9014$ , figure 2.8). No significant survival differences were demonstrated when patients were stratified by staining category.

Nuclear CA IX expression was not found to be of prognostic significance with respect to initial response to RT, local recurrence or disease-specific survival.



**Figure 2.8:** Kaplan-Meier survival curve for disease-specific survival following treatment with RT, stratified by CA IX status. No significant difference is demonstrable ( $p = 0.9014$  by log rank).

In the final multivariate analysis, 4 factors retained independent prognostic significance for reduced survival; pre-treatment ureteric obstruction, lack of response to radiation therapy at three-month cystoscopy, local recurrence and metastatic spread ( $p=0.005$ ,  $p=0.009$ ,  $p=0.001$ ,  $p=0.04$  respectively).



## 2.4 DISCUSSION

Previous studies of CA IX as a marker of hypoxia in bladder cancer have been undertaken using relatively small numbers of tumours, which are often heterogeneous with respect to tumour stage and treatment modality. Collectively, they provide no clear consensus as to the prognostic value, or clinical utility of CA IX immunostaining in major patient groups. Furthermore, no previous studies have examined the relationship between CA IX expression and outcome after radical RT (as monotherapy) which is widely used in the treatment of muscle-invasive disease. The present study, involving a large homogeneous series of 110 patients, provides a definitive assessment of the prognostic value of CA IX immunostaining in this important group of patients.

The abundance of CA IX immunostaining in luminal and perinecrotic areas of the tumour is consistent with previous studies in bladder cancer [134-136] and supports the notion that CA IX localises to areas of tumour hypoxia. The observation that CA IX immunostaining localised to the nucleus in 25 cases is interesting and not reported elsewhere. Although nuclear staining was not found to be of prognostic significance using the outcome measurements employed in this series, further work is required to determine whether this subgroup represents a distinct phenotype.

In our series, CA IX immunostaining was found not to be of prognostic significance with respect to response to RT in patients with muscle-invasive bladder cancer. This is to be contrasted with previous reports of this endogenous marker in other tumour models [131-

133], in which CA IX expression has been associated with adverse prognosis and radioresistance. Pre-treatment ureteric obstruction, lack of response to RT at three-month cystoscopy, local recurrence and metastatic spread were all identified as adverse prognostic indicators ( $p=0.005$ ,  $p=0.009$ ,  $p=0.001$ ,  $p=0.04$  respectively). This is in keeping with previous studies [13, 14, 23] and provides validation of this series.

This investigation, like any other immunohistochemical study of bladder cancer, may be hindered by the fact that tumours are often extensive, multifocal and heterogeneous, containing numerous biologically different populations within a single neoplasm (discussed in 1.3.2 [92, 93]). Single paraffin embedded tissue sections may not be representative of the hypoxic response elsewhere in the tumour.

There is growing evidence that superficial and invasive bladder cancer exhibit significant biological differences, particularly in the expression of hypoxia-inducible genes. There is differential regulation of VEGF in non-invasive compared with invasive cell lines. The expression of VEGF mRNA has been shown to be greater in superficial compared with invasive bladder cancer [139]. In addition, CA IX expression has been shown to be greater in superficial than invasive disease [134]. Paradoxically, these differences in HIF-1-induced gene expression between superficial and invasive tumours suggest relatively less activation of hypoxia regulated transcription pathways in invasive tumours.

Another explanation for the lack of association between CA IX expression and tumour response to RT may arise from mounting evidence that different intracellular pathways

may modify HIF-1 $\alpha$  expression and/or expression of its target proteins; thus introducing a compounding variable. The phosphatidylinositol 3-kinase (PI3K), mitogen-associated protein kinase (MAPK) [140, 141] and Smad pathways are known to influence HIF-1 $\alpha$  expression independently of hypoxia and these pathways are overactive in a wide range of tumour models. Epidermal growth factor receptor (EGFR) is a member of the *c-erb B* family of tyrosine kinase receptors and is commonly over expressed in bladder cancer cells [142]. In prostate cancer cell lines, EGF stimulation induces HIF-1 $\alpha$  via the PI3K pathway independently and additively to hypoxia [143]. Recent evidence suggests that the effects of the PI3K pathway on HIF-1 $\alpha$  activation may be cell line-specific and of a smaller magnitude to the effects of hypoxia [144]. Nevertheless, the compounding effect of interactions between the hypoxia response pathway and other cell signalling pathways should be borne in mind when immunohistochemical studies using hypoxia-related markers are undertaken.

Importantly, the present findings may highlight the importance of mechanisms other than hypoxia in dictating outcome after RT in this disease. In 1.4.2.3, repopulation is discussed as a potential cause of RT failure in muscle-invasive bladder cancer. Immunostaining using Ki67 as a marker of cellular proliferation has been successfully applied to identify rapidly dividing cell populations within tumours and in a retrospective analysis [99] patients with very low proliferating tumours seem to achieve better local control after fractionated RT compared with other patients. However clinical studies, in which duration of treatment has been modified to account for repopulation, have cast doubt over the relative importance of repopulation as a cause of RT failure [79].

Furthermore, whilst cellular reassortment is an interesting concept, there is no current experimental or clinical evidence to support that this has an influence on RT outcome for muscle-invasive bladder cancer.

It seems appropriate, therefore, for attention to turn to the concept of IRS as a potential cause of RT failure. Differences in IRS of tumour cells are well recognized in other human tumour models and these differences relate to clinical radiocurability [80]. IRS as a cause of radioresistance in human muscle-invasive bladder cancer is the focus of chapters 3 and 4.

## **2.5 CONCLUSIONS**

The distribution of CA IX expression in paraffin-embedded tissue sections seen in this series is consistent with previous studies in bladder cancer. However, CA IX immunostaining in tissue sections from patients with muscle-invasive bladder cancer does not provide significant prognostic information with respect to local control and survival following radical RT. The clinical utility of CA IX immunostaining in this subgroup of patients is therefore limited.

# **CHAPTER 3: APPLICATION OF THE ALKALINE COMET ASSAY IN MEASURING INTRINSIC BLADDER CANCER CELL RADIOSENSITIVITY**

### **3.1 INTRODUCTION**

#### **3.1.1 General Background**

Current available evidence suggests that IRS may be a major determinant of cellular response to ionizing radiation. This being the case, a rapid, accurate and reproducible means of measuring this parameter is required, in order to assess its relative importance in the outcome of RT for muscle-invasive bladder tumours *in vivo*. The limitations of established techniques that measure inherent cellular radiosensitivity are discussed in 1.3.4.3.

Single cell gel electrophoresis (SCGE) or ‘comet assay’ has evolved over the last twenty years as a rapid and very sensitive fluorescent microscopic method that allows examination of DNA damage and repair at the level of individual cells. In 1978, Ryberg and Johanson described a method of single cell analysis based on the differential lysis of irradiated cells in alkali; an approach that was modified later for flow cytometry by first encapsulating cells in agarose beads before irradiation and alkaline lysis [145]. The observation that the treatment of cells with 2M NaCl and anionic detergents generated ‘nucleoids’ composed of 50-100kb loops of DNA attached to a proteinaceous matrix, led to the development of the ‘halo’ assay [146]. DNA strand breaks lead to the relaxation of supercoiled DNA, allowing expansion of the ‘halo’ of DNA loops from the nuclear matrix proteins of individual cells. In 1984, Ostling and Johanson introduced the novel concept of performing electrophoresis on individual cells embedded in agarose and lysed

to produce nucleoids. Intact DNA remained in the nucleoid, whereas damaged, negatively-charged DNA migrated in the electrophoretic field towards the anode [147]. This gave rise to a characteristic ‘comet’ formation after which the assay was later named [148]. This approach conferred greater sensitivity, allowing the resolution of subpopulations of cells that showed different amounts of damage, i.e. heterogeneity of response. Furthermore, a few thousand cells provided an adequate sample for analysis, such that when cell numbers are limited, this may be the only practical method for DNA damage analysis.

### **3.1.2 The Alkaline Comet Assay**

The comet assay can be carried out in either neutral or alkaline conditions. A limitation of the ‘neutral’ version of the assay is that it is relatively insensitive to DNA damage arising from lower (more clinically relevant) doses of irradiation. To detect DSBs, which occur 25-40 times less frequently than SSBs, higher doses and more extensive lysis is required. The limit of sensitivity for detecting DSBs is of the order of 5 Gy [149]. A recent study in which the neutral comet assay was used to measure DSBs reported an association between DSB formation and survival in bladder tumour cells [109]. However, the correlations were weak and one cell line appeared falsely radiosensitive at the high doses (30Gy) involved. This highlights the importance of conducting predictive tests of radiosensitivity at clinically relevant doses.

These problems have been addressed by more complete protein lysis, accomplished by including an alkaline lysis step before or during electrophoresis [150]. These alkaline conditions cause denaturation of duplex DNA, allowing individual strands to separate and migrate independently, therefore allowing comet formation from SSBs. In addition, the use of alkali causes strand breakages at alkali-labile sites. In the alkaline comet assay, DSBs account for less than 5% of the total damage. However, the mechanisms proposed to account for variation in the yield of radiation-induced DSB damage formation are also expected to vary the yield of SSBs [151]. Consequently, the extent of radiation-induced SSB formation (detected by the ACA) can be considered a surrogate marker of DSB formation.

<b>Advantages of the (Alkaline) comet Assay</b>	
•	Rapid
•	Inexpensive
•	Sensitive at clinical doses of radiation
•	Simple
•	Low cell numbers required
•	Demonstrates differential response within cell population
•	Slides can be dried/stored for later analysis
•	Can be automated

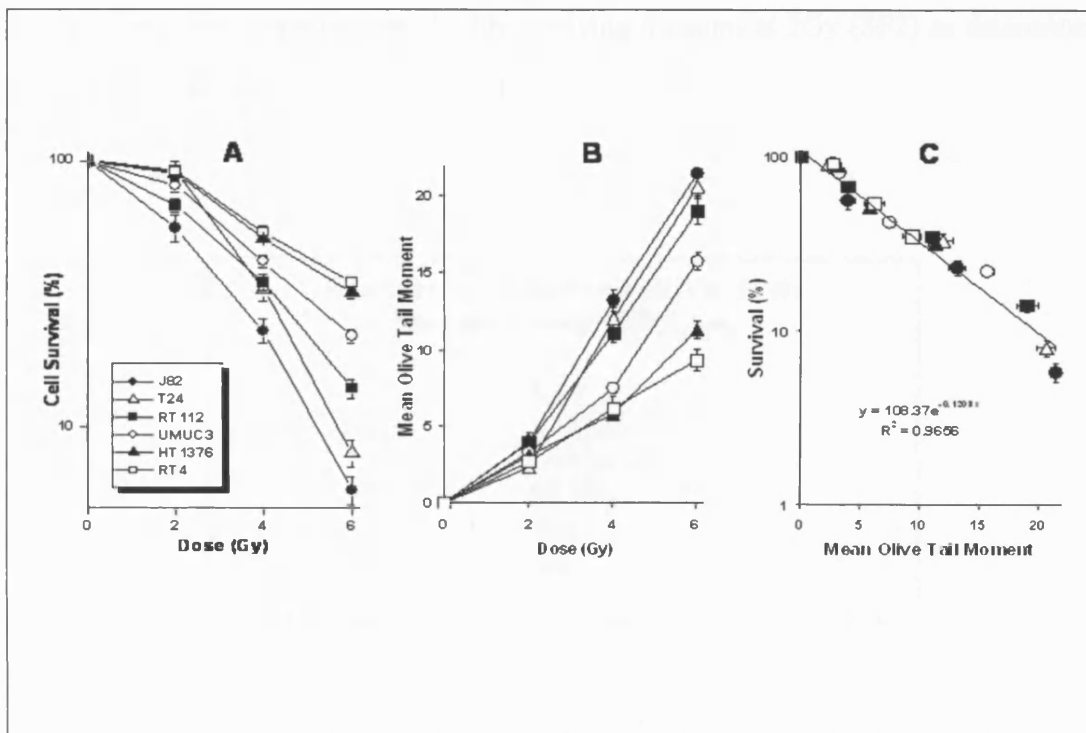
**Table 3.1:** Advantages of the Alkaline Comet Assay, indicating its potential application as a clinical test of intrinsic cellular radiosensitivity.



### 3.1.3 The Alkaline Comet Assay and Bladder Cancer

We have previously applied the ACA to examine radiation response, as determined by mean Olive Tail Moment (discussed later), in a panel of six bladder cancer cell lines derived from high-grade TCCs (RT112, UM-UC-3, HT1376, J82, T24 and RT4, figure 3.1 B) [111]. These responses were compared to clonogenic cell survival over the same dose range (0-6 Gy, figure 3.1 A), where survival was defined as the number of colonies formed following X-ray exposure. The relationship between the measures of mean Olive tail moment for initial comet formation, as measured by ACA, and the measures of clonogenic cell survival are shown in figure 3.1 C, where measurements for all six cell lines are collated. An exponential trend line was applied and correlation coefficients derived ( $R^2 = 0.9656$ ).

These data indicate a strong inverse correlation between clonogenic cell survival and ACA measurements over a dose range of 0-6Gy, indicating that measurements of initial comet formation following ionising radiation accurately predict clonogenic cell survival. In addition, the six cell lines are characterised such that a ‘rank order’ of radiosensitivity is established (Table 3.2).



**Figure 3.1:** (A) The radiation cell survival curve responses for the six bladder cancer cell lines investigated, over a dose range of 0 - 6 Gy, as determined by clonogenic assay. (B) The extent of initial comet formation (mean Olive tail moment), for six bladder cancer cell lines (0 - 6 Gy), as determined by ACA. (C) The relationship between mean Olive tail moment for initial comet formation and clonogenic cell survival. From AL Moneef *et al.*, 2003 [111].

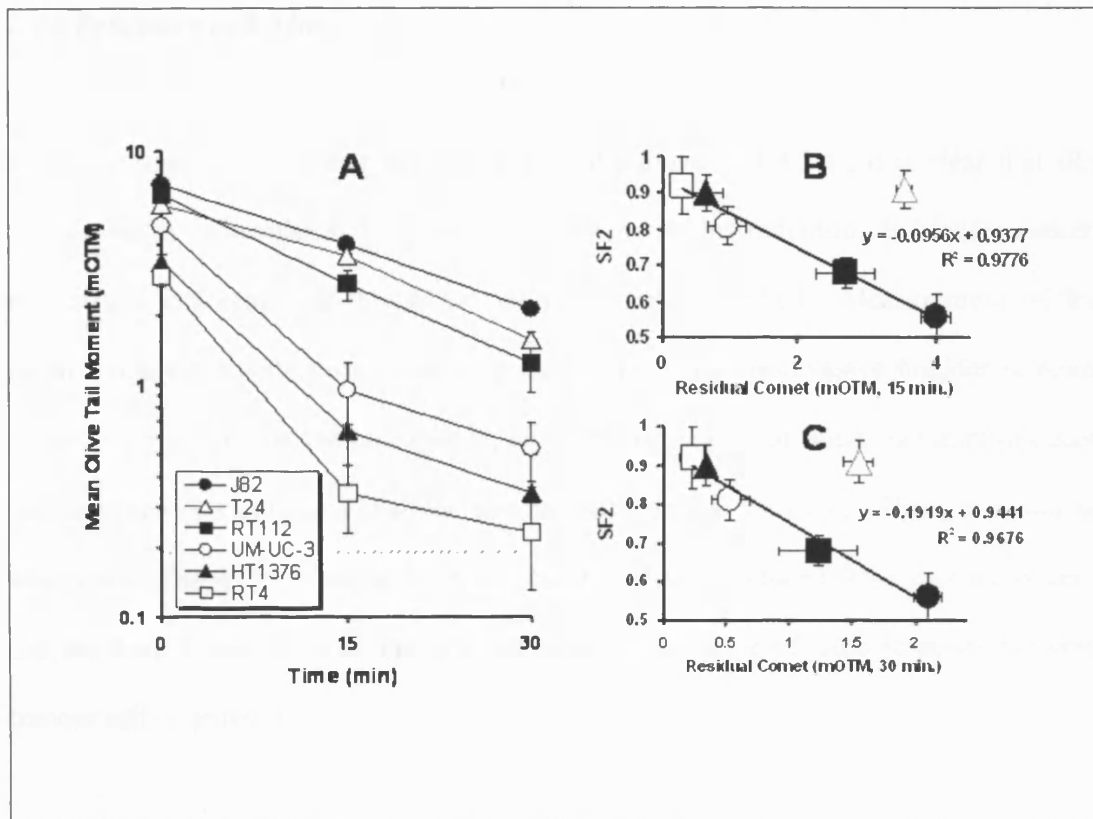
The ability of each of the six cell lines under evaluation to repair radiation-induced damage was also examined in this analysis. Irradiated cells (on slides) were incubated in repair medium for pre-determined time periods (15 and 20 minutes after irradiation to 2.5Gy), before using ACA to assess residual damage levels (Figure 3.2 A). These

measurements were then compared with surviving fractions at 2Gy (SF2) as determined by clonogenic analysis.

Rank Order of Radiosensitivity in six Bladder Cancer Cell Lines	
	RT4
	HT1376
	UM-UC-3
	RT112
	T24
	J82

**Table 3.2:** Rank order of radiosensitivity (most resistant to least resistant) in a panel of six bladder cancer cell lines, as determined by ACA and clonogenic survival.

When collated, ACA measurements for five of the six cell lines (J82, RT112, UM-UC-3, HT1376 and RT4) did correlate with SF2 values, with  $R^2$  values of 0.9776 and 0.9676 respectively (Figure 3.2 B & C). However, inclusion of the data for T24 (Figure 3.2 B & C (open triangles)) significantly worsened these correlations, giving rise to  $R^2$  values of 0.39934 and 0.5097 respectively.



**Figure 3.2:** (A) The extent of damage repair as determined by ACA, after irradiation (2.5 Gy). Each data point is the mean of three independent experiments  $\pm$  SE (dashed line = mean 'background' level for unirradiated/control cells). (B & C) The correlation between ACA measurements of residual damage at (B) 15 and (C) 30 minutes, as determined by ACA, and the determined SF2 values for the five cell lines J82, RT112, UM-UC-3, HT1376 & RT4. Data points for T24 (open grey triangles) were excluded from the analysis. From AL Moneef *et al.*, 2003 [111].

Data from these repair studies indicates that measurements of immediate comet formation post-irradiation provide the most accurate prediction of clonogenic cell survival and intrinsic radiosensitivity *in vitro*. This assertion is supported by similar independent work in bladder cancer and colorectal cell lines [110, 112, 113].

### **3.1.4 Summary and Aims**

From previous work in other tumour models (discussed in 1.4.2.4), it is clear that IRS may be highly influential in the tumour response to ionising radiation. In bladder cancer, the relative importance of this factor has yet to be established. Measurement of this parameter may provide prognostic information about muscle-invasive bladder tumours treated with ionising radiation. Indeed, ACA measurements of initial radiation-induced damage predict clonogenic bladder tumour cell survival *in vitro*. Here, we aim to examine the feasibility of using ACA to measure radiation-induced DNA damage in cells derived from human bladder tumours and to assess the range of radio-response between tumour cell preparations.

## **3.2 MATERIALS AND METHODS**

### **3.2.1 Patient Selection and Tumour Collection**

Ethical approval was obtained for the acquisition of tumour specimens from patients undergoing TURBT for suspected muscle-invasive transitional cell carcinoma of the bladder. Such patients were identified from departmental waiting lists and flexible cystoscopy reports were sought to assess the likelihood of muscle-invasive disease. Additional information was obtained from ultrasound scan reports and magnetic resonance imaging (MRI) reports where available. Patients were approached for consent

to the study at their visit for pre-admission assessment, in advance of their admission. Consent forms (appendix II) were filed in the medical records.

TURBT specimens were obtained at the time of surgery, labelled and placed immediately in ice-cold RPMI 1640 culture medium. Samples were then transported, on ice, to the laboratory for further analysis. Actual disease stage and grade were determined later by hospital histopathology services.

### **3.2.2 Tumour Disaggregation & Preparation of Cell Suspensions**

Specimens were processed in a class 1 tissue culture vacuum hood at the University of Leicester. TCC specimens and transport media were transferred to Petri dishes. Areas of necrotic material or diathermy damage were dissected free and removed. Remaining tissue was chopped finely between crossed scalpel blades and transferred to a universal container. Collagenase IV 0.05% solution (Boehringer, Mannheim, Germany) was added and the mixture was incubated at 37°C for 20 minutes in a shaking water bath. Suspended cells were removed, washed twice by centrifugation with phosphate buffered saline (PBS) and filtered by passing through a 120 gauge filter grid (Locker Wire Weavers Ltd. UK). Cells were counted on a haemocytometer (Weber, UK). Cellular viability was assessed using Trypan blue exclusion (BDH Chemicals Ltd., UK). Tumours yielding cell suspensions with less than 60% viability were excluded from

further analysis. Suspensions with sufficient viability were then immediately processed for human epithelial antigen (HEA) enrichment.

### **3.2.3 Enrichment of Cell Suspensions using HEA MicroBeads**

#### **3.2.1.1 General**

All materials were from Miltenyi Biotech, Germany. Cells suspensions were enriched with respect to their tumour cell content using magnetic cell sorting (MACS) HEA MicroBeads and separation kit, in accordance with the manufacturers' instructions. HEA is known to be widely expressed in tumour cells of epithelial origin [152]. For MACS separation, cells were magnetically labeled with HEA MicroBeads and separated in a column which was placed in the magnetic field of a MACS separator. The magnetically labeled HEA<sup>+</sup> cells were retained in the column whilst unlabeled HEA<sup>-</sup> cells flowed through. After removal of the column from the magnetic field, the retained HEA<sup>+</sup> cells were eluted as the positively selected cell fraction.

#### **3.2.3.2 HEA Labelling**

Cells (to a maximum of  $5 \times 10^7$ ) were resuspended in 300µl of degassed separation buffer (PBS pH 7.2, 0.5% bovine serum albumin, 2 mM EDTA). 100 µl of FcR Blocking

Reagent was added to block non-specific binding and mixed well. 100 µl of MACS HEA MicroBeads were added, mixed well and incubated at 6-12°C for 30 minutes. The cells were washed in 10-20 x the labelling volume of buffer and centrifuged at 300 x g for 10 minutes. The supernatant was then removed and the pellet resuspended in 500 µl of separation buffer, before proceeding to magnetic separation.

### **3.2.3.3 Magnetic Separation**

An MS+/RS+ column type was selected and placed in the magnetic field of a MACS separator. The column was prepared by washing with 500 µl of separation buffer. The cell suspension was applied to the column after passing through a pre-separation 30 µm filter and the negative cells were allowed to pass through. The column was then rinsed with 4 x 500 µl of buffer. The column was removed from the separator, 1 ml of buffer applied and positively selected cells were flushed out using a plunger. The separation step was then repeated, using only the eluted positive cells. Cell numbers and viability were assessed in the two fractions as described earlier.



### **3.2.4 Assessment of Cell Enrichment by Immunohistochemistry and Immunocytochemistry**

At the time of collection, a sample of tumour tissue acquired at TURBT was placed in 10% neutral buffered formalin (NBF). The remaining available tissue was transported back to the laboratory, disaggregated and enriched using the methods described above.

#### **3.2.4.1 Tumour Tissue Analysis**

Formalin-fixed tissue was paraffin-embedded. Tissue sections of 4µm thickness were cut onto glass slides that were previously treated with 2% APES (in methanol) and dried overnight at 37°C to promote section-to-slide adhesion. These sections were then serially stained using  $\alpha$ -actin, cytokeratin 8, cytokeratin 18 and vimentin (BD Biosciences, USA), to determine stromal versus tumour cell staining patterns.

#### **3.2.4.2 Analysis of Separated Cell Fractions**

Samples of HEA+ and HEA- fractions were fixed in methanol and 10% NBF separately. Cells were then centrifuged and resuspended in diluent to prepare smears. Smears were prepared on slides previously treated with 2% 3-aminopropylethoxysilane (in methanol) and dried overnight at 37°C to promote adhesion. Smears prepared from both fractions

were assessed by immunocytochemistry using antibodies to  $\alpha$ -actin, cytokeratin 8, cytokeratin 18 and vimentin, to determine the relative amounts of tumour cells versus stromal/other cell types found in the two fractions.

### **3.2.5 The Alkaline Comet Assay**

#### **3.2.5.1 General**

The modified 'slide' version of the Alkaline Comet Assay (ACA) [109, 110] was used, in an effort to minimise the opportunity for repair of radiation-induced damage before cell lysis. Experiments were carried out under reduced light, avoiding exposure to either daylight or overhead fluorescent strip lights. When using cell lines, cell lines were harvested at 75% confluence. When using cell suspensions derived from TURBT specimens, slides were prepared from a control cell line (Raji, American Tissue Culture Collection), irradiated and analysed in parallel, to correct for inter-experimental variation. Slides were prepared on a level surface and left to solidify on ice, protected by aluminium foil. All chemicals were obtained from Sigma, UK.

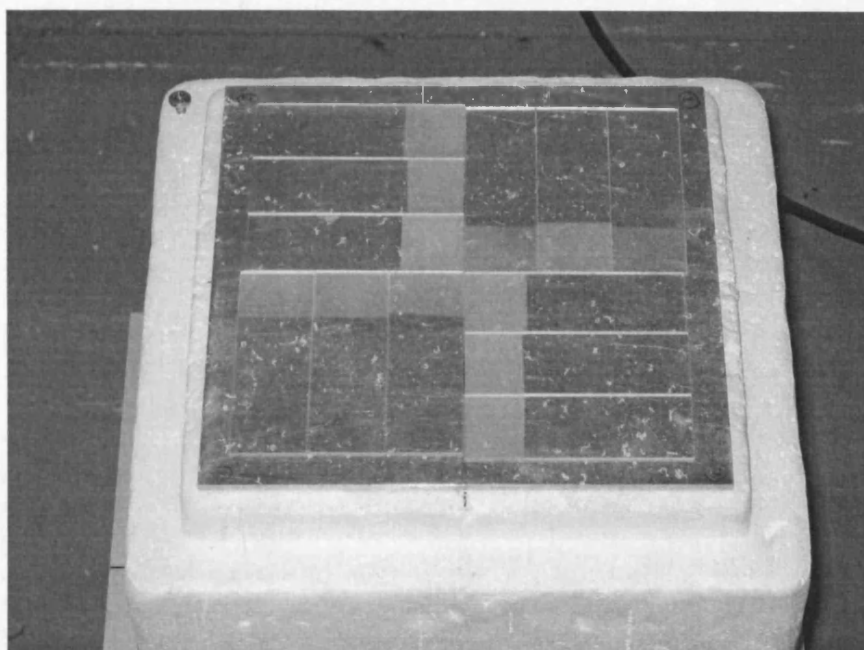
### **3.2.5.2 Slide Preparation**

Cell suspensions were aliquoted into eppendorf tubes to provide an optimal  $4 \times 10^4$  cells per slide (all dose points performed in duplicate). Cells were pelleted by centrifugation at 300 g for 5 minutes and the supernatant discarded. 100  $\mu$ l of 0.6% molten normal melting point agarose (NMA) was pipetted onto fully frosted Dakin microscope slides and a 22 mm x 22 mm coverslip applied immediately. Slides were allowed to solidify for 5 minutes, before removing the coverslips. 80  $\mu$ l of 0.6% low melting point agarose (LMA) was added to each eppendorf and pipetted gently to resuspend the cells. The LMA cell suspension was then pipetted onto the NMA layer prepared previously and a fresh coverslip applied. After solidification, the coverslips were removed and the slides placed on ice and in darkness for transfer to the irradiator.

### **3.2.5.3 Irradiation**

Slides were irradiated using the Pantak DXT300 x-ray machine in the Department of Genetics, University of Leicester. The source was operated at 300 kV and calibrated to deliver  $2.6 \text{ Gy min}^{-1}$  to slides placed at a distance of 40.5 cm from the source. Slides were positioned on a 15 x 15 cm aluminium plate in the configuration shown in figure 3.3 and the plate placed in thermal contact with ice in a container positioned underneath, as shown in figure 3.4. Slides were irradiated at 2, 4 and 6 Gy with the time of exposure adjusted to provide the level of irradiation required. After irradiation, slides were placed

in a Coplan jar containing ice cold, pre-prepared lysis buffer (2.5 M NaCl, 100 mM Na<sub>2</sub>EDTA, 10 mM tris, pH 10 with 1% Triton-X added fresh), and incubated overnight at 4°C. Control slides (0 Gy) were not irradiated, but placed immediately into lysis solution.

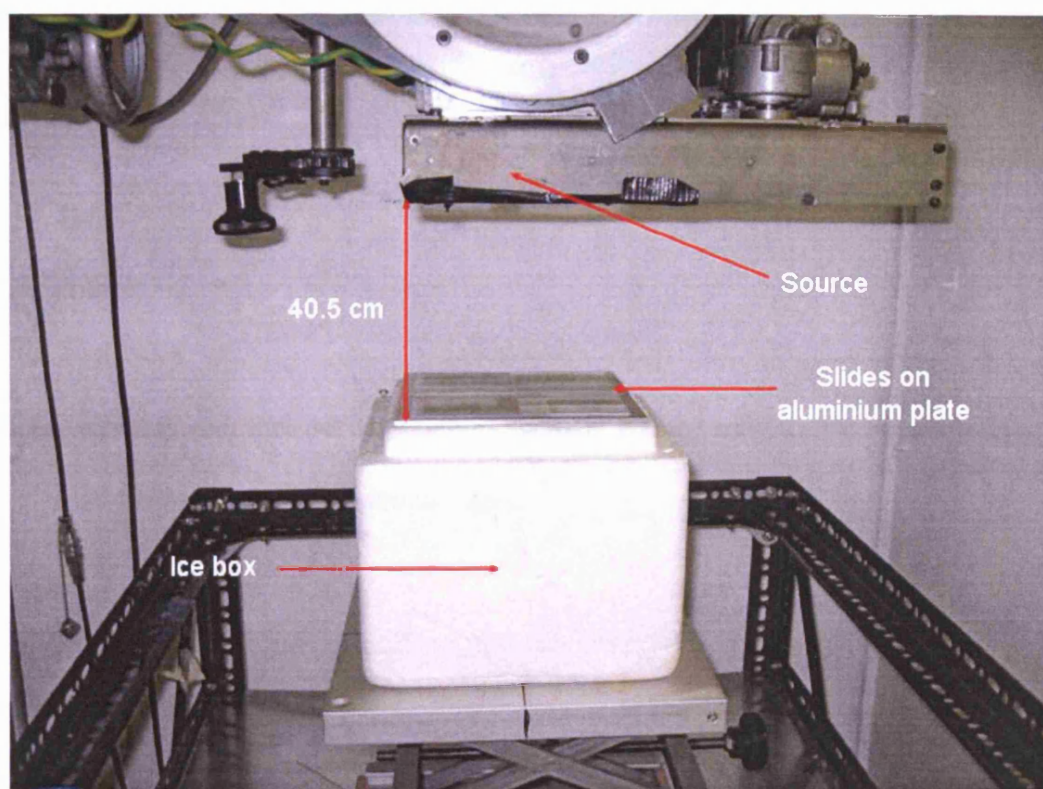


**Figure 3.3:** Slide configuration for irradiation

#### **3.2.5.4 Electrophoresis**

Slides were removed from lysis solution and drained on tissue paper. They were then placed in an electrophoresis tank filled with electrophoresis buffer (300 mM NaOH, 1 mM Na<sub>2</sub>EDTA, pH 13, 4°C). The tank was placed on a tray and surrounded by ice.

Slides were positioned side by side close to the anode end and left for 20 minutes with the power switched off, to permit DNA unwinding.



**Figure 3.4:** Irradiator setup

The power supply was set at 25 V and the current to 300 mA by adjusting the buffer level. Electrophoresis was allowed to proceed for 20 minutes. Slides were then drained and transferred to Petri dishes.

### **3.2.5.5 Neutralisation**

Neutralisation buffer (0.4 M tris, pH 7.5) was added dropwise to cover the slides, and left for 5 minutes. This process was then repeated; the slides removed and allowed to drain prior to staining.

### **3.2.5.6 Staining**

Slides were stained with 50  $\mu\text{l}$  ethidium bromide ( $20\text{ }\mu\text{g ml}^{-1}$ ) and a coverslip applied. Image analysis was performed immediately.

### **3.2.5.7 Image analysis**

Slides were examined using an epifluorescent microscope at x 400 magnification, with a 100 W mercury lamp, an excitation filter (515-535 nm) and barrier filter (590 nm). For each slide, a total of 50 comets were examined. Duplicate slides were examined for each radiation dose. Comets were analysed using purpose-designed image analysis software (Komet 4.0, Kinetic Imaging Ltd., UK.). The software allowed calculation of several comet parameters. Olive tail moment (fraction of tail DNA  $\times d$ , where  $d$  = distance between profile centres of gravity for DNA in the head and DNA in the tail) was selected as the parameter that best represented extent of comet formation.

### **3.3 RESULTS**

#### **3.3.1 Enrichment of Cell Suspensions using HEA MicroBeads**

##### **3.3.1.1 Immunohistochemistry using Paraffin-Embedded Tissue Sections**

In formalin fixed, paraffin embedded tissue sections from the bladder tumour being evaluated, immunohistochemical staining with cytokeratins 8 and 18 was noted to be highly specific for transitional cell epithelium/tumour cells (figure 3.5). Conversely, staining with vimentin was highly sensitive for stromal cells (figure 3.6).

##### **3.3.1.2 Tumour Disaggregation and HEA Cell Selection**

Following removal of necrotic tissue, mechanical and enzymatic disaggregation as described in 3.2.2, viability was measured at 76 %, with a viable cell count of  $1.71 \times 10^6$ . After HEA cell selection,  $1.79 \times 10^5$  cells were collected as the negative fraction, whilst  $1.14 \times 10^6$  cells were eluted as the positive fraction.

### **3.3.1.3 Erythrocyte Count in Eluted Fractions**

HEA positive and negative fractions stained with haematoxylin and eosin were examined to determine the proportion of erythrocytes per cell population (figures 3.7 & 3.8). Over 4 randomly selected fields studied for each fraction, erythrocytes constituted 42.03 % of the cells observed in the HEA negative fraction and 14.68 % of those counted in the HEA positive fraction.

### **3.3.1.4 Cytokeratins 8/18 and Vimentin Immunocytochemistry**

The abundance of cytokeratin 8/18 and vimentin immunostaining (figures 3.9-3.12) in the two cell fractions (expressed as the percentage of cells staining over 4 fields at x 20 magnification) is shown in table 3.3., together with the proportion of the total cell population selected as HEA negative or positive.

## **3.3.2 ACA Response in Cells Derived from Human Tumour Specimens**

### **3.3.2.1 Experimental Success Rates**

A total of 29 patient tumour samples were obtained at the time of TURBT. Of these, 22 (76%) were fully analysed using the method described above. Time from resection to the



start of experimentation was approximately 2 hours. Unsuccessful experiments were due to poor cellular viability (<60%), insufficient cell numbers, or excessive diathermy damage.

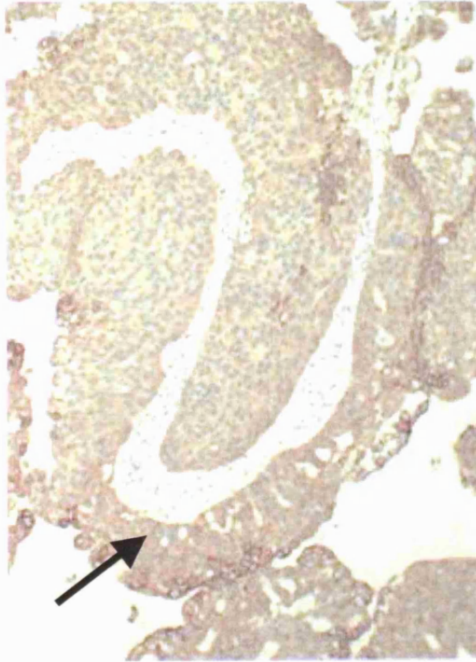
	HEA Positive	HEA Negative
<b>Proportion of Cell Population (%)</b>	86.43	13.57
<b>Cytokeratin 8/18 Abundance (%)</b>	89.12	63.80
<b>Vimentin Abundance (%)</b>	5.86	11.47

**Table 3.3:** The proportion of the total cell population selected as HEA negative or positive and abundance (% of cells staining over 4 fields at x 20 magnification) of Cytokeratin 8/18 and Vimentin immunostaining in the two cell fractions.

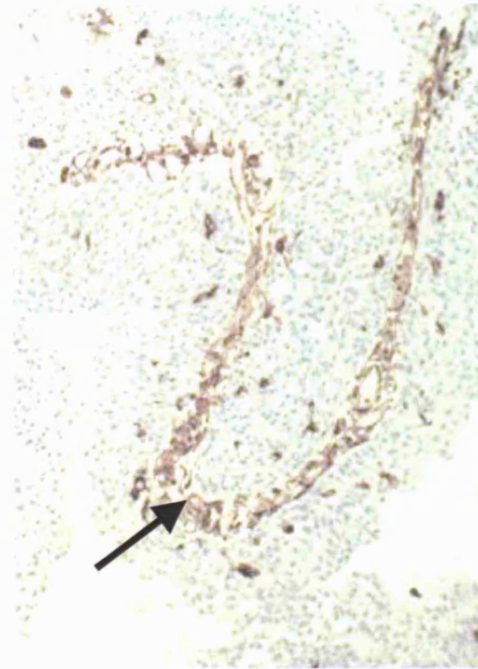
### 3.3.2.2 Patient/Tumour Data

Of the 22 tumour samples analysed, 5 (23%) were from females, 17 (77%) from males. Median patient age at the time of TURBT was 78 years, (range = 56–89 years). Cell counts in the positive fraction varied considerably, from  $3.41 \times 10^5$  to  $1.38 \times 10^7$  (mean =

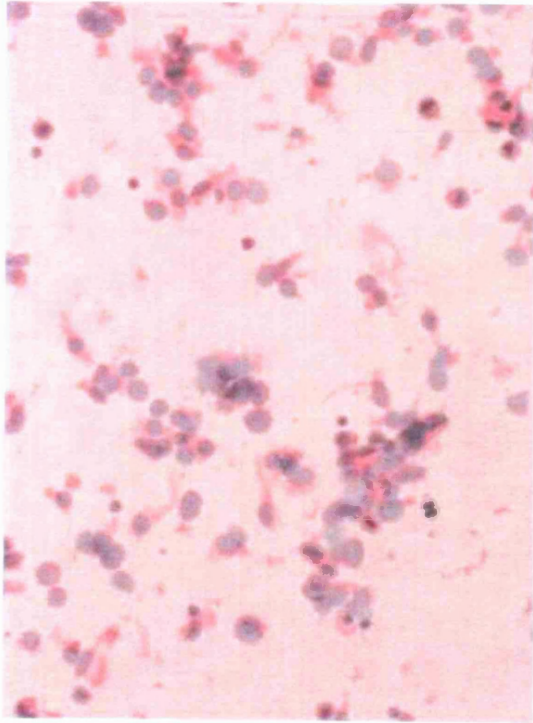
$5.74 \times 10^6$  cells). Negative fraction cell counts ranged from  $3.40 \times 10^4$  to  $3.21 \times 10^6$  (mean =  $3.24 \times 10^5$  cells). Overall viability ranged from 60 to 85% in successful experiments (mean = 69.2%). The 22 tumours included 3 of pathological stage Ta, 4 of T1, 7 of T2, 4 of T4 and 4 unknown/other (table 3.4).



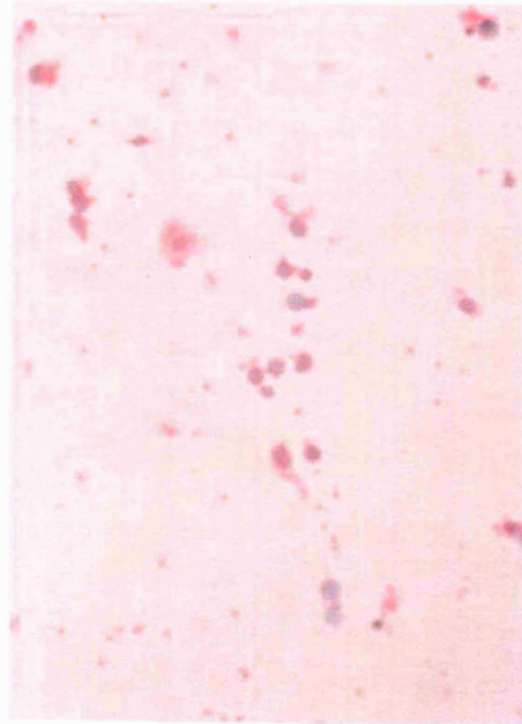
**Figure 3.5:** NBF cytokeratins 8/18 (arrow indicates transitional cell epithelium)



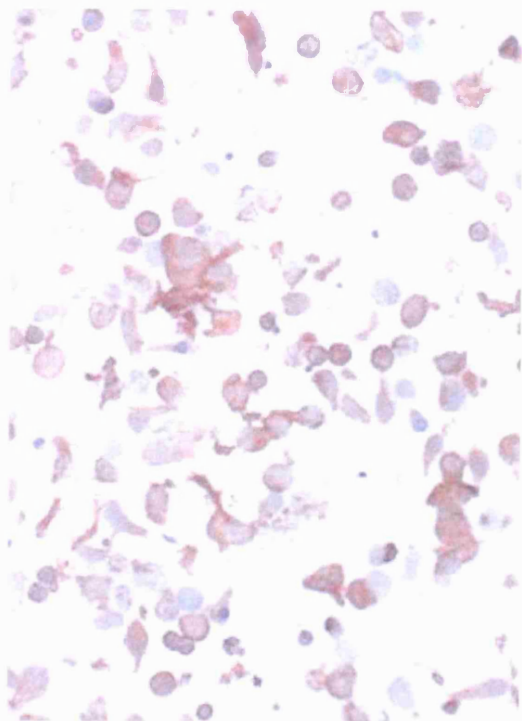
**Figure 3.6:** NBF vimentin (arrow indicates stromal tissue)



**Figure 3.7:** Methanol fixation HEA +ve population (H & E, x20)



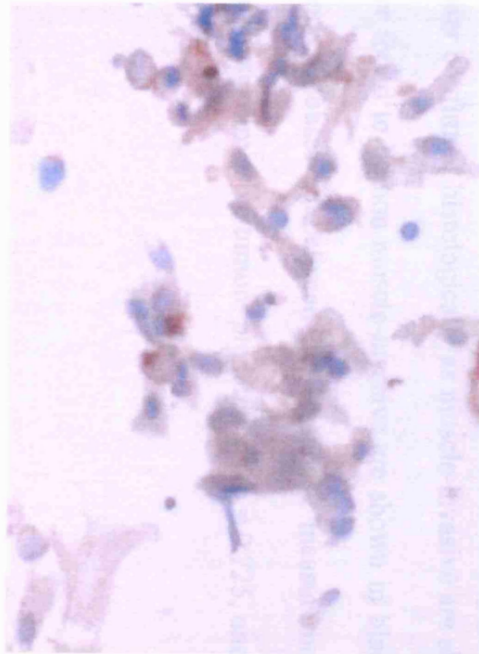
**Figure 3.8:** Methanol fixation HEA -ve population (H & E, x20)



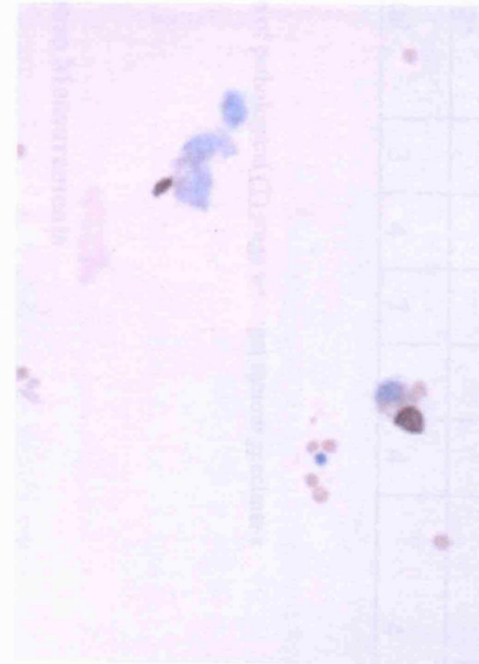
**Figure 3.9:** Cytokeratins 8/18 HEA+ve population (x20)



**Figure 3.10:** Cytokeratins 8/18 HEA-ve population (x20)



**Figure 3.11:** Vimentin HEA+ve population (x20)



**Figure 3.12:** Vimentin HEA-ve population (x20)

Pathological (T) Stage	Ta	T1	T2	T3	T4	Unknown/Other
Total	3	4	7	0	4	4

**Table 3.4:** Distribution of pathological (T) stage amongst tumour specimens

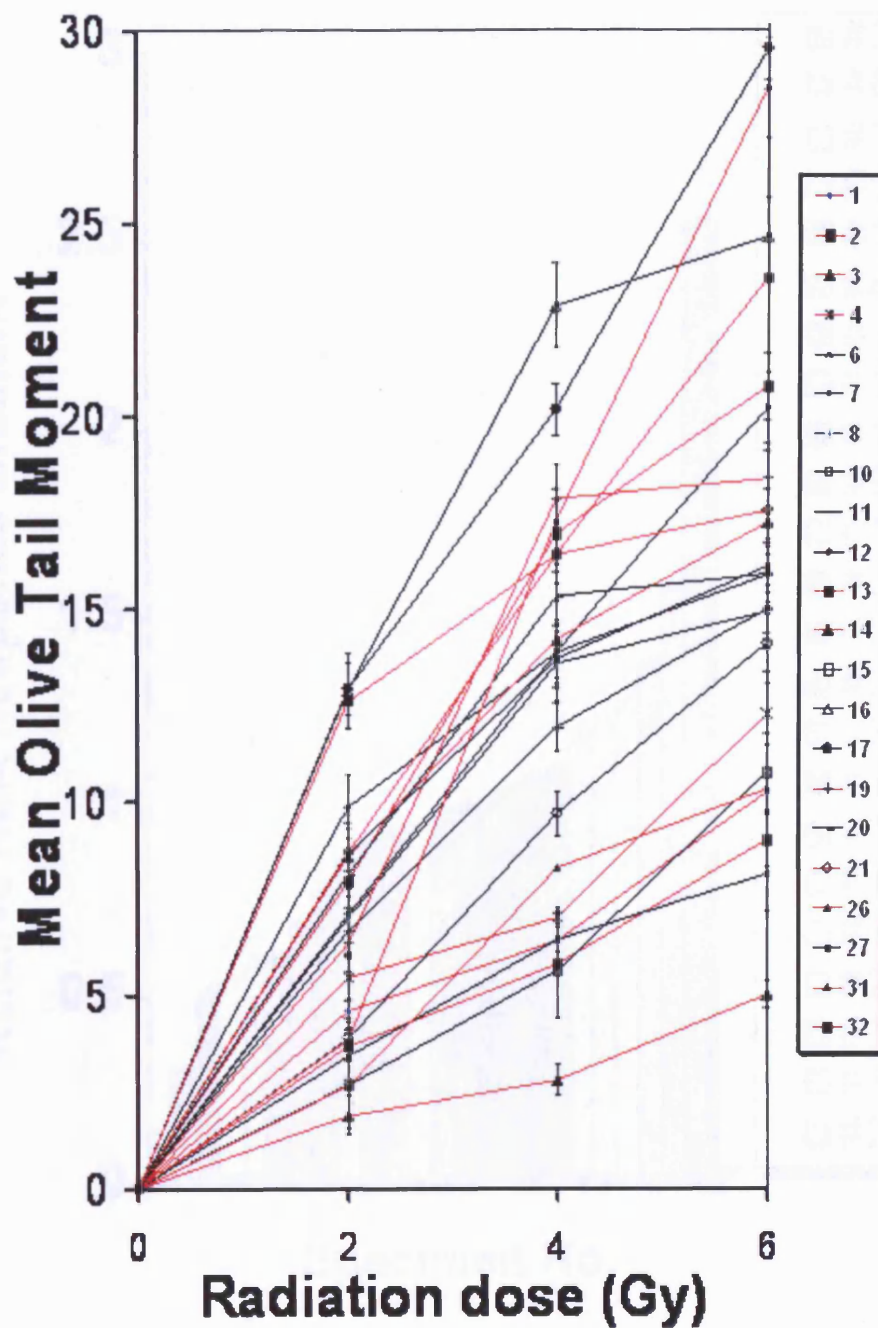
### 3.3.2.3 Dose-response Measurements

22 tumours were analysed using ACA. Measurements of immediate comet formation (Olive Tail Moment) for each tumour analysed are shown at doses of 2, 4 and 6 Gy (figure 3.13), expressed as the mean value of 100 cells per dose (50 cells per slide in duplicate). Considerable spread of response between tumour samples is observed at all doses. Tumours later confirmed histologically as muscle-invasive are shown in red ( $n = 11$ ). Tumours later found to be non-muscle-invasive are shown in black ( $n = 11$ ). No significant difference is observed between dose-response measurements for muscle-invasive versus non-muscle-invasive tumour preparations ( $p = 0.725$ , one-way ANOVA).

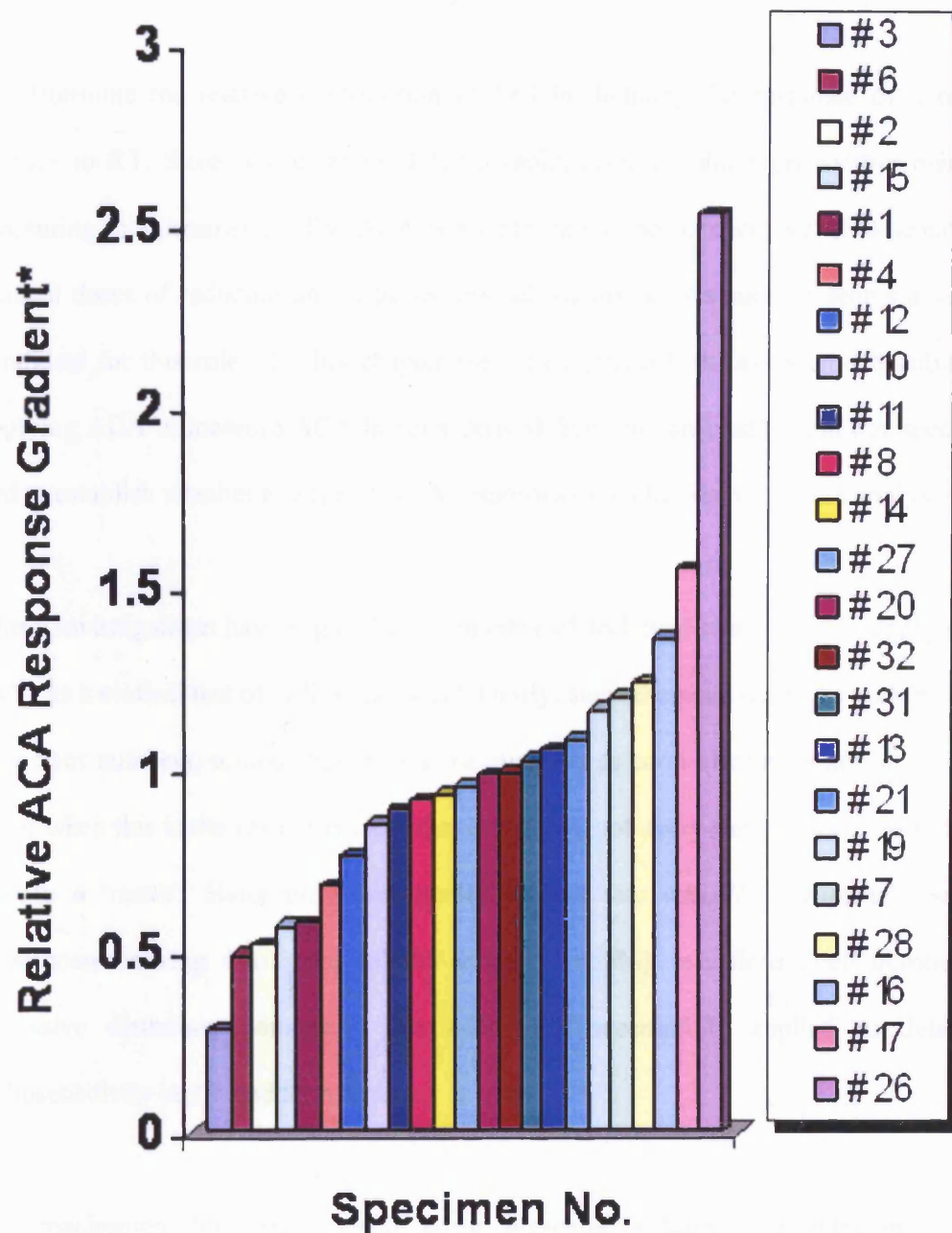
#### **3.3.2.4 Relative Dose-Response Measurements**

In an effort to account for inter-experimental variability, responses were adjusted against dose-response gradients obtained for a control cell line (Raji), irradiated and processed in parallel (discussed in 3.2.6.1). The range of adjusted responses obtained in this way is shown in figure 3.14. A greater than 8-fold difference is observed between the lowest and highest adjusted response. No significant difference is observed between adjusted responses for muscle-invasive versus non-muscle-invasive tumour preparations ( $p = 0.922$ , one-way ANOVA). When responses were compared for tumour preparations, before and after adjustment, the 'rank-order' of response was maintained in all but 3 cases ( $R^2 = 0.8945$  for adjusted response versus initial response).





**Figure 3.13:** Relative spread of dose response curves for 22 tumours evaluated. Tumours later confirmed histologically as muscle-invasive are shown in red. Tumours later found to be non-muscle-invasive are shown in black.



**Figure 3.14:** The range of relative ACA response gradients in the tumour specimens analysed, where  
 \*relative ACA response gradient = gradient of dose-response curve for the tumour specimen/gradient of  
 dose response curve for control (Raji) cell line analysed concurrently.

### **3.4 DISCUSSION**

To determine the relative contribution of IRS in dictating the response of a bladder tumour to RT, there is a clear need for a rapid, accurate and reproducible means of measuring this parameter. The ACA is simple, rapid and inexpensive. It is sensitive at clinical doses of radiation and requires low cell numbers. As such, it seems a suitable candidate for this role. In this chapter we aimed primarily to assess the feasibility of applying ACA to measure ACA in cells derived from human bladder tumour specimens and to establish whether a range of ACA responses exists between tumours analysed.

These investigations have highlighted a number of technical challenges in applying the ACA as a clinical test of radiosensitivity. Firstly, since the assay requires viable cells in sufficient numbers, tumour tissue must be analysed as soon after resection as possible. Even when this is the case, it is clear that at present, not every sample obtained will give rise to a 'result'. Here, the experimental success rate was 76%, with unsuccessful experiments arising from poor cellular viability (<60%), insufficient cell numbers, or excessive diathermy damage. The ACA was successfully applied to determine radiosensitivity in 22 bladder tumours.

The requirement for 'fresh' viable tissue presented a further difficulty in that the pathological stage and therefore the optimal treatment of the patient concerned were unknown at the time of resection. The clinical opinion of the operating surgeon and the results of preceding staging investigations (e.g. magnetic resonance imaging (MRI)) were

sought in an effort to focus on patients with only muscle-invasive tumours. Despite these measures, both muscle-invasive and non-muscle-invasive tumours and indeed other histological tumour types were analysed. This would have important economic implications if the assay were applied, in its current form, as a clinical test.

A further consideration is the presence of cells other than malignant tumour cells in the cell suspension used for the ACA. The magnetic cell separation technique outlined in 3.2.3 was used since it fulfilled the requirement to deliver viable cells after separation. This is to be contrasted with other techniques such as laser-capture micro dissection. Magnetic cell separation was used in tandem with resection of the most exophytic part of the tumour for disaggregation and ACA analysis. A limited assessment of this approach was undertaken using immunocytochemistry with cytokeratins 8/18 and vimentin as cytological markers of tumour cells derived from transitional cell epithelium and cells from underlying stromal tissue, respectively. In the tumour specimen examined these antibodies were highly specific for their target cell types. Immunocytochemical analysis of the separated fractions indicated relative enrichment of the HEA positive fraction with respect to tumour cells. However, the assumption that this relative enrichment is achieved in all samples studied may be invalid. This information might be obtained by assessment of enrichment for a greater number of tumour specimens. In addition, considerable disruption to cellular integrity was observed on the immunocytochemistry slides, which may have resulted from the preceding mechanical and enzymatic digestion in preparing the cell suspensions. This could have implications for the effectiveness of subsequent cell sorting by cell surface antigen recognition. Finally, although HEA is

known to be widely expressed in tumour cells of epithelial origin [152], its expression pattern in invasive tumours, and in cells that have undergone mesenchymal transformation, is unknown.

As discussed in 2.4, superficial and invasive bladder cancer may exhibit important biological differences, for example, with differential regulation of VEGF in non-invasive compared with invasive cell lines. The expression of VEGF mRNA has been shown to be greater in superficial compared with invasive bladder cancer [139]. In addition, CA IX expression has been shown to be greater in superficial than invasive disease [134]. A potential limitation of selecting the most exophytic part of the tumour for ACA analysis is that it may not represent the behaviour of the deeper ‘advancing front’ of the tumour.

The ACA measurements for the tumour samples analysed to date indicate a wide range of responses at clinical doses of ionizing radiation. This is highly important since these differences may reflect actual differences in IRS, which in turn may affect tumour response to RT *in vivo*. Of course, a confounding variable is inter-experimental variability. In an effort to account for this, a control cell line was irradiated and analysed in parallel with the test sample. Dose-response gradients for each tumour sample were expressed as relative to the gradient of the corresponding dose-response curve for the control cell line (as an absolute value). Reassuringly, a wide range of values is again observed, supporting the contention that this is representative of genuine differences in IRS. An alternative approach to correcting for inter-experimental variation, which is currently under investigation, involves the use cells grown in the presence of BrdU.

These can be mixed with the test cells and embedded on the same slides prior to irradiation, but may be readily visualized and analysed separately through a different filter [153]. This would therefore constitute an ‘internal control’ and potentially enhance the accuracy of the assay further.

Given the potential importance of IRS in dictating the response of tumours to RT, further work is required to determine whether ACA measurements of IRS, such as those presented here, correlate with tumour outcome after RT, as represented by tumour response 3-6 months after treatment and ultimately, patient survival. This question will be addressed by ongoing research in our laboratory.

## **5.5 Conclusions**

The ACA shows considerable promise as a means of assessing IRS in cells derived from human bladder tumour specimens, however further optimization of the assay conditions in terms of experimental success rates and reproducibility may be required. These preliminary data indicate that a wide range of ACA responses are observed between tumour specimens, which may represent actual differences in IRS.

## **CHAPTER 4: THE NUCLEAR MATRIX, COMET FORMATION AND INTRINSIC CELLULAR RADIOSENSITIVITY**

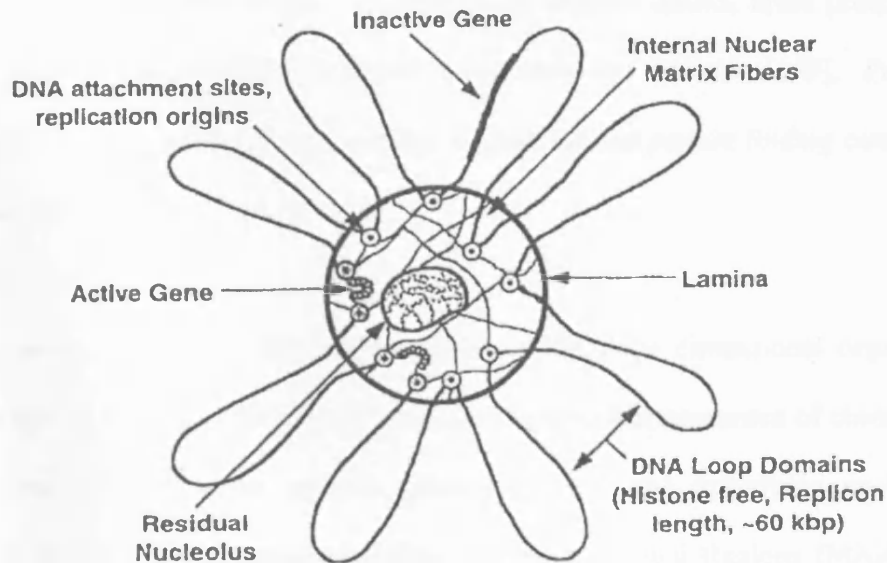
## **4.1 INTRODUCTION**

### **4.1.1 General Background**

There is mounting evidence that structures within the nucleus involved in DNA organization contribute to the balance between repair and fixation of DSBs [154]. One hypothesis proposed to explain this association is that the interactions between the DNA and the nuclear matrix may govern the isolation characteristics between contiguous supercoiled loops of DNA, thereby affecting the stability of DNA in the presence of radiation-induced DNA damage and influencing repair. This is consistent with the view that repair of DNA damage following irradiation not only requires functional repair enzymes, but also adequate access of damaged sites to those enzymes [155].

The nuclear matrix is a macromolecular structure that defines the three dimensional shape of the nucleus. It may be defined as the non-chromatin protein and ribonuclear protein network remaining after the nuclear membrane, cytoskeletal and chromatin elements have been removed by detergents, DNase digestion and high ionic strength buffers. During alkaline comet assay measurements of radiation-induced DNA damage, cells embedded on slides are placed in ice cold lysis buffer immediately after irradiation. This step isolates the nuclear structures involved in comet formation; an entity that can be operationally defined as a nucleoid body, consisting of nuclear matrices and associated superhelical DNA loops (Figure 4.1).





**Figure 4.1:** Schematic diagram of the nucleoid body [126].

#### 4.1.2 The Nuclear Matrix

The nuclear matrix proteins (NMPs) account for approximately 10% of all nuclear proteins (1-5% of the total cell protein) [156]. The NMPs include the lamina/nuclear pore complex proteins and heteronuclear RNA together with hundreds of relatively low abundance proteins comprising the internal nuclear matrix network [157]. The first NMPs to be characterized were the lamins A, B and C which define the nuclear membrane and peripheral nuclear matrix [158]. More recently, proteomic studies have shed further light on the protein content of the nuclear matrix [159-161]. These studies

have indicated that a common set of nuclear matrix proteins exists in nuclei isolated from different cell types and tissues. In addition to nuclear lamins, these proteins include actin, heterogeneous nuclear riboproteins, numatrin and vimentin [159]. Further work has confirmed the presence of molecular chaperones and protein folding catalysts in the nuclear matrix [160].

The principal function of the nuclear matrix is the three dimensional organization of chromatin fibers [162] which in turn governs the spatial arrangement of chromatin. The chromatin structure forms periodic associations with the underlying nuclear matrix through nucleoprotein interactions called Matrix Associated Regions (MARs) forming 50-100kb loops of DNA [163, 164].

#### **4.1.3 The Nuclear Matrix and Gene Transcription**

Initially, the function of the nuclear matrix was felt to be purely architectural. However, this topographical organization is now known to have further important ramifications in terms of DNA replication, gene organization and expression. MAR proteins effectively cause isolation of specific DNA domains, thereby allowing separate regions to be altered topologically to influence gene activity. Actively transcribed genes are associated with the bases of the loops, near MARs [165]. It is thought that these regions are immobilized on the nuclear matrix by multiple dynamic attachment sites. The transcription machinery, specific transcription factors and enzymes (such as histone acetyltransferase

and deacetylase) are thought to mediate the dynamic attachments between actively transcribed chromatin and the nuclear matrix [163, 166]. In the past, three types of NMPs were described in cultured cells; those that were present in all cells irrespective of type, those that were specific to a particular type of cell, for example, epithelial cells and those specific to a particular anatomical site or disease process. Most known NMPs are common to all cell types and physiological states, but several are tissue and cell-type specific [167].

#### **4.1.4 NMPs and Cancer Development**

Because NMPs are known to play a central role in DNA organization and the regulation of gene expression, it seems probable that these proteins are important in the development of cancer. Indeed, one characteristic that is common to all cancer cells is abnormal nuclear shape and such alterations are regarded as a pathological marker of transformation. Nuclear shape reflects the internal nuclear structure and processes and is determined in part by the nuclear matrix.

It is now recognized that aberrations in the nuclear matrix contribute to the development of cancer, but the nature of these aberrations remains incompletely understood. Certainly, cancer development has been found to be associated with the expression of several new nuclear matrix proteins, with a reduction or cessation in the expression of others. This is the case in breast, colon and head and neck [168-170]. Partin *et al.* [171]

reported for the first time a prostate cancer-specific NMP (PC-1). More recent work from the same laboratory [172] has identified a further protein (YL-1), whose expression is correlated with pathological stage.

#### **4.1.5 NMPs and Bladder Cancer**

The NMP composition of bladder tumours has been compared with that of normal bladder samples obtained from organ donors [124]. In this study, the NMP composition of several human bladder cancer cell lines was examined. 2-dimensional electrophoresis was applied to resolve bladder cancer specific NMPs. Specifically, six NMPs were found in tumour samples but not in normal tissues and three were present in normal tissue but were absent in tumour tissue. Five of the six cancer-specific NMPs were detectable in all bladder cancer cell lines.

In a separate study, three of these proteins, BLCA-1, 4 and 6 were microsequenced and specific antibodies raised [173]. Using immunocytochemistry and immunoblotting, expression of the BLCA-4 protein was demonstrated in the cancerous and adjacent normal-looking regions of bladder tumours, but not in the normal tissue of donors with no evidence of disease. This is consistent with the 'field defect' model of urothelial cancer, whereby some of the earliest pre-neoplastic changes can be detected in normal-appearing adjacent tissues. This concept has also been observed in liver metastases from colorectal tumours, where NMP composition was able to distinguish between normal

liver tissue and metastatic cells. Furthermore, normal-looking tissue adjacent to metastases altered its NMP profile compared with hepatocytes elsewhere [174]. As in many cancers, the early diagnosis of bladder cancer is pivotal to successful curative treatment. These reports suggest that comparative studies of nuclear matrix proteins could be of extreme importance in the search for early detection tools.

BLCA-4 is also detectable in the urine of bladder cancer patients [175]. Using a urine-based immunoassay, BLCA-4 levels were determined in 54 patients with histologically proven bladder cancer and 51 healthy volunteers. BLCA-4 levels were found to be significantly higher in bladder cancer patients and this technique yielded sensitivity of 94.6% and specificity of 100%.

Following the observation that another nuclear matrix protein, NMP-22, was frequently found in the urine of patients with urothelial cancers [176], an ELISA based on two monoclonal antibodies (302-18 and 302-22) was developed to quantify NMP-22 in stabilized voided urine samples and is widely used in diagnosing bladder cancer. Although the sensitivity is reputedly better than urinary cytology [177], the clinical utility of this technique as a screening tool will require further in-depth evaluation, involving, for example, patients with urothelial inflammation or other benign urological disease.

#### 4.1.6 NMPs and Radiation-induced Damage

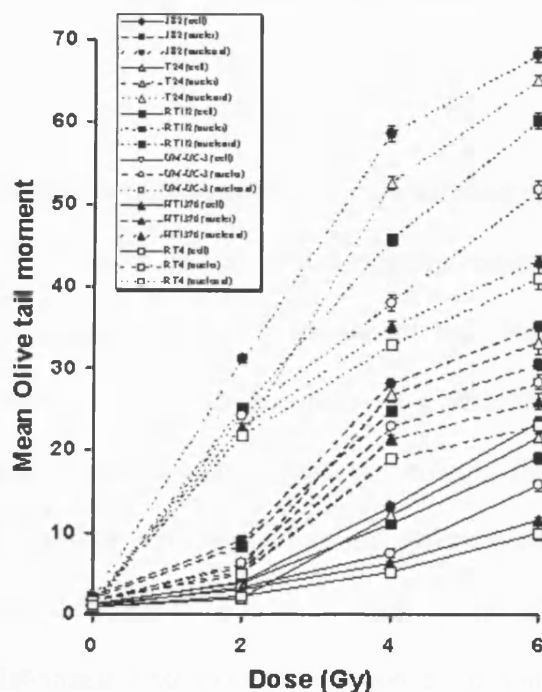
DNA loops in nucleoid bodies form ‘negatively’ supercoiled structures [178]. When nucleoids are exposed to ethidium bromide they initially relax and expand, before contracting in size or ‘rewinding’ as ‘positive’ supercoiling is introduced into individual DNA loops [179]. Loops that have been damaged by irradiation fail to contract to the same extent and the nucleoids remain unwound and relaxed. Alterations in nuclear matrix proteins and/or their binding affects this DNA loop ‘rewindability’ in the presence of DNA damage [180]. Malyapa *et al.* [126] investigated the composition of NMPs and DNA supercoiling characteristics of cell lines of differing radiation sensitivity. They compared wild type Chinese hamster ovary cell lines with a repair deficient, radiosensitive cell line and a radioresistant sub-type, bearing human chromosome 5. The degree of inhibition of loop rewinding was greater in the radiation-sensitive cells compared to radiation resistant ones. High-resolution 2-dimensional electrophoresis was used to determine NMP composition in these cell lines of differing radiosensitivity. 13 NMPs were found to be absent in the radiosensitive cell line compared with wild type. In the radioresistant sub-type cell lines, 5 of these proteins were restored. These results were consistent with the hypothesis that DNA-nuclear matrix anchoring contributes to radiosensitivity by modulating the extent to which supercoiling changes in one DNA loop domain affect the reparability of DSBs in adjacent domains and that this in turn is governed by differences in NMP composition.

Using tumour cells obtained from patients with muscle-invasive bladder cancer prior to radiotherapy, Vaughan *et al.* [103] examined how differences in DNA supercoiling related to outcome after radiotherapy. Cells were irradiated *in vitro* and nucleoid supercoiling behaviour was determined by exposure to ethidium bromide and subsequent flow cytometry. Two predominant forms of supercoiling behaviour were observed as determined by the magnitude of light scattering relative to unirradiated controls. Nucleoids either scattered either approximately 10% (type I) or 35% (type II) more light than controls. Patients with residual disease after completing radical RT showed more type I behaviour (84%), compared with those who achieved disease remission (39%). It was proposed that the ability of nucleoids to adopt positive supercoiling after irradiation was governed by a stronger association between individual DNA loops and the nuclear matrix.

Using a panel of six bladder cancer cell lines, we have previously investigated the effects of irradiation, as determined by the ACA, on intact cells, their corresponding isolated cell nuclei and then nucleoid bodies isolated using the lysis conditions described earlier\* (figure 4.2). These studies demonstrated greater comet formation in isolated nucleoid bodies than in nuclei, which in turn was greater than that observed in the intact parent cell, at each dose of X-irradiation (0-6Gy). This was the case in all six cell lines studied. Moreover, the same 'rank order' of initial comet formation was retained at each dose. These data clearly indicate that a feature of the nucleoid body governs the extent of comet formation, which in turn reflects intrinsic cellular radiosensitivity.

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\* AL Moneef *et al.*, unpublished data



**Figure 4.2:** The extent of initial comet formation, as measured by mean Olive tail moment, for prepared nucleoid bodies vs. isolated nuclei vs. intact cells of the six cell lines investigated, over a dose range of 0 - 6 Gy, as determined by the ACA.

#### 4.1.7 Summary and Aims

The role of the nuclear matrix in dictating the spatial organisation of DNA is well recognized. Current opinion suggests that the nuclear matrix proteins play a significant role in malignant transformation and radiation response in bladder cancer. It is also becoming increasingly clear that in bladder cancer, like many other malignancies, NMPs could provide valuable substrates for cytopathological screening. NMPs have been



shown to be shed by tumours as cells die and they are detectable in the serum and urine of cancer patients [181].

It is clear from earlier studies (AL Moneef *et al.*, unpublished data) that in bladder cancer cell lines, the nucleoid body is critical in dictating the extent of comet formation and thereby may directly influence intrinsic radiosensitivity. Nuclear matrix proteins are implicated in this role and further work is required to explain this association. We aim to examine the nuclear factors that influence comet formation (and therefore radiosensitivity). This will be investigated by using Pulsed Field Gel Electrophoresis and the H2AX immunoassay, to determine DSB rates in a panel of bladder cancer cell lines and examine the relationship with comet formation as demonstrated by the ACA. In addition, a comparative proteomic analysis of bladder cancer cell nuclei will be undertaken, in cell lines of differing radiosensitivity, to establish whether nuclear factors (such as nuclear matrix composition) may influence comet formation and therefore radiosensitivity.

## **4.2 MATERIALS AND METHODS**

### **4.2.1 Cell lines and Culture Conditions**

Six cell lines were used, all derived from high-grade invasive bladder tumours; RT112, UM-UC-3, HT1376 (American Tissue Culture Collection) and J82, T24, RT4 (gift from

Professor Kilian Mellon, Professor of Urology, Leicester General Hospital). All cell lines were maintained as a monolayer by subcultivation, in Dulbecco's Minimum Essential Medium (Sigma, UK), supplemented with 10% Fetal Calf Serum. Cells were harvested at 75% confluence, using 1 x trypsin at 37°C, washed in prewarmed PBS and counted using a haemocytometer. Viability was determined using trypan blue exclusion.

Five of the six cell lines were used in attempting H2AX immunoassay (4.3.4) and pulse-field gel electrophoresis (carried out concurrently, see 4.2.3) due to difficulties in establishing a satisfactory monolayer for RT4. Three of the six cell lines (J82, RT112 and HT1376) were later used in clonogenic analysis to confirm their relative radiosensitivities prior to proteomic studies. For reasons of simplicity, two of these, J82 and HT1376 were used in the preliminary comparative proteomic study, representing the most radiosensitive and radioresistant cell lines respectively.

#### **4.2.2 Clonogenic Assay**

Three cell lines (J82, RT112 and HT1376) were assessed using the clonogenic assay, to confirm the 'rank order' of clonogenic survival (3.1.3). Plating efficiencies were calculated and seeding densities derived as shown below. Cells were seeded in 60 x 15 mm Petri dishes and incubated at 37°C for 4 hours. The cells were then irradiated at 2, 4 and 6 Gy (as in 3.2.4.3), with unirradiated controls processed in parallel and then

incubated at 37°C for 14 days. Colonies were fixed with 3:1 ethanol:acetic acid and stained with crystal violet (Sigma, UK). Plating efficiency was defined as the number of colonies scored as a percentage of viable, unirradiated control cells plated. The surviving fraction, defined as the ratio of colonies formed to cells plated (with correction for plating efficiency (PE), was calculated using the formula:  $SF = \text{colonies counted} / \text{cells seeded} \times (PE/100)$ .

Cell line	Plating Efficiency (%)	Seeding Densities
J82	65	0 Gy = 100, 2 Gy = 300, 4 Gy = 500, 6 Gy = 800
RT112	45	0 Gy = 200, 2 Gy = 600, 4 Gy = 800, 6 Gy = 1000
HT1376	45	0 Gy = 200, 2 Gy = 600, 4 Gy = 800, 6 Gy = 1000

**Table 4.1:** Plating efficiencies and seeding densities used for clonogenic analysis.

### 4.2.3 Pulse Field Gel Electrophoresis

#### 4.2.3.1 General

Pulse Field Gel Electrophoresis (PFGE) was used to quantify radiation-induced DSB rates in the cell lines. Experiments were performed at the MRC Radiation and Genome Stability Unit (RAGSU), Harwell, Didcot, Oxfordshire. PFGE was carried out for J82, T24, UM-UC-3, RT112 and HT1376.

#### **4.2.3.2 Labelling and Irradiation**

Cells were split into 12.5 cm<sup>2</sup> cell culture flasks with 3 ml of DMEM. 30 µl of <sup>3</sup>H Thymine was added to each flask and placed in the incubator at 37°C for 24 hours. Fresh media was added, containing 0.3 ml free Thymine per flask (0.1 ml Thymine/ml medium) and left for 1 hour. The flasks were then placed on a bed of ice and transported to the irradiator (calibrated to deliver 3Gy min<sup>-1</sup>). Cells were irradiated at 7, 14 and 20 Gy with the time of exposure adjusted to provide the level of irradiation required.

#### **4.2.3.3 Preparation of Agarose Plugs**

Media was removed and the cells washed with PBS, before harvesting with 1 ml 1 x trypsin and counting. Cells were then transferred to universal tubes on ice and 1 ml of fresh medium added. Cells were then pelleted by centrifugation at 1,750 rpm (10°C). After extracting the supernatant, pellets were resuspended in 1,000 µl LMA solution (0.4 g LMA, 50 ml PBS) and transferred to ice cold plug moulds (25-30 000 cells per plug in 100 µl suspension, 10 plugs per dose). After allowing the plugs to set at 4°C, they were transferred to universal tubes containing 6 ml proteinase K lysis solution (0.6 g n-Lauroyl-sarcosine, 30 ml 0.5M EDTA, 15 mg Proteinase K (all Sigma, UK)), returned to the refrigerator for 1 hour, then placed in the incubator (37°) overnight.

#### 4.2.3.4 Pulse Field Gel Preparation

Agarose solution for the pulse field gel (150 ml TBE buffer, 1.2 g agarose) was poured into the gel tray on a flat surface. The combs were removed and one plug placed in each well, before sealing with an agarose plug. The gel was transferred to a CHEF Mapper tank filled with 2 L of 0.5 x TBE and any air bubbles removed. The cooler was set to 16°C, cooler timer to 100 minutes, run time to 96 hours and the programme started. After 96 hours the gel was placed in a tray and covered with 500ml 0.5 x TBE. For staining, 35 µl Ethidium bromide was added to the tray and incubated for 1 hour at 4°C.

#### 4.2.3.5 Scintillation Counting

After checking DNA bands by UV light, the gels were cut as shown in figure 4.3, and then individual squares were added to scintillation vials containing 0.5ml HCl solution and melted. 3ml of scintillation fluid was added to each vial and vortexed. The scintillation counts were used to calculate the Fraction of Activity Released (FAR) from each well during PFGE as a result of DSBs. FAR was calculated using the following equation:

$$\text{FAR (\%)} = \frac{\text{Counts in Lane}}{\text{Total Counts (well and lane)}} \times 100$$

	0Gy	7Gy	14Gy	20Gy
1				
2				
3				
4				
5				

**Figure 4.3:** Gel cutting for scintillation vials.

#### 4.2.4 H2AX Immunoassay

The H2AX immunoassay was used in conjunction with PFGE in an effort to ascertain low dose radiation-induced DSB rates between cells of differing radiosensitivity as characterized by ACA and clonogenic survival measurements. Five cell lines were examined in this way (UM-UC-3, T24, RT112, HT1376 and J82). H2AX was also undertaken at RAGSU, Harwell, Didcot, Oxfordshire. Cells were seeded in adapted Hosterfan dishes to form a near-confluent monolayer at the time of the experiment. Cells

were irradiated at 0.5, 1 and 2 Gy and placed immediately on ice. Cold medium was then removed and replaced with fresh media at 37°C, before placing the samples in the incubator at 37°C for 30 min to allow foci development. Cells were then washed with PBS and fixed with 1ml 3% paraformaldehyde for 1 hour at room temperature or 4°C overnight. After fixation, cells were washed with PBS (x 3) and permeabilised with 1ml 1% Triton-X for 10 minutes. Cells were washed again and 1 ml of blocking agent (1% fish skin gelatin and 1% bovine serum albumin) applied for 1 hour. After removing the blocking agent, primary antibody solution (Rabbit anti-phospho-H2AX (Upstate Technology, USA) diluted 1:200) was added for 2 hours at room temperature. Cells were then washed in PBS (x 3), 1 ml of blocking agent added for a further 1 hour and the secondary antibody (FITC goat anti-rabbit (Stratech) diluted 1:100) added for 1 hour. Cells were then washed with PBS (x 2), stained with Vectorsheild (PI) and a coverslip added prior to image capturing.

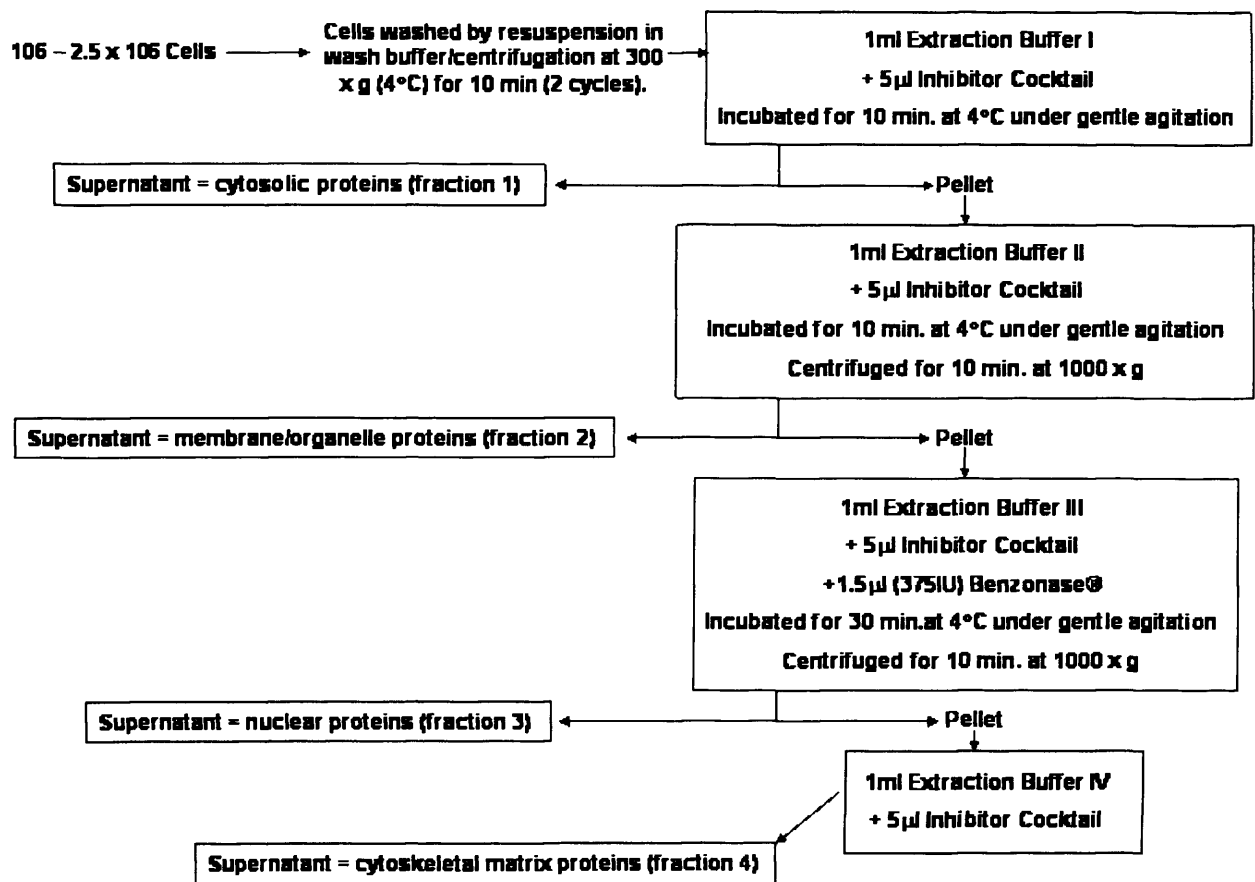
#### **4.2.5 Sub-cellular Proteome Extraction**

Further experiments were undertaken to examine the protein content of the cell nucleoids (particularly nuclear matrix proteins) with a view to examining their potential role in dictating comet formation and therefore IRS. Sequential extraction of the cell proteome was achieved using the Sub-cellular Proteome Extraction Kit (S-PEK, Calbiochem, UK), in accordance with the manufacturers' instructions. The protocol described below was

applied to up to  $1 \times 10^6$  cells. For greater numbers, buffer amounts were scaled accordingly. The extraction protocol is summarised in Figure 4.4.

Cells were harvested at 75% confluence using 1 x trypsin and transferred to 15 ml Falcon tubes. Cells were pelleted by centrifugation at 300 x g, 4°C, for 10 minutes. The supernatant was removed and discarded. The pellet was resuspended in 2 ml ice-cold Wash buffer and transferred to a microcentrifuge tube. Cells were pelleted at 300 x g, the supernatant removed and the wash step repeated. Cells were resuspended in a mixture of ice-cold extraction buffer I and 5 µl of protease inhibitor cocktail and incubated at 4°C for 10 minutes under gentle agitation. Insoluble material was sedimented at 1000 x g, 4°C, for 10 minutes. The supernatant was removed as fraction 1 (cytosolic proteins). Pelleted material was resuspended in a mixture of ice-cold extraction buffer II (1 ml) and 5 µl of protease inhibitor cocktail and incubated at 4°C under gentle agitation, for 30 minutes. Insoluble material was then sedimented at 6000 x g for 10 minutes. The supernatant was removed as fraction 2 (membrane/organelle proteins). Sedimented material was then resuspended in a mixture of ice-cold extraction buffer III (500 µl), 5 µl of protease inhibitor cocktail and 1.5 µl ( $\geq 375$  U) Benzonase<sup>®</sup> and incubated at 4°C under gentle agitation, for 10 minutes. Insoluble material was sedimented at 6800 x g, 4°C, for 10 minutes. The supernatant was removed as fraction 3 (nuclear proteins). Insoluble material was resuspended in a mixture of 500 µl extraction buffer IV (room temperature) and 5 µl of protease inhibitor cocktail and retained as fraction 4 (cytoskeletal matrix proteins). Fractions were divided into 100 µl aliquots and stored at -20°C for future use.





**Figure 4.4:** Schematic representation of protein extraction protocol (adapted from S-PER (Calbiochem UK) manufacturers' instructions).

#### **4.2.6 Protein Precipitation**

Samples obtained as described in 4.2.5 were subject to methanol precipitation to remove organic soluble contaminants (e.g. detergents, lipids) prior to 1 and 2-dimensional polyacrylamide gel electrophoresis (2-D PAGE). 3 volumes of ice cold 100% methanol were added to the extract and proteins were allowed to precipitate at -20°C for at least 2 hours.

#### **4.2.7 Bio-Rad Protein Assay**

1ml of diluted dye reagent (Bio-Rad, UK) was added to 5 µl of protein sample and vortexed, before transferring to a cuvette and incubating for 5 minutes. Absorbance at 595 nm was measured in a spectrophotometer, using two preparations for each sample to provide a mean. Concentrations were derived from a standard curve of protein concentration against optical density.

#### **4.2.8 Analysis of Sub-cellular Protein Extracts by 1-Dimensional Electrophoresis**

Samples from all fractions extracted using the S-PEK kit were evaluated with 1-dimensional tris-glycine SDS polyacrylamide gel electrophoresis, to generate protein 'profiles' for each cell line. After protein assay (4.2.7), samples were suspended in

sample buffer (100 mM tris, pH 8.8, 4% SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol, with dithiothreitol (DTT) 200 mM added fresh). 16cm x 18cm 8% vertical resolving gels were prepared with 1.5 mm spacers (30% acrylamide mix, 1.5 M tris (pH 8.8), 10% SDS, 10% ammonium persulfate and TEMED (0.01%)) with 5% stacking gels (30% acrylamide mix, 1.0 M tris (pH 6.8), 10% SDS, 10% ammonium persulfate & 0.01% TEMED). 20 µg of sample was loaded per lane, together with marker proteins and electrophoresis was run initially at 30 mA for approximately 5 hours.

#### **4.2.9 Coomassie staining**

After electrophoresis gels were fixed by soaking in fixing solution (50% ethanol, 10% acetic acid) overnight. Fixing solution was aspirated off and the gel washed twice in washing solution (50% methanol, 5% acetic acid, 30 minutes per wash). Washing solution was then aspirated, and Coomassie stain (Bio-Rad, UK) added (prepared as per manufacturer's instructions). Gels were stored in 5% acetic acid (in water) until further use.

#### **4.2.10 Western Blotting**

##### **4.2.10.1 General**

Western blotting was employed to establish the purity of the 4 subcellular protein fractions extracted using the method described in 4.2.5. A panel of 4 commercially available antibodies (representing the 4 derived fractions) were used: lamin A,  $\alpha$ -tubulin, Nucleolin and  $\alpha$ -complex V (BD Biosciences, USA). The most radiosensitive (J82) and most radioresistant (HT1376) cell lines were used for this purpose. Western blotting was also used to validate the findings of tandem capillary liquid chromatography/mass spectrometry (MS/MS, 4.2.12), using antibodies to vimentin, calnexin and  $\beta$ -catenin (BD Biosciences, USA).

##### **4.2.10.2 Electrophoresis**

8% resolving gels with 5% stacking gels were prepared using 1.5 mm spacers as in 4.2.8. Lysates in sample buffer were placed in boiling water for denaturation for 10 minutes and then placed on ice before loading. The electrophoresis tank was filled with electrophoresis buffer (15.1 g tris-base, 94 g glycine, 950 ml deionised water, 50 ml 10% SDS). 20  $\mu$ g of protein in sample buffer (4.2.8) was applied to each well together with 10  $\mu$ l of marker protein solution. The power supply was set to supply a constant voltage of 115 V (~ 15 mA) and electrophoresis performed for 60-90 minutes.

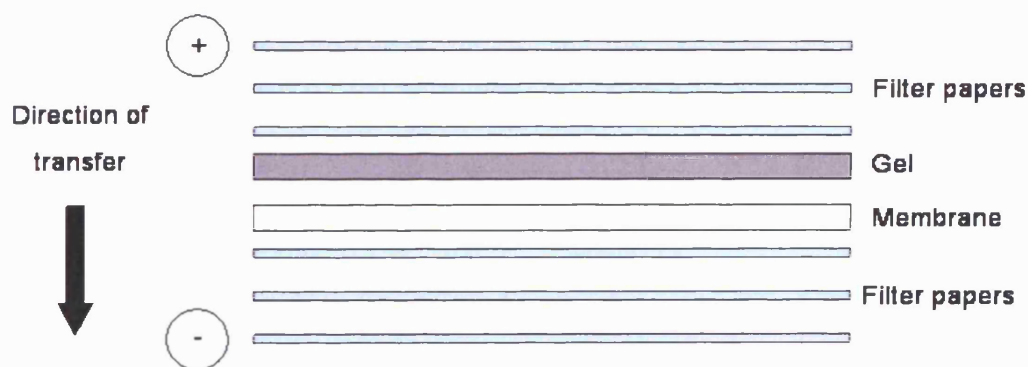
#### **4.2.10.3 Gel Transfer**

The Bio-Rad wet blotting apparatus (transfer kit) was assembled in accordance with the manufacturers' instructions. Filter papers (x 6) and nitrocellulose membranes were cut to size. The membrane was soaked in methanol and excess discarded. Filter papers were covered with blotting buffer (10 x stock solution = 58.1 g tris-base, 29.3 g glycine, 37.5 ml deionised water, made up as 1 x working solution with 200 ml methanol and 720 ml deionised water). The gel was removed from the electrophoresis apparatus and placed on the membrane and filters as shown in figure 4.5. A further 3 filter papers were placed on top of the gel and the layers were rolled gently to exclude air bubbles. The layers were then transferred to the transfer apparatus and screwed tight. The power supply was set to a constant 200 mA (~10 V) and run for 60-90 minutes. After transfer, the membrane was placed in a dish with 0.1% ponceau stain (Sigma, UK) for 5 minutes under gentle agitation and then air dried.

#### **4.2.10.4 Western Blot Staining**

The membrane was soaked in methanol for 1 minute and transferred to TBS buffer (18 ml tris-Cl pH8.0 (1 M), 24.5 ml NaCl (5 M), 900 ml deionised H<sub>2</sub>O and 900 µl TWEEN 20) under gentle agitation. The membrane was then placed in milk blocking agent (2.5 g (5%) milk powder in 50 ml TBS buffer) for 60 minutes under gentle agitation. The membrane was then transferred to an A4 coverslip and 3 sides were sealed. Primary

antibody solution was made up to the appropriate concentration, added to the coverslip and the fourth side sealed after removing any air bubbles. The membrane was left in the coverslip for 60 minutes, removed and washed in TBS-T buffer (4 x 10 minutes). The secondary antibody solution (1:2000 (10 ml TBS-T milk buffer: 5  $\mu$ l secondary agent)) was then applied and mixed for 45 minutes before washing in TBS-T (4 x 10 minutes). The membranes were laid out on Clingfilm and the tertiary agent (Amersham Biosciences, UK) applied for 1 minute, before an image was recorded using autoradiography.



**Figure 4.5:** Assembly of wet blotting apparatus.

#### **4.2.11 2-Dimensional Polyacrylamide Gel Electrophoresis (2-D PAGE)**

Fractions 3 and 4 extracted from J82 and HT1376 were further assessed using 2-D PAGE with a view to resolving nucleoid proteins and performing a comparative proteomic analysis.

##### **4.2.11.1 Sample Preparation**

All materials were from Amersham Biosciences, UK. Protein extracts were resuspended in 8 M urea, 4% CHAPS, 60 mM DTT, 2% Pharmalyte<sup>TM</sup> and 0.002% bromophenol blue. 200 µg of protein was loaded for isoelectric focusing (4.2.11.2) per sample.

##### **4.2.11.2 Isoelectric Focusing (IEF)**

IEF was performed on a Multiphor II unit. 13 cm ready-made Immobilized pH Gradient (IPG) strips (pH range 3-10) were selected for an overview of protein composition. 250 µl rehydration solution (8 M urea, 0.5% (w/v) CHAPS, 0.2% (v/v) IPG buffer, 0.002% bromophenol blue) was applied per strip and the strip overlaid with 3 ml of DryStrip cover fluid to minimize evaporation and urea crystallization. Strips were rehydrated for 10 hours. After positioning the strips in the aligner and covering with Drystrip Cover

Fluid, samples were applied near the anode, using a cup loading technique. IEF was commenced initially for 1 minute at 300 V and then allowed to proceed for 5 hours at 3500 V. After IEF, IPG strips were stored at -70°C prior to second dimension electrophoresis.

#### **4.2.11.3 Second Dimension SDS-PAGE**

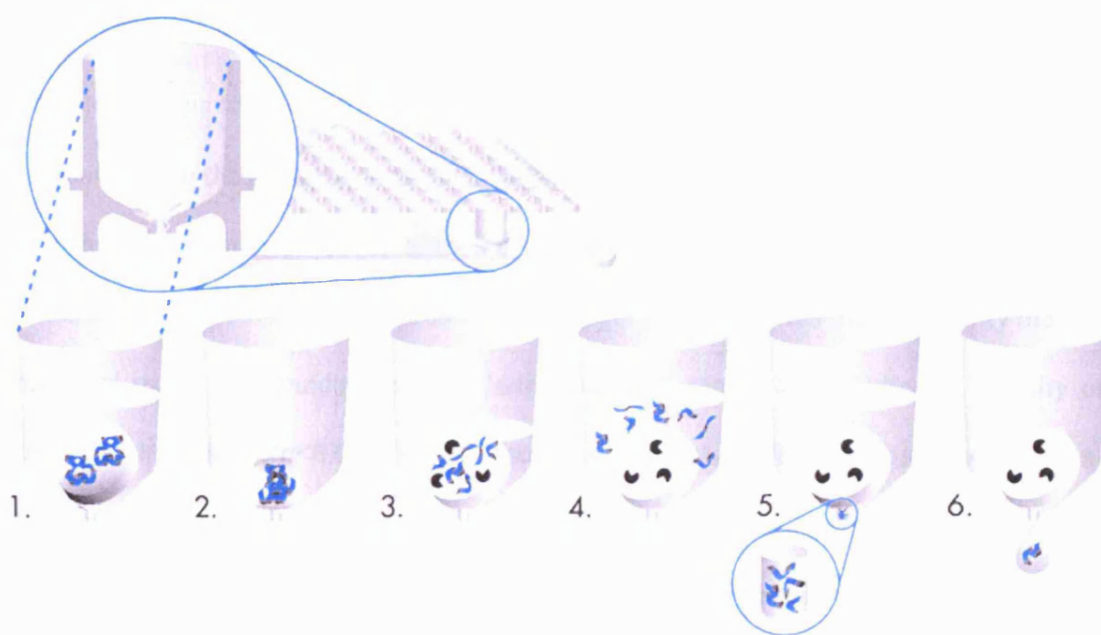
18 x 16 cm (1.5mm thickness) 8% vertical gels were cast (30% acrylamide mix, 1.5 M tris (pH 8.8), 10% SDS, 10% ammonium persulfate and TEMED (0.01%)). IPG strips were equilibrated (in separate tubes) initially in 10 ml equilibration buffer (2% SDS, 50 mM tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 0.002% bromophenol blue) with 100 mg DTT for 15 minutes, and then for a further 15 minutes in 10ml equilibration buffer with 250 mg iodoacetamide. After equilibration, the tank was filled with electrophoresis buffer (25 mM tris-base, 192 mM glycine, 0.1% SDS) and IPG strips applied to the top of the gel, together with a marker protein/agarose plug (marker proteins mixed with an equal volume of molten 1% agarose solution). Electrophoresis was run initially at 15 mA for 15 minutes followed by 30 mA for approximately 5 hours until complete. Coomassie staining was performed as described in 4.2.9.



#### 4.2.12 Tandem Capillary Liquid Chromatography/Mass Spectrometry (MS/MS)

##### Analysis of Nuclear Proteins (from 1-D Gels)

Since 2-D resolution of the samples was incomplete (see 4.4), protein fractions 3 and 4 extracted from J82 and HT1376 were examined further using liquid chromatography/mass spectrometry analysis in a further effort to perform a comparative study of nucleoid proteins. 1-D profiles were again generated as described in 4.2.8. Areas of interest (see figure 4.12) were excised manually and divided into 1 mm strips for further analysis.



**Figure 4.6:** Digestion of gel fragments excised for further analysis (1 = destaining, 2 = fragment shrinking with acetonitrile, 3 = trypsin digestion, 4 = peptide extraction and washing, 5 = peptide capture on C<sub>18</sub>, 6 = elution in MS compatible buffer). From Millipore In-Gel Digest Kit, technical information (Millipore, USA).

Each sample was destained and sequentially digested, under the same conditions, using trypsin-based Montage In-Gel Digest Kit system (Millipore USA, figure 4.6). 4 µg of digested peptide mixture in MS-compatible buffer was loaded onto a Waters Nano-Eze 3 µm biphasic (strong cation exchange/reversed phase) capillary column and washed with a mixture of buffer A (5% acetonitrile, 0.1% formic acid and 95% DDI H<sub>2</sub>O) and B (95% acetonitrile, 0.1% formic acid and 5% DDI H<sub>2</sub>O), initially at a ratio of 93:7% (A:B), increasing biphasically to 20:80%. Tandem (MS/MS) was performed using a Micromass Q-TOF 2<sup>TM</sup> mass spectrometer.

#### **4.2.13 Data Set Processing**

MS/MS datasets were searched using the mascot (SwissProt) ([www.matrixscience.co.uk](http://www.matrixscience.co.uk)) database, using the associated statistical software. Ion search parameters were adjusted to include only human proteins, allowing for 2 missed cleavages and modification by oxidation. Mowse ion scores represented  $-10\log(P)$ , where P was the probability that the observed match is a random event. Individual ions scores > 32 indicated identity or extensive homology ( $p < 0.05$ ). Protein scores were derived from ions scores as a non-probabilistic basis for ranking protein hits. A small correction was applied to reduce the contribution of low-scoring random matches. This correction was a function of the total number of molecular mass matches for each query and the width of the peptide tolerance window. Results with a protein score of > 45 were deemed to be significant. After searches were complete, results from all digests were collated and proteins detected in corresponding fractions from both cell lines (i.e. 'matched') were identified. Keratins

and unmatched proteins were also highlighted. Basic information regarding function and subcellular localization of identified proteins was obtained from SwissProt datasheets prior to formal literature searches.

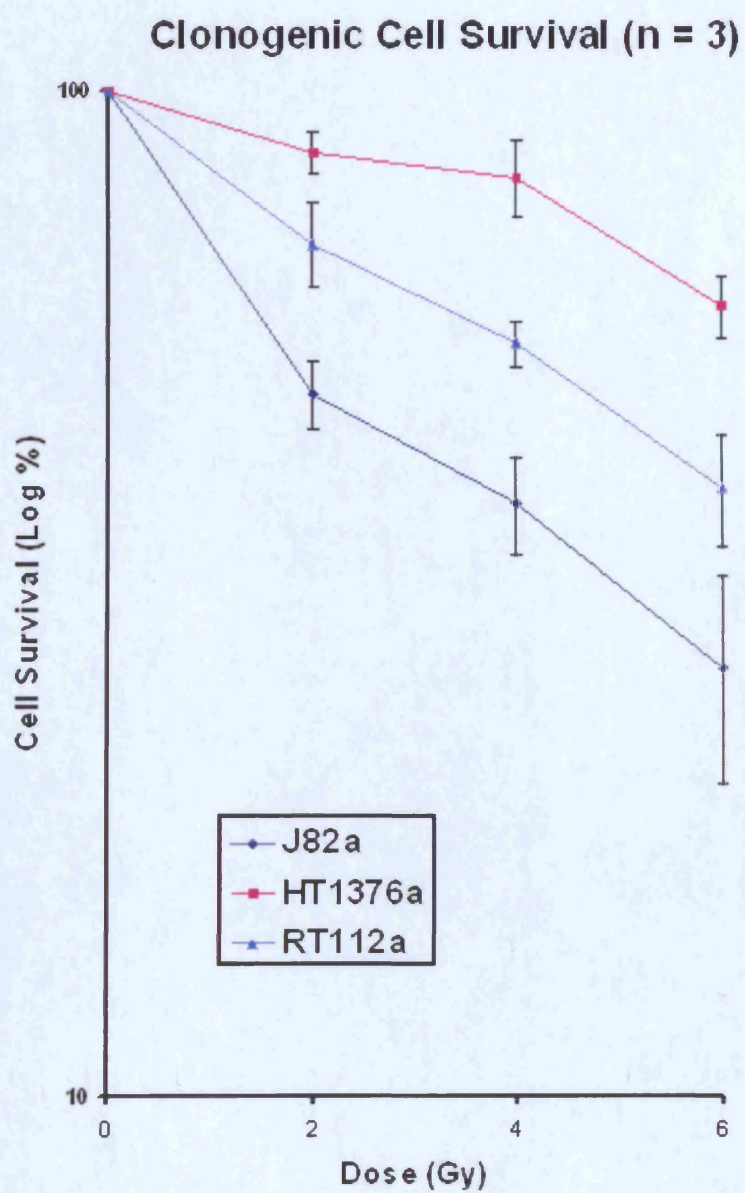
## **4.3 RESULTS**

### **4.3.1 Clonogenic Cell Survival**

Clonogenic cell survival curves were generated (figure 4.5) for J82, RT112 and HT1376. At all dose points, clonogenic cell survival was lowest in J82, followed by RT112, with HT1376 giving the highest cell survival values ( $n = 3$ ).

### **4.3.2 Pulse Field Gel Electrophoresis (PFGE)**

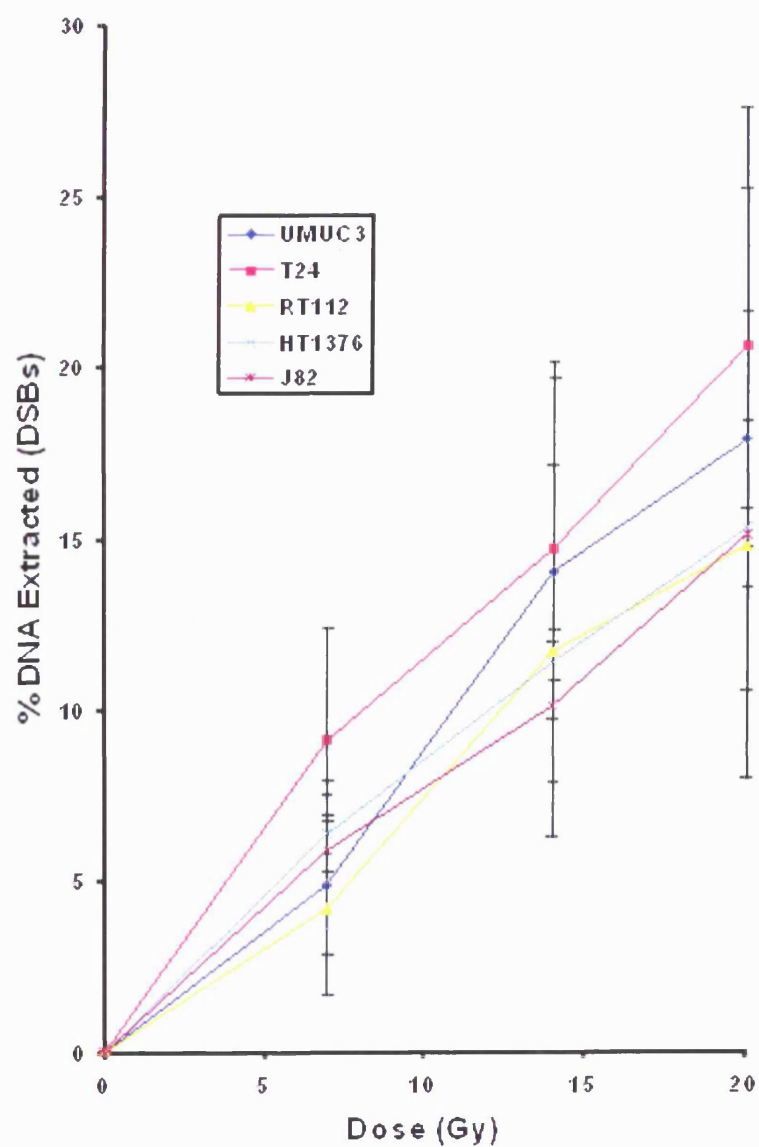
Five cell lines (UM-UC-3, T24, RT112, HT1376 and J82) were analysed using PFGE (figure 4.6), at doses of 7, 14 and 20 Gy ( $n = 3$ ). No significant differences were observed between dose responses over the dose range examined ( $p = 0.750$ , one-way ANOVA).



**Figure 4.5:** Clonogenic cell survival curves for the bladder cancer cell lines J82, HT1376 and RT112. A

'rank order' of radiosensitivity is established and is consistent at all dose points.

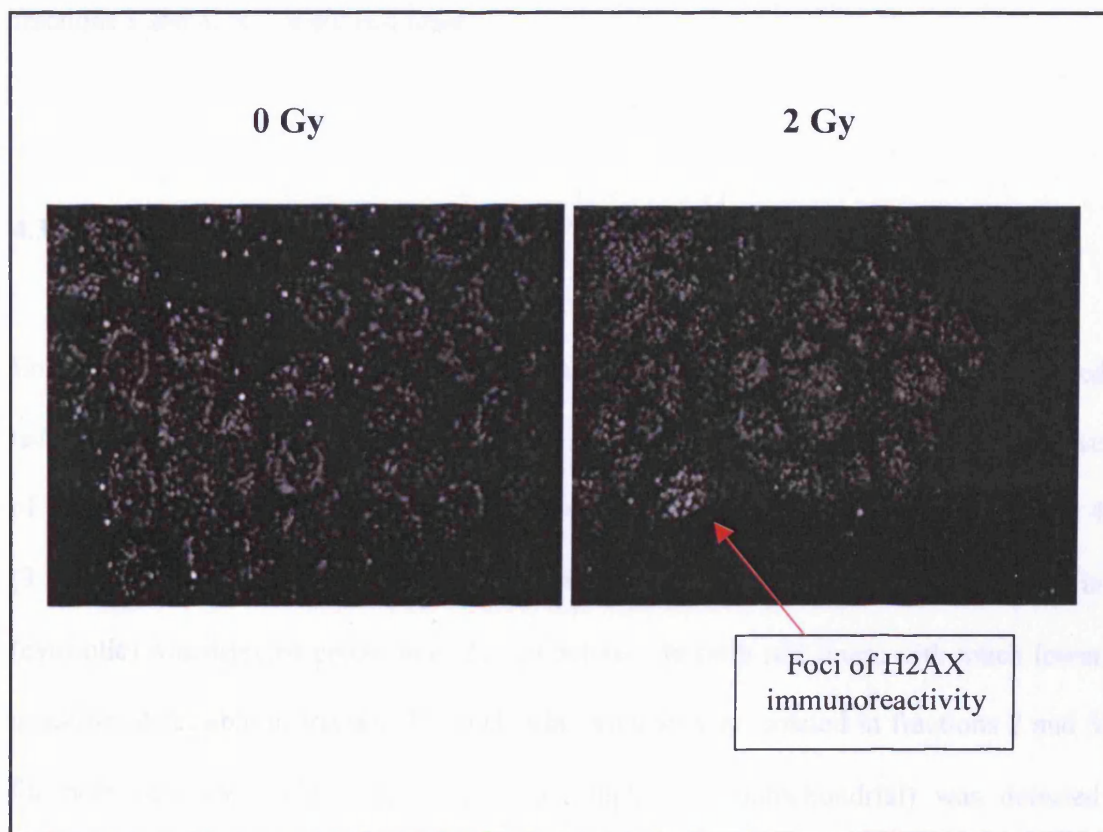
### Pulse-Field Gel Electrophoresis (n=3)



**Figure 4.6:** Pulse-field gel electrophoresis dose response curves for 5 bladder cancer cell lines (UM-UC-3, T24, RT112, HT1376 and J82) over 0-20 Gy. No demonstrable differences in double strand break rates are observed ( $p = 0.750$ , one-way ANOVA).

#### 4.3.3 H2AX Immunoassay

The same five cell lines (UM-UC-3, T24, RT112, HT1376 and J82) were assessed by examining H2AX immunoreactivity over a dose range of 0-2 Gy (increments of 0.5, 1 and 2Gy). Foci of H2AX immunoreactivity (figure 4.7) were not reproducibly elicited in the cell lines studied and unfortunately, no meaningful data were generated (discussed further in 4.4).



**Figure 4.7:** Images captured of RT112 cells before and after irradiation at 2 Gy, demonstrating foci of H2AX immunoreactivity within the nuclei.

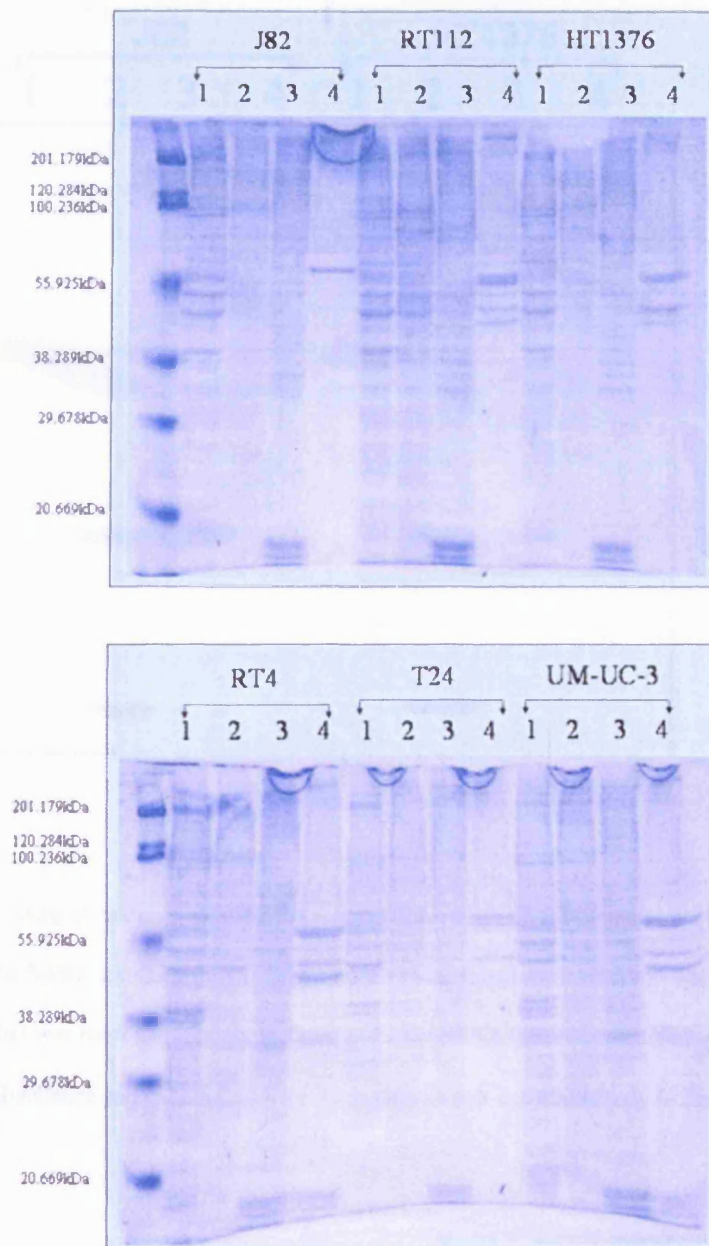
#### **4.3.4 Extraction of Subcellular Protein Fractions for Proteomic Analysis**

The Sub-cellular Protein Extraction Kit (S-PEK) was used to generate 4 lysates per cell line for further proteomic analysis (fraction 1 = cytosolic proteins, fraction 2 = membrane/organelle proteins, fraction 3 = soluble nuclear proteins and fraction 4 = nuclear/cytoskeletal matrix proteins (figure 4.4)). 1-dimensional polyacrylamide gel electrophoresis was used to generate profiles for 4 subcellular fractions for all six cell lines (figures 4.8 and 4.9). Appreciable differences are observed between the profiles for fractions 3 and 4, for all six cell lines.

#### **4.3.5. Assessment of Protein Extraction by Western Blotting**

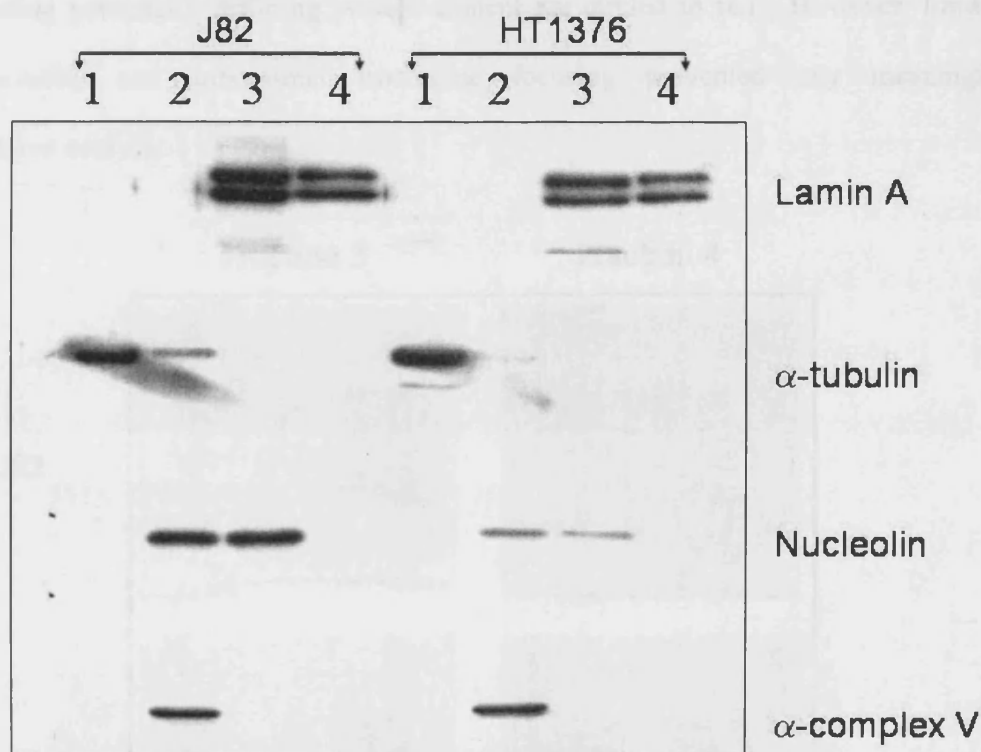
Enrichment of the subcellular fractions with respect to their target proteins was assessed using Western analysis (figure 4.10), employing antibodies from proteins representative of each subcellular fraction. Lamin A (nuclear matrix) was detected in fractions 3 and 4 (3 > 4, both J82 and HT1376) but was not demonstrated in fractions 1 and 2.  $\alpha$ -tubulin (cytosolic) was detected predominantly in fraction 1 in both cell lines, with much lower quantities detectable in fraction 2. Nucleolin (nuclear) was isolated in fractions 2 and 3 for both cell lines (J82 > HT1376).  $\alpha$ -complex V (mitochondrial) was detected exclusively in fraction 2 for both cell lines).





**Figures 4.8 & 4.9:** 1-dimensional polyacrylamide gel electrophoresis profiles of 4 subcellular fractions derived from six bladder cancer cell lines (J82, RT112, HT1376, RT4, T24 and UM-UC-3) using the S-PEK kit (4.2.4). Fraction 1 = membrane/organelle proteins, fraction 2 = cytosolic proteins, fraction 3 = soluble nuclear proteins and fraction 4 = nuclear/cytoskeletal matrix proteins. Appreciable differences are observed between the profiles for fractions 3 and 4, for all six cell lines.



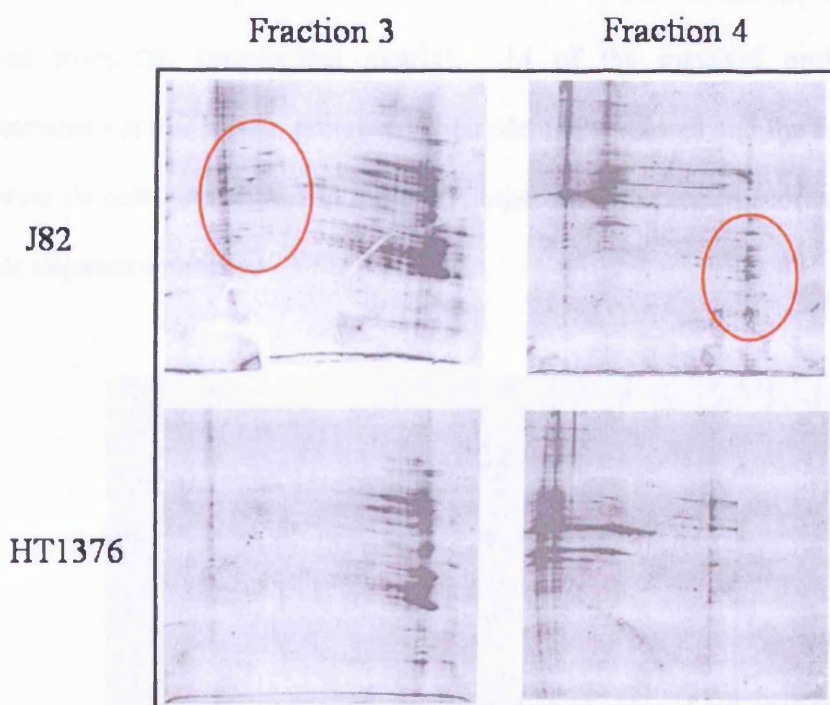


**Figure 4.10:** Western blots demonstrating the relative abundance of 4 proteins in the 4 subcellular fractions isolated using the S-PEK kit. Lamin A (nuclear matrix) was found exclusively in fractions 3 & 4 (3>4).  $\alpha$ -tubulin (cytosolic) was most abundant in fraction 1. Nucleolin (nuclear) was demonstrable in fractions 2 and 3 in equal abundance and  $\alpha$ -complex V (mitochondrial) was detectable only in fraction 2).

#### 4.3.6 2-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE)

Since lamin A was detected in both fractions 3 and 4 for both cell lines, both were used for further comparative analysis. 2D-PAGE was used in an effort to resolve the proteins in these fractions. Examples of gels obtained are shown in figure 4.11. Areas

representing potentially differing protein content are circled in red. However, limited reproducibility and suboptimal isoelectric focusing prevented any meaningful comparative analysis.

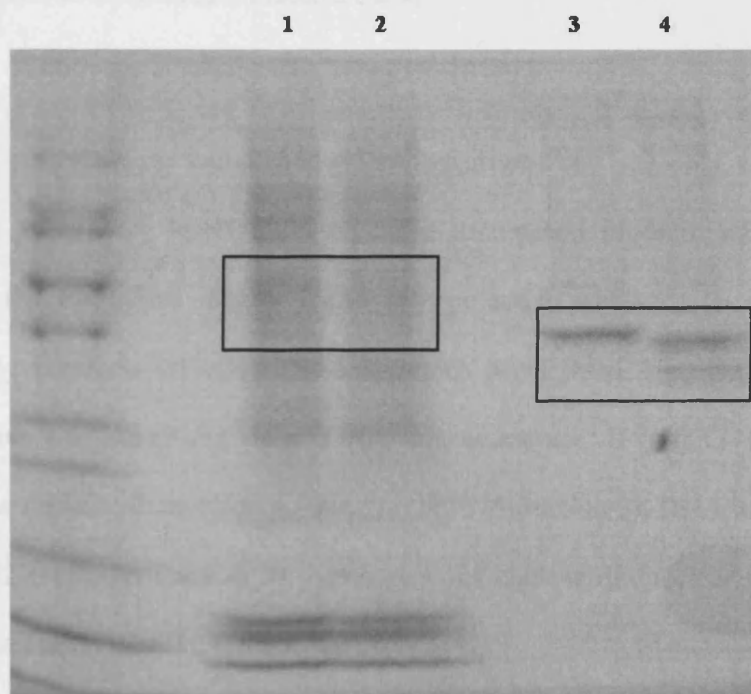


**Figure 4.11:** Comparative 2-dimensional poly acrylamide gel electrophoresis (horizontal plane = isoelectric resolution, vertical plane = molecular weight) of fractions 3 & 4 derived from J82 and HT1376. Potential areas of differential protein content are circled in red.

#### 4.3.7 Liquid Chromatography/Mass Spectrometry Analysis of Nuclear Proteins

Because of the problems encountered with 2D-PAGE, 1-D gels were generated, from which a comparative proteomic analysis was undertaken by excising areas potentially

representing differential protein content (figure 4.12). 11 digests were generated from the gel areas indicated, from which a total of 218 proteins were identified from peptide sequences, as defined by a protein score of  $> 45$  (table 4.2). Of these, 108 were matched (found in corresponding fractions from both cell lines). A further 49 were keratins (derived from the cytoskeletal matrix). 14 of the matched proteins were well characterized nuclear matrix proteins. Their identities, masses and the fractions in which they were detected are shown in table 4.3, together with protein scores and numbers of peptide sequences matched.



**Figure 4.12:** 1-dimensional polyacrylamide gel electrophoresis of fractions 3 & 4 derived from J82 and HT1376 (1 = J82 fraction 3, 2 = HT1376 fraction 3, 3 = J82 fraction 4 and 4 = HT1376 fraction 4). Areas of apparent differential protein content (indicated in black rectangles) were cut and analysed as described in 4.2.12).

<b>Digests</b>	<b>Proteins Identified*</b>	<b>Keratins</b>	<b>Unmatched</b>
22	218	49	61

**Table 4.2:** Proteins identified (\*as defined by a Protein score of >45) from the gel areas indicated in figure 4.12. Of 218 proteins identified, 49 were keratins and 61 proteins were unmatched in the digests derived from J82 (fractions 3 & 4) and HT1376 (fractions 3 & 4).

61 identified proteins were unmatched (identified in one cell line only, table 4.2). The function and subcellular localization of these unmatched proteins was reviewed as recorded in the SwissProt database and any potential relevance to radiosensitivity determined by literature review. These approach highlighted 5 proteins of particular interest (table 4.4; Appendix III); DNA topoisomerase II (HT1376 fraction 3), heterogeneous nuclear ribonuclear protein H (HT1376 fraction 3), DNA mismatch repair protein MSH2 (HT1376 fraction 3), heterogeneous nuclear ribonuclear protein L (J82 fraction 4) and Lamin B2 (J82 fraction 4).

<b>Protein</b>	<b>Mass (D)</b>	<b>J82</b>	<b>HT1376</b>
<b>SM1A - Structural maintenance of chromosome 1-like 1 protein</b>	143144	Fraction: 3 Protein score: 272 MPs: 11	Fraction: 3 Protein score: 734 MPs: 19
<b>SFB2 - Scaffold attachment factor B2</b>	107408	Fraction: 3 Protein score: 200 MPs: 5	Fraction: 3 Protein score: 121 MPs: 5
<b>SFB1 - Scaffold attachment factor B1</b>	102578	Fraction: 3 Protein score: 190 MPs: 7	Fraction: 3 Protein score: 133 MPs: 5
<b>SMC3 - Structural maintenance of chromosome 3</b>	141454	Fraction: 4 Protein score: 368 MPs: 10	Fraction: 4 Protein score: 657 MPs: 16
<b>ROU - Heterogeneous nuclear ribonucleoprotein U</b>	90423	Fraction: 3 Protein score: 157 MPs: 3	Fraction: 3 Protein score: 44 MPs: 2
<b>MAT3 – Matrin 3</b>	94565	Fraction: 3 Protein score: 94 MPs: 5	Fraction: 3 Protein score: 429 MPs: 11
<b>RA21 - Double-strand-break repair protein rad21 homolog</b>	71645	Fraction: 3 Protein score: 61 MPs: 2	Fraction: 3 Protein score: 82 MPs: 2
<b>ROR - Heterogeneous nuclear ribonucleoprotein R</b>	70899	Fraction: 4 Protein score: 397 MPs: 16	Fraction: 4 Protein score: 415 MPs: 12
<b>ROQ - Heterogeneous nuclear ribonucleoprotein Q</b>	69590	Fraction: 4 Protein score: 118 MPs: 4	Fraction: 4 Protein score: 484 MPs: 10
<b>THO1 - THO complex subunit 1</b>	75619	Fraction: 4 Protein score: 48 MPs: 5	Fraction: 4 Protein score: 5619 MPs: 138
<b>LAMA - Lamin A/C</b>	74067	Fraction: 3 Protein score: 811 MPs: 14	Fraction: 3 Protein score: 2 MPs: 62
<b>ROM - Heterogeneous nuclear ribonucleoprotein M</b>	77418	Fraction: 3 Protein score: 549 MPs: 16	Fraction: 3 Protein score: 371 MPs: 11
<b>LP2A - Lamina-associated polypeptide 2 isoform alpha</b>	75315	Fraction: 3 Protein score: 116 MPs: 5	Fraction: 3 Protein score: 223 MPs: 7
<b>LAM1 - Lamin B1</b>	66237	Fraction: 4 Protein score: 365 MPs: 9	Fraction: 4 Protein score: 351 MPs: 9

**Table 4.3:** Nuclear matrix proteins matched in corresponding fractions from both cell lines, as identified by MS/MS. Mass (D), protein score and numbers of peptide sequences matched are also shown.

<b>Protein</b>	<b>Cell Line/Fraction</b>	<b>Mass (D)</b>	<b>Peptide Sequences Matched</b>	<b>Protein Score</b>	<b>Function‡</b>
<b>TP2B – DNA Topoisomerase II</b>	HT1376 Fraction 3	182567	4	111	Control of topological states of DNA
<b>ROH1- Heterogeneous nuclear ribonuclear protein H</b>	HT1376 Fraction 3	49198	1	65	Component of heterogeneous nuclear ribonuclear complexes
<b>MSH2 - DNA Mismatch Repair Protein MSH2</b>	HT1376 Fraction 3	104677	1	59	Binds to DNA containing mismatched nucleotides
<b>ROL - Heterogeneous nuclear ribonuclear protein L</b>	J82 Fraction 4	60149	1	48	Component of heterogeneous nuclear ribonuclear complexes
<b>LAM2 – Lamin B2</b>	J82 Fraction 4	67647	2	46	Provides framework for the nuclear envelope and interacts with chromatin

**Table 4.4:** Proteins of interest amongst the 61 unmatched proteins in the lysates derived from J82 and HT1376, including mass values and protein scores. (‡ Function as described in SwissProt database (discussed further in 4.3, see also Appendix III)).

#### 4.3.8 Validation of MS/MS Datasets by Western Blotting

Unmatched proteins vimentin, calnexin and  $\beta$ -catenin were selected for validation experiments (figure 4.13) on the basis of certainty of identification (table 4.5) and local

availability of antibodies. Both vimentin and calnexin were more abundant in the J82 lysates compared with HT1376, in keeping with the MS/MS datasets. Western blotting for  $\beta$ -catenin demonstrated that it was slightly more abundant in HT1376, in keeping with the MS/MS datasets, but was detectable in both lysates. Lamin A was identified in both cell lines by MS/MS (table 4.3). By Western analysis, this protein was detectable in Fraction 4 of both cell lines by Western blotting (figure 4.14).

<b>Protein</b>	<b>Cell Line/Fraction</b>	<b>Mass</b>	<b>Peptide Sequences Matched</b>	<b>Protein Score†</b>
VIME - Vimentin	J82 Fraction3	53522	3	212
CALX – Calnexin	J82 Fraction 3	67526	4	120
CTNB – Beta-catenin	HT1376 Fraction 4	85442	1	73

**Table 4.5:** 3 proteins were differentially detected in the fraction3/4 lysates from J82 and HT1376. These proteins were used as ‘marker proteins’ for validation by Western analysis. They were selected on the basis of accuracy of peptide profile and availability of antibodies locally (†Protein scores derived from the ion scores, as described in 4.2.13).

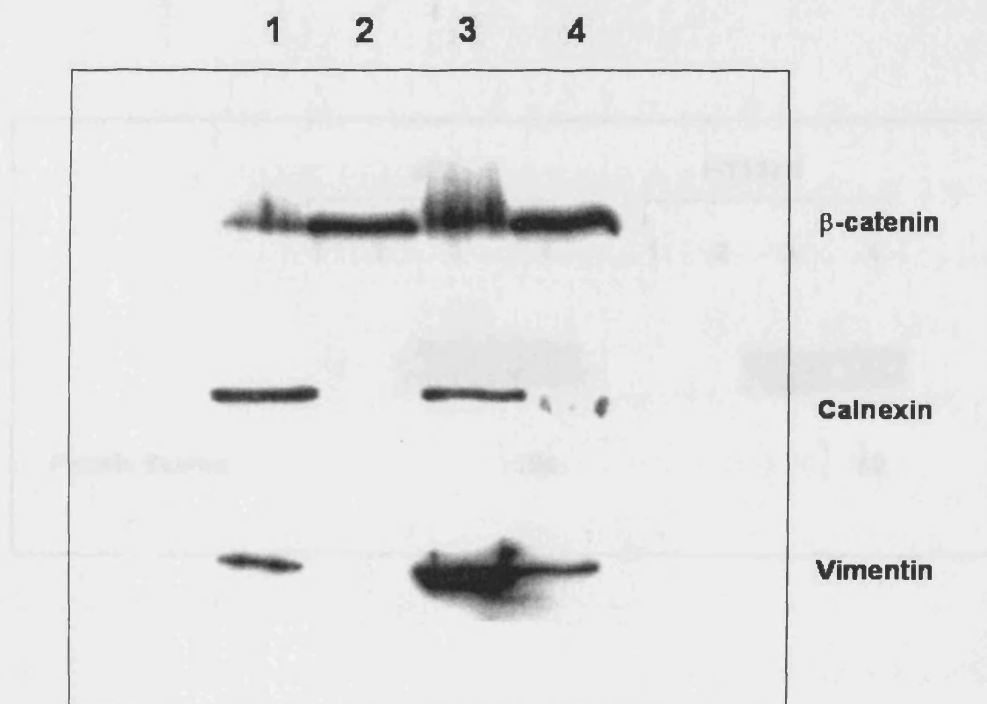
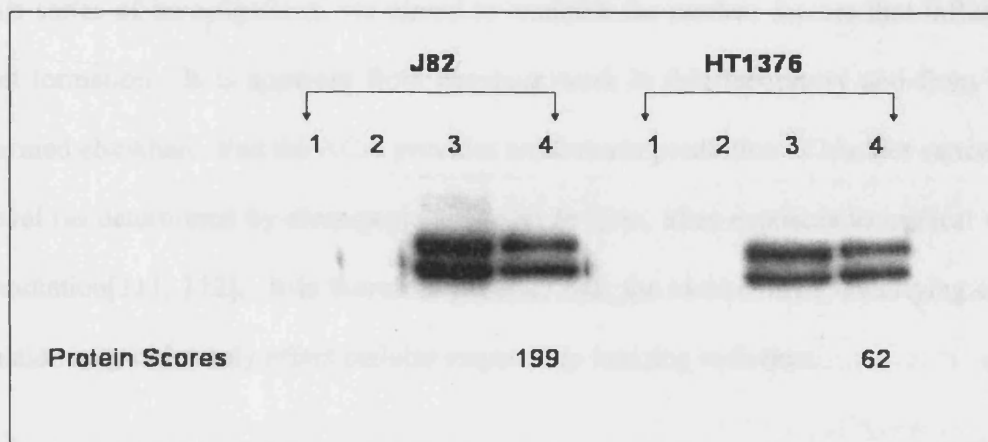


Figure 4.13: Validation of MS/MS datasets using Western blotting (1 = J82 fraction 3, 2 = HT1376 fraction 3, 3 = J82 fraction 4 and 4 = HT1376 fraction 4). Both vimentin and calnexin were more abundant in the J82 lysates compared with HT1376, in keeping with table 4.3. However Western blotting for  $\beta$ -catenin was more equivocal, being relatively abundant in both J82 and HT1376 lysates.



#### 4.4 DISCUSSION



**Figure 4.14:** Further validation of MS/MS data sets. Lamin A was detectable in Fraction 4 of both cell lines by Western blotting and was identified in these fractions by Tandem Capillary Liquid Chromatography/Mass Spectrometry (MS/MS, protein scores as shown).

#### 4.4 DISCUSSION

In this series of investigations, we aimed to examine the nuclear factors that influenced comet formation. It is apparent from previous work in this laboratory and from work performed elsewhere, that the ACA provides an accurate prediction of bladder cancer cell survival (as determined by clonogenic analysis) *in vitro*, after exposure to clinical doses of irradiation[111, 112]. It is therefore possible that the mechanisms underlying comet formation may ultimately affect cellular response to ionizing radiation.

As was discussed in chapter 3 (3.1.1), comet formation requires that damaged, negatively-charged DNA migrates in the electrophoretic field towards the anode [147]. When duplex DNA is denatured in alkaline conditions, individual strands can separate and migrate independently, allowing comet formation from SSBs. The alkaline conditions also cause strand breakages at alkali-labile sites. Since the mechanisms proposed to account for variation in the yield of radiation-induced DSB damage formation would also be expected to vary the yield of SSBs [151], we have regarded SSBs to be a surrogate marker of DSB formation.

One hypothesis to explain the inverse correlation between extent of comet formation immediately after irradiation and cell survival is that radiosensitive cell lines acquire more DNA damage (DSBs *and* SSBs) than radioresistant cell lines, after exposure to the same dose of ionizing radiation. To explore this possibility, we aimed to quantify DSB (the radiation-induced lesion directly associated with subsequent cell death) rates in cell

lines of differing radiosensitivity using PFGE and the H2AX immunoassay. PFGE was undertaken for five bladder cancer cell lines (figure 4.6). Interestingly, no demonstrable differences were observed in FAR (as a marker of DSB formation) between cell lines at the doses examined. Though this suggests that radiation-induced DSB rates are not significantly different between these cell lines, a clear limitation of this particular study is that PFGE was undertaken at doses in excess of those used in fractionated RT. The H2AX immunoassay was therefore attempted in an effort to quantify DSB rates in these cell lines at lower doses. Unfortunately these experiments were unsuccessful, possibly reflecting the need for further optimization of the assay conditions for these cell lines. Nevertheless, the relationship between radiation dose and DSB rate is widely accepted to be linear, at 40 DSB/cell/Gy [182]. Indeed, in recent studies using the H2AX immunoassay, DSB rates immediately after irradiation were found to be equal in different irradiated cell lines [183, 184]. If SSBs are accepted as a surrogate of DSB formation, this suggests that factors other than the absolute amount of DNA damage may be responsible for differences in the extent of comet formation between cell lines of differing radiosensitivity.

Previous work has indicated that interactions between DNA and the nuclear matrix may govern the isolation characteristics between contiguous loop domains, thereby affecting the stability of DNA in the presence of radiation-induced DNA damage and influencing repair. In Chinese hamster ovary cell lines [126], it has been suggested that DNA-nuclear matrix anchoring may contribute to radiosensitivity, by allowing or not allowing supercoiling changes in one DNA loop domain to affect the reparability of DSBs in

adjacent domains and that this in turn is governed by differences in NMP composition. In bladder cancer, Vaughan *et al.* [103] identified that differences in DNA supercoiling related to outcome after radiotherapy and proposed that the ability of nucleoids to adopt positive supercoiling after irradiation was governed by a stronger association between individual DNA loops and the nuclear matrix. From earlier experiments with ACA (AL Moneef *et al.* unpublished data; see 4.1.6), it is clear that a feature of the nucleoid body governs the extent of comet formation immediately after irradiation (which in turn reflects radiosensitivity), providing ample justification for analysis of NMP composition between bladder cancer cells of differing radiosensitivity.

A major challenge in undertaking such analysis is the preparation of suitable sub-cellular protein preparations. This was successfully achieved using the techniques described in 4.2, and the relative purity with respect to the target subcellular protein fractions was confirmed by Western analysis. Three cell lines were initially selected for further proteomic analysis after confirming their differing radiosensitivity by clonogenic studies (figure 4.5), but later it was decided, for simplicity, to focus on simply the most radioresistant and radiosensitive cell lines for this preliminary comparative analysis. Western blotting for the well characterized nuclear protein lamin A, demonstrated its presence in both fractions 3 and 4 of these cell lines and therefore both were used in subsequent analyses.

Achieving satisfactory 2-dimensional resolution of these protein fractions was extremely problematic. Prominent vertical streaking at the point of application of the sample during

IEF suggested sample aggregation or precipitation and horizontal streaking may have resulted from ionic impurities in the sample. It was not possible, during the time available, to achieve gels of adequate quality to justify an attempt at comparative analysis or MS identification of proteins. Future work in this area may require further sample purification steps, targeted IEF using more stringent pH ranges and optimization of protein loading.

When applying mass spectrometry to proteins resolved by 2D-PAGE, the analyzed proteins are homogeneous or simple mixtures of proteins and are relatively straightforward to process. An alternative strategy, known as 'shotgun proteomics', reduces reliance on protein fractionation and follows careful proteolytic digestion of the protein mixtures to produce a collection of peptides analyzed by on-line mass spectrometry [185]. A further modification involves coupling liquid chromatography with mass spectrometry to identify proteins in mixtures using tandem mass spectral database searching. 'Shotgun' proteomic analysis is now commonly used to identify proteins in biological experiments and to determine protein localization, protein complexes and modifications [186, 187] [188, 189]. Due to the difficulties in achieving satisfactory 2-dimensional resolution, this approach was applied to analyse samples excised from 1-dimensional profiles generated from the two fractions of interest (3 and 4) for J82 and HT1376.

Clearly, J82 and HT1376, though both derived from high grade bladder tumours, are inherently different cell lines. As such, any nuclear matrix or other proteins that are unique to one or other of these cells may not be of prognostic significance, despite their

well characterized differences in terms of radiosensitivity. Furthermore, this ‘shotgun’ approach does not provide any quantitative information other than to indicate, with varying degrees of accuracy, the presence or absence of a protein within a complex mixture. Nevertheless, the findings of the comparative analysis undertaken here are of considerable interest.

A total of 14 ‘matched’ NMPs (table 4.3) were identified in the lysates from both cell lines. This indicated that the correct subcellular fractions were being targeted, although the possibility of other relevant proteins being isolated in the other fractions (1 and 2) cannot be excluded. The ‘unmatched’ proteins of interest included the NMPs heterogeneous nuclear ribonuclear protein L (Hnrnp L), heterogeneous nuclear ribonuclear protein H (Hnrnp H) and lamin B2. As nuclear matrix proteins, they may well interact with and contribute to the spatial organization of superhelical DNA, although no specific association with radiosensitivity has been previously identified for these proteins.

In addition, the MS/MS datasets included two further proteins of considerable interest. The first of these, DNA topoisomerase II (topo II), was identified in fraction 3 of HT1376 (protein score 111, 4 matched peptides). Topo II has been shown to be required for chromatin condensation and chromosomal segregation during mitosis, and its isoform topo II $\alpha$  is linked with active cell proliferation in mammalian cells [190]. Giocanti *et al.* [191] demonstrated a radiosensitizing effect of the topo II poison etoposide. Rapidly repairable radiation-induced DNA damage was fixed into lethal lesions by etoposide,

giving rise to a supra-additive interaction under concomitant radiation-drug exposure. Furthermore, cells in G2 phase arrest following radiation were hypersensitive to the cytotoxic effect of etoposide. It was proposed that topoisomerase II alpha was closely involved in a rapid DNA repair pathway operating in all phases of the cell cycle, and particularly involved in DNA repair acting within radiation-induced G2 block. Studies with human fibroblast cell lines have shown a time and dose-dependent increase in DNA topoisomerase II abundance after  $\gamma$ -irradiation, again implicating this enzyme in the cellular response to ionizing radiation [192]. It is therefore interesting to note that ACA measurements of repair (figure 3.2.2, [111]) showed HT1376 to be much more repair proficient than J82.

Nakopoulou *et al.* [193] studied the expression of topo II $\alpha$  in bladder cancer using immunohistochemistry and examined its relationship with biological behaviour of tumours. By multivariate analysis, high topo II $\alpha$  expression was found to be predictive of worse survival ( $p = 0.0047$ ). Raised topo II $\alpha$  expression also correlated with high tumour grade and invasion of the muscularis propria ( $p = 0.027$  and  $0.013$  respectively).

DNA Mismatch Repair Protein MSH2 was also only identified in the MS/MS datasets from HT1376 (protein score 59, 1 matched peptide sequence). The role of this enzyme in the cellular response to ionizing radiation has been studied by examining the responses of MSH2-deficient mouse cells to X-rays [194]. In this study, loss of MSH2 function was associated with reduction in cell survival following irradiation, the absence of either MRE11 or RAD51 relocalization and a higher level of X-ray-induced chromosomal

damage, especially in G2-phase cells. The fact that this enzyme was not detectable in the radiosensitive J82 MS/MS dataset supports the hypothesis that this enzyme is important in the cellular response to ionizing radiation.

Western blotting was again applied to validate the MS/MS datasets. Vimentin,  $\beta$ -catenin and calnexin were selected for this purpose on the basis of high protein scores and the availability of suitable antibodies locally. Both vimentin and calnexin were more abundant in the J82 lysates compared with HT1376, in keeping with the corresponding datasets. Western blotting for  $\beta$ -catenin demonstrated that it was slightly more abundant in HT1376, in keeping with the MS/MS datasets, but was detectable in both lysates. . This may highlight a potential limitation of the ‘shotgun’ approach to this comparative analysis in terms of its sensitivity.

The fact that several of the proteins of interest identified here are known to be associated with radiation response provides valid proof of principle for this proteomics approach. These results will serve as a basis for future work to confirm the differential abundance of these NMPs and repair enzymes and to determine their prognostic significance in terms of cellular and tumour response to ionizing radiation.

## **4.5 CONCLUSIONS**



Previous work has indicated that a feature of the nucleoid body dictates the extent of comet formation following ionizing radiation, which in turn reflects cellular response to irradiation. DSB rates are not demonstrably different in cells of differential radiosensitivity (as measured by PFGE), indicating that factors other than the absolute amount of DNA damage may underlie differences in the extent of comet formation between cell lines of differing radiosensitivity. Comparative proteomic analysis of nuclear proteins from a radioresistant and radiosensitive cell line suggests differences in their constituent NMPs and repair mechanisms. However, further work is required to evaluate the prognostic importance of these proteins.

## **CHAPTER 5: FINAL SUMMARY**

## 5 FINAL SUMMARY

RT is the mainstay of bladder-preserving, potentially curative treatment for muscle-invasive bladder cancer. Unfortunately, many patients who are treated with RT fail to respond; indicating that radioresistance in muscle-invasive bladder tumours is a major problem. Since an alternative form of primary intervention is available, in the form of radical cystectomy, there is a clear need for predictive information regarding the response of bladder tumours to RT, to enhance patient selection for RT-based bladder preservation. This thesis has sought to identify and explore those factors that, based on current evidence, are most likely to account for the variation in radiosensitivity between muscle-invasive bladder tumours. The volume of published work centered on radiosensitivity and the prediction of radiation response in human cancer is considerable and yet, at present, no predictive tests are available to the clinician.

The mechanisms of radioresistance in malignant tumours are widely accepted to be represented by the five ‘Rs’ of radiobiology (discussed in 1.4.2.1); repair, reoxygenation, repopulation, reassortment and intrinsic cellular radioresistance. The key question however, is the relative contribution of each in determining RT outcome. In bladder cancer, current evidence implicates both hypoxia and IRS as potentially important factors in dictating tumour response to RT. There is presently little convincing evidence to suggest that differences in repopulation rates or reassortment account for the variable responses to RT.

In this study, the prognostic significance of hypoxia within bladder tumours was assessed, with regard to outcome after RT, using the novel hypoxia-related marker, CA IX. CA IX was widely expressed in this series of paraffin-embedded tissue sections and the distribution of staining was consistent with previous studies in bladder cancer. However, CA IX immunostaining in tissue sections from patients with muscle-invasive bladder cancer did not yield prognostic information with respect to local control and survival following radical RT. This may indicate that hypoxia is not a significant determinant of RT outcome, or that factors other than hypoxia may modulate its expression. Certainly, the clinical utility of CA IX immunostaining in this sub-group of patients is limited.

Attention was therefore focused on IRS as a potential cause of radioresistance in muscle-invasive bladder cancer. An important problem in this particular area is the difficulty in effectively measuring IRS, in a way that might ultimately be applicable to treatment planning. Recently, however, the ACA has emerged as a potentially suitable technique to address this. Therefore, a further aim of this thesis was to examine differences in IRS between tumour cell preparations derived from TURBT specimens, using the ACA. The data presented in chapter 3 supports the use of the ACA in assessing IRS in cells derived from human bladder tumour specimens. In particular, a wide range of ACA responses are observed between tumour specimens, which may represent actual differences in IRS. However, further optimization of the assay conditions to improve experimental success rates and reproducibility may be required. Further work is necessary to determine

whether ACA responses correlate with outcome after RT and this study is currently ongoing in this laboratory.

Given the differences in the extent of comet formation observed between cell lines of differing radiosensitivity *in vitro*, and indeed in cells derived from TURBT specimens, further work was undertaken to examine the nuclear factors that influence comet formation and therefore radiosensitivity, such as DSB rates and nuclear protein content. Investigation of the latter has led to the application of proteomic technology. Previous work has indicated that it is a feature of the nucleoid body that dictates the extent of comet formation following ionizing radiation, which in turn reflects cellular response to irradiation. It is interesting to note, therefore, that DSB rates are not demonstrably different in cells of differential radiosensitivity (as measured by PFGE), indicating that factors other than the absolute amount of DNA damage may underlie differences in the extent of comet formation. Furthermore, comparative proteomic analysis of nuclear proteins from a radioresistant and radiosensitive cell line suggests potentially important differences in their constituent NMPs and repair capabilities. Further work is warranted to evaluate the prognostic importance of these proteins.

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## **APPENDICES**

## **Appendix I: Related Publications and Research Abstracts**

### **PUBLICATIONS:**

AL Moneef, M., Sherwood, B.T., Bowman, K.J., Kockelbergh, R.C., Symonds, R.P., Steward, W.P., Mellon J.K. and Jones, G.D.D. Measurements by the Alkaline Comet Assay Predict Bladder Cancer Cell Radiosensitivity. *British Journal of Cancer*. (89) 2271-2276 2003

Sherwood, B.T., Jones, G.D.D., Kockelbergh, R.C., Steward, W.P., Mellon, J.K. and Symonds, R.P. Concomitant Chemotherapy for Muscle-invasive Bladder Cancer: The Way Forward for Bladder Preservation? *Clinical Oncology*. In press Jan 2004

Sherwood, B.T., Richardson, D., Colquhoun, A.J., Jones, G.D.D., O'Byrne K.J., Kockelbergh, R.C., Symonds, R.P., Steward, W.P. and Mellon, J.K. Carbonic Anhydrase IX (CAIX) Expression and Outcome after Radiotherapy for Muscle-invasive Bladder Cancer. *International Journal of Rad Onc Biol Phys*. Submitted Jan 2005.

### **RESEARCH ABSTRACTS**

Sherwood, B.T., AL Moneef, M., Kockelbergh, R.C., Symonds, R.P., Steward, W.P. Mellon, J.K., and Jones, G.D.D. Evaluation of the Alkaline Comet Assay as a Predictive Test of Bladder Cancer Cell Radiosensitivity. *Urological Research Society Meeting, Royal College of Surgeons of England, UK, January 2003. BJU International 92: (7) 826; 2003.*

Sherwood, B.T., AL Moneef, M., Kockelbergh, R.C., Symonds, R.P., Steward, W.P. Mellon, J.K., and Jones, G.D.D. Alkaline Comet Assay Predicts Bladder Cancer Cell Radiosensitivity. *Association of Radiation Research Annual Meeting, St. Catherine's College, Oxford, UK, March 2003.*

Sherwood, B.T., AL Moneef, M., Bowman, K.J., Kockelbergh, R.C., Symonds, R.P., Steward, W.P., Mellon J.K. and Jones, G.D.D. Alkaline comet formation best predicts bladder cancer cell radiosensitivity. *Biological Basis of Sensitivity to Ionising Radiation International Workshop, DKFZ, Heidelberg, Germany, May 2003.*

Sherwood, B.T., AL Moneef, M., Bowman, K.J., Kockelbergh, R.C., Symonds, R.P., Steward, W.P., Mellon J.K. and Jones, G.D.D. The Alkaline Comet Assay and Therapy for Bladder Cancer. *3<sup>rd</sup> Institute of Cancer Studies Annual Meeting, University of Leicester, UK, May 2003.*

Sherwood, B.T., AL Moneef, M., Bowman, K.J., Kockelbergh, R.C., Symonds, R.P., Steward, W.P., Mellon J.K. and Jones, G.D.D. The Alkaline Comet Assay as a

Predictive Test in Bladder Cancer Radiotherapy. *Institute of Genetics, University of Leicester, UK, July 2003.*

AL Moneef, M., Sherwood, B.T., Bowman, K.J., Kockelbergh, R.C., Symonds, R.P., Steward, W.P., Mellon J.K. and Jones, G.D.D. Comet Assay Analysis of Bladder Cancer Cell DNA Substrates. *5<sup>th</sup> International Comet Assay Workshop, University of Aberdeen, Scotland, August 2003*

Sherwood, B.T., AL Moneef, M., Kockelbergh, R.C., Symonds, R.P., Steward, W.P. Mellon, J.K., and Jones, G.D.D. Alkaline Comet Analysis of Bladder Cancer Cell Radiosensitivity. *Urological Research Society Meeting, Royal College of Surgeons of England, UK, January 2004.*

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AL Moneef, M., Sherwood, B.T., Bowman, K.J., Kockelbergh, R.C., Symonds, R.P., Steward, W.P., Mellon J.K. and Jones, G.D.D. Comet Assay Measurements of Cis-platin and Mitomycin C-induced Crosslink Formation Predicts Bladder Cancer Cell Chemosensitivity *in vitro*. *American Association of Cancer Research 95<sup>th</sup> Annual Meeting, Orlando, FL, USA, March 2004. Proceedings of the American Association for Cancer Research* **45**, 356 (abstract 1545).

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Sherwood, B.T., AL Moneef, M., Kockelbergh, R.C., Symonds, R.P., Steward, W.P., Jones, G.D.D. and Mellon, J.K. Can the Alkaline Comet Assay Predict Outcome of Radiotherapy for Muscle-invasive Bladder Cancer? *British Association of Urological Surgeons, Harrogate, UK, June 2004. BJU International* **93** (4) 85; 2004.

AL Moneef, M., Sherwood, B.T., Bowman, K.J., Kockelbergh, R.C., Symonds, R.P., Steward, W.P., Mellon, J.K., McKelvey-Martin, V.J., McKeown, S.R. and Jones, G.D.D., Alkaline comet assay measurements of cancer cell radiosensitivity & chemosensitivity. 'Prognostic Testing in Cancer' a one day meeting of the BIR's Radiation and Cancer Biology Committee, British Institute of Radiology, 3<sup>rd</sup> November 2004, London.



## Measurements using the alkaline comet assay predict bladder cancer cell radiosensitivity

MAL Moneef<sup>1</sup>, BT Sherwood<sup>1,2</sup>, KJ Bowman<sup>1</sup>, RC Kockelbergh<sup>2</sup>, RP Symonds<sup>3</sup>, WP Steward<sup>3</sup>, JK Mellon<sup>2</sup> and GDD Jones<sup>\*,1</sup>

<sup>1</sup>Department of Cancer Studies and Molecular Medicine, Hodgkin Building, University of Leicester, Lancaster Road, PO Box 138, Leicester LE1 9HN, UK;

<sup>2</sup>University Division of Urology, Leicester General Hospital, Gwendolen Road, Leicester LE5 4PW, UK; <sup>3</sup>University Department of Cancer Studies and Molecular Medicine, Osborne Building, Leicester Royal Infirmary, Leicester LE1 5WW, UK

In the UK, the two main treatments of invasive bladder cancer are radiotherapy or cystectomy. However, ~50% of patients undergoing radiotherapy fail to respond. If tumour radiosensitivity could be predicted in advance, it may be possible to improve control rates significantly by selecting for radiotherapy those patients whose tumours are radiosensitive. Additionally, patients who would benefit from surgery would be identified earlier. The alkaline comet assay (ACA) is a sensitive method for the detection of DNA strand break damage in cells. In the present study, using six bladder cancer cell lines of differing radiosensitivities, cell survival was compared to the manifestation of radiogenic DNA damage as assessed by ACA. For all the cell lines, the extent of comet formation strongly correlates with cell killing ( $R^2 > 0.96$ ), with a greater response being noted in radiosensitive cells. In repair studies, measures of residual damage correlate with survival fraction at 2 Gy ( $R^2 > 0.96$ ), but for only five of the cell lines. Finally, cells from human bladder tumour biopsies reveal a wide range of predicted radiosensitivities as determined by ACA. Overall, these studies demonstrate ACA to be a good predictive measure of bladder cancer cell radiosensitivity at low dose, with potential clinical application.

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**Keywords:** bladder cancer; comet assay; radiosensitivity; DNA damage; radiotherapy

In the UK, bladder cancer is a common urological malignancy, affecting more than 12 500 individuals each year and causing nearly 5 000 deaths per annum (CRC, 2001). In all, 90% of bladder cancers are transitional cell carcinomas (TCCs) and ~30% of bladder tumours are muscle invasive at presentation, a feature that is associated with significant risk of metastasis (30–60%). Patients with organ-confined, muscle-invasive tumours, (T2/3/4a) who are deemed fit, are considered for potentially curative treatment in the form of radical surgery (cystectomy) or radiotherapy. Radical cystectomy (removal of the bladder and urinary diversion) is associated with significant morbidity and a mortality rate of ~2% (Skinner *et al*, 1988; Studer *et al*, 1995). Furthermore, the necessary creation of an abdominal wall stoma (ileal conduit) may impact on quality of life, and erectile dysfunction is virtually universal. The outcome following radical cystectomy varies with tumour stage, with an overall 5-year survival of ~40%.

Radical radiotherapy (RT) is the mainstay of bladder-sparing treatment regimens. It avoids the trauma of major surgery and is considered a primary treatment for patients deemed unfit for surgery. However, RT is itself associated with dose-related complications arising from bowel and bladder being included in the radiation field. Furthermore, while for ~50% of patients radical RT results in effective local tumour control and acceptable

bladder function (Quilty *et al*, 1986; Jenkins *et al*, 1989), the remaining patients suffer local recurrence. For this latter group, the decision to treat with RT is disadvantageous, as these patients have been unnecessarily exposed to additional risks of ionising radiation. In addition, the time taken to recognise treatment failure (3–6 months) may provide further opportunity for metastatic spread, before secondary treatment (salvage cystectomy) is undertaken. Also, the morbidity in patients undergoing salvage cystectomy has been found to be significantly higher than in patients undergoing primary cystectomy (Rosario *et al*, 2000). Consequently, if a patient's bladder tumour RT response could be predicted in advance, RT could be promoted in patients with tumours that are predicted to respond. Conversely, patients with nonresponsive tumours could be identified and offered surgery at an earlier stage. In this way, the overall local control rates could be improved.

While much work has been undertaken to develop assays capable of predicting tumour response to RT, none has been successfully applied to clinical practice. However, *ex vivo* measures of the surviving fraction of tumour cells at 2 Gy (SF2) suggest that intrinsic radiosensitivity (IRS) is a significant factor in determining tumour radiocurability (West, 1995; West *et al*, 1997). However, the SF2 assay fails to provide information on a time scale appropriate for treatment planning and also suffers from limited success rates; in an unpublished study, <10% of bladder tumours gave rise to colonies on soft agar (McKeown, McKelvey-Martin and Ho, personal communication). In addition, the relationship between clonogenic survival and clinical response is

\*Correspondence: Dr GDD Jones; E-mail: gdj2@le.ac.uk

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far from proven. The limitations of SF2 have stimulated research into methods to provide a more rapid and complete measure of IRS.

DNA is the most important cellular target for the lethal effects of ionising radiation, with double-strand breaks (DSBs) proposed to be the principal lesions responsible for radiogenic cell killing (Ward, 1988; Iliakis, 1991). Unfortunately, the relative yield of radiogenic DSBs is low, and high radiation doses tend to be required to produce measurable levels; doses that are far greater than those used clinically. Indeed, while a recent study using the neutral comet assay to measure DSBs reports an association between DSB manifestation and survival (Price *et al*, 2000), the correlations were not definite, with one cell line yielding a false impression of radiosensitivity at the high doses (30 Gy) required for the assay. This highlights a benefit of conducting predictive tests of radiosensitivity at clinically relevant doses.

In contrast with DSBs, the yield of radiation-induced single-strand breaks (SSBs) is far greater (Ward, 1988), and can be readily measured at low clinically relevant doses of radiation. Furthermore, the mechanisms that are proposed to vary the yield of radiation-induced DSBs formation are also expected to vary the yield of radiation-induced SSBs (Ward, 1990). Consequently, the extent of radiation-induced SSB formation can be considered a valid surrogate marker of radiogenic DSB formation.

The alkaline comet assay (ACA) is a highly sensitive method for the assessment of SSBs and alkali labile sites (ALSs), and can readily detect levels of damage induced by clinically relevant doses of radiation (Singh *et al*, 1994; Singh, 1996). In a previous study of just three bladder cancer cell lines, an inverse correlation was obtained between clonogenic survival and mean tail moment (TM) for comet formation, suggesting that ACA could potentially be used to predict the radio response of the single cell lines (McKelvey-Martin *et al*, 1998).

In the present study, we report our evaluation of ACA as a measure of bladder cancer cell radiosensitivity *in vitro* using a panel of six bladder cancer cell lines, and demonstrate that the extent of comet formation best reflects bladder cancer cell radiosensitivity; these results are supported by two independent, parallel studies using colorectal tumour cells (Dunne *et al*, 2003) and bladder tumour cells (McKeown *et al*, 2003). We also report on a preliminary study to determine the differing ACA radio response of epithelial cells isolated from human bladder tumour biopsies.

## MATERIALS AND METHODS

### Cell lines and culture conditions

The six bladder cell lines used in this study (RT112, UM-UC-3, HT1376, J82, T24 and RT4; derived from high-grade TCCs) were purchased from American Tissue Culture Collection (Manassas, USA) and the European Collection of Cell Cultures (Salisbury, UK). All cells were cultured as monolayers in exponential growth by subcultivation. The RT112 and HT1376 cells were cultured in Eagle's minimal essential medium (EMEM), with 10% foetal calf serum (FCS), 1% nonessential amino acid and 1% penicillin-streptomycin. For the J82 cell line, 1% sodium pyruvate and 1% glutamax were also added. The UM-UC-3 cell line was cultured in EMEM with 10% FCS, 1% penicillin-streptomycin and 1% sodium pyruvate. The T24 cell line was cultured in McCoy's medium with 10% foetal calf serum and 1% penicillin-streptomycin. The RT4 cell line was subcultivated in RPMI medium with 10% foetal calf serum, 1% penicillin-streptomycin and 1% sodium pyruvate. All the cell lines used in the present study were tested for mycoplasma contamination and were determined to be free of contamination ('Mycoplasma Experience', Reigate, Surrey, UK). Cells were harvested at 70–80% confluence by washing with prewarmed 37°C phosphate-buffered saline (PBS) and then trypsinisation

(0.1% trypsin, 0.4% EDTA in PBS) at 37°C. Culture medium was added to the cells before centrifugation at 1500 r.p.m. for 5 min. The pellet was resuspended and incubated in culture medium (37°C, 30 min) at  $1 \times 10^5$  cells ml<sup>-1</sup> (for ACA and clonogenic assay) before use. All cultures were tested for viability following this procedure by trypan blue exclusion and was consistently found to be >99%.

### Alkaline comet assay

**'Cell-slide' preparation** Radiation-induced SSB and ALS DNA damage were assessed using a modified version of ACA, whereby the cells were irradiated 'set' in agarose gels on microscope slides ('cell-slides'). This modified version of the comet assay increases sensitivity by minimising the opportunity for repair of induced damage prior to cell lysis (McKeown *et al*, 2003).

The ACA technique used was adapted from the initial protocol of Singh *et al* (1988). Briefly, dakin-frosted slides were covered with 100 µl of 0.6% normal melting point agarose (dissolved in Ca- and Mg-free PBS at 45°C), and the agarose was allowed to solidify under a coverslip on ice and then the coverslips were removed. Aliquots (1 ml) of harvested cells containing  $1 \times 10^5$  cells in culture medium were then centrifuged at 1500 r.p.m. for 5 min. The pellets were resuspended in 80 µl of 0.6% low melting point agarose (dissolved in Ca- and Mg-free PBS at 37°C), layered onto the normal melting point agarose and allowed to solidify under a fresh coverslip on ice. For 'repair-slides' equal volumes (40 µl) of cell suspension (in RPMI medium) and 1.2% of low melting point agarose (dissolved in RPMI medium containing 20% FCS held at 37°C) were mixed, layered and allowed to solidify under a fresh coverslip on ice. All the steps described were conducted under a reduced light level to prevent additional DNA damage.

**X-ray irradiations** Cell-slides were irradiated on ice, using a Pantak DXT300 X-ray machine (Radiotherapy Unit, Leicester Royal Infirmary) operated at 300 kVp (HVL of 3.5 mm Cu). Up to 12 slides are placed flat, in a prescribed manner, on an aluminium sheet in thermal contact with ice, 0.8 cm from a 50 cm FSD, 20 cm square, normal therapy applicator. The dose rate (0.98 Gy min<sup>-1</sup>) and uniformity of the field ( $\pm 10\%$ ) were previously determined for this configuration using thermoluminescent dosimeters (not presented). Duplicate or triplicate slides were irradiated with a dose of 2, 4 and 6 Gy to generate an immediate damage dose response. Additional duplicate or triplicate slides irradiated with 2.5 Gy were used to monitor repair at 15 and 30 min.

**Lysis and electrophoresis** For studies of immediate damage, slides were irradiated and then placed immediately in cold lysis buffer (2.5 M NaCl, 100 mM Na<sub>2</sub>EDTA, 10 mM Tris, pH 10 and 1% Triton X-100 (added fresh), 4°C) for a minimum of 1 h. For repair studies, after irradiation the 'repair-slides' were incubated in growth media at 37°C for 15 or 30 min and then placed in lysis buffer. After lysis, the slides were drained and placed in a horizontal gel electrophoresis tank, surrounded by ice and filled with fresh cold electrophoresis buffer (300 mM NaOH, 1 mM Na<sub>2</sub>EDTA, pH 13) to a level of ~0.25 cm above the slides. Slides were kept in the high pH buffer for 20 min, to allow DNA unwinding. Electrophoresis was then carried out for 20 min at 25 V and 300 mA. The slides were then drained and flooded slowly with three changes of neutralisation buffer (0.4 M Tris, pH 7.5) for 5 min each and then stained with 50 µl of ethidium bromide (20 µg ml<sup>-1</sup>) and covered with a coverslip for immediate analysis.

**Comet image capture and analysis** A total of 50 cells per slide were analysed to give a representative result for the population of cells (Price *et al*, 2000). Comet image capture and analysis utilised Komet software (Version 4, Kinetic Imaging Ltd, Bromborough, UK) and an epifluorescence microscope (Olympus BH2) fitted with

an excitation filter of 515–535 nm, a barrier filter of 590 nm and a 100 W mercury lamp, and operated at a magnification of  $\times 200$ . The olive tail moment (OTM) was selected as the parameter that best reflected DNA damage (defined as the fraction of tail DNA (TDNA) multiplied by the distance between the profile centres of gravity for DNA in the head and tail). OTM was measured from three independent experiments, each containing duplicate or triplicate measures and presented as the mean  $\pm$  s.e.

### Clonogenic assay

Cells from exponentially growing culture were seeded in appropriate numbers in  $60 \times 15 \text{ mm}^2$  Petri dishes with 10 ml of appropriate culture medium. Following 4 h incubation at  $37^\circ\text{C}$ , cells were irradiated in the dishes with 2, 4 and 6 Gy. Non-irradiated cultures were processed in parallel. Dishes were subsequently incubated at  $37^\circ\text{C}$  in humidified 95% air, 5%  $\text{CO}_2$  atmosphere for 2–3 weeks. Cells were then fixed with 3:1 ethanol:acetic acid and stained with crystal violet. Colonies were counted for the control and dose groups, and each experiment was performed on at least three separate occasions in triplicate. The SF, presented as the mean  $\pm$  s.e., was defined as the ratio of colonies formed to cells plated (with correction for plating efficiency (PE)), and calculated using the formula:  $\text{SF} = \text{colonies counted} / (\text{cells seeded} \times (\text{PE}/100))$ . PE was expressed as the number of colonies scored as a percentage of the number of viable control cells plated. This was  $> 35\%$  for all the cell lines.

### Human bladder samples

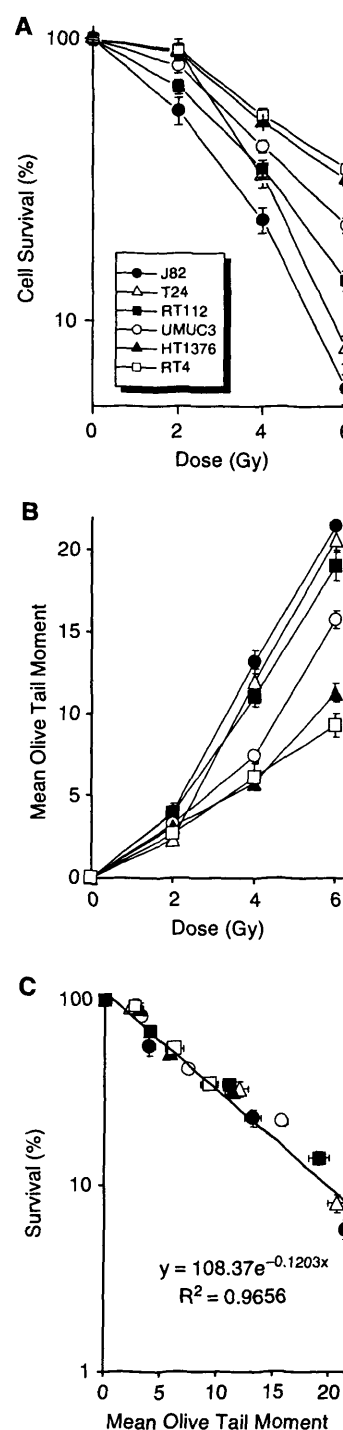
Samples of human bladder cancer tissue were acquired from eight patients undergoing transurethral resection for suspected muscle-invasive bladder cancer. Muscle invasion was confirmed by subsequent histological analysis. Acquisition of tissue specimens was approved by the local ethical committee. Samples were transported to the laboratory in culture media (10 ml) immediately following resection. Only exophytic areas of the tumour were sampled, avoiding subepithelial layers of the bladder wall. The tumour material was finely chopped and placed in 20 ml of collagenase ( $1 \text{ mg ml}^{-1}$ ) in a shaking water bath at  $37^\circ\text{C}$  for 20 min. The sample was then filtered through a nylon mesh (120-gauge filter) and the filtrate was washed three times with PBS. The resulting single cells were counted and the viability was assessed by trypan blue exclusion. (typically this was  $> 80\%$ ; samples with lower levels of viability were discarded).

To reduce infiltrating cells contaminating the cell suspension, cells expressing human epithelial antigen (HEA) were selected via a biomagnetic technique using HEA microbeads according to the supplier's instructions (Miltenyi Biotech, Bisley, UK). HEA is widely expressed on cells of epithelial origin, including associated tumour cells (Moldenhauer *et al*, 1987).

To account for variation between experiments, additional slides were prepared using an established lymphoblastoid cell line (Raji) and these were irradiated and analysed in parallel.

## RESULTS

Figure 1A shows the radiation cell survival responses for the six bladder cancer cell lines investigated, over a dose range of 0–6 Gy, as determined by clonogenic assay. The determined SF2 values are as follows: RT4  $0.92 \pm 0.08$ ; T24  $0.91 \pm 0.054$ ; HT1376  $0.9 \pm 0.052$ ; UM-UC-3  $0.81 \pm 0.051$ ; RT112  $0.68 \pm 0.04$ ; J82  $0.56 \pm 0.062$ . The studied cell lines exhibit a range of radiogenic sensitivities, with J82 being the most sensitive to radiation-induced cell killing, and RT4 and HT1376 being the most resistant. Notably, one cell line, T24, by virtue of its unusual dose–response curve, with a



**Figure 1** (A) The radiation cell survival curve responses for the six bladder cancer cell lines investigated, over a dose range of 0–6 Gy, as determined by clonogenic assay. Survival was determined as the number of colonies formed following X-ray exposure. (B) The extent of initial comet formation, as measured by mean OTM, for the six bladder cancer cell lines, over a dose range of 0–6 Gy, as determined by ACA. For (A and B) each data point is the mean of three independent experiments  $\pm$  s.e. (C) The relationship between the measures of mean OTM for initial comet formation, as measured by ACA, and the measures of clonogenic cell survival, for all six bladder cancer cell lines at 0, 2, 4 and 6 Gy. The data are fitted with an exponential trend line and the slope and correlation coefficient are deduced.

prominent shoulder and steep exponential, demonstrates relative radioresistance at a low dose (2 Gy) and relative radiosensitivity at higher doses.

Figure 1B compares the measures of initial comet formation (as assessed by mean OTM) for the six bladder cancer cell lines, over a dose range of 0–6 Gy, as determined by ACA. The plots reveal clear dose–response curves for each cell line, with the most radiosensitive cell line (J82) displaying the highest values (at 6 Gy: OTM 21.5; TM 67.5; % tail DNA (%TDNA) 69%), and the most radioresistant cell line (RT4) the lowest (at 6 Gy: OTM 9.3; TM 19.0; %TDNA 25.5). At 6 Gy, the rank order for initial comet formation matches the rank order of cell killing for all six cell lines (J82 > T24 > RT112 > UM-UC-3 > HT1376 > RT4). Furthermore, the T24 cell line exhibits a relatively low measure of mean OTM at a low dose (2 Gy), but relatively greater measures at higher doses (4 and 6 Gy). Using the ACA method as described, at 2 Gy there is a significant difference for the measures of mean OTM between the more radiosensitive (J82 and RT112) and radioresistant (T24 and RT4) cell lines ( $P < 0.05$ , Tukey one-way ANOVA).

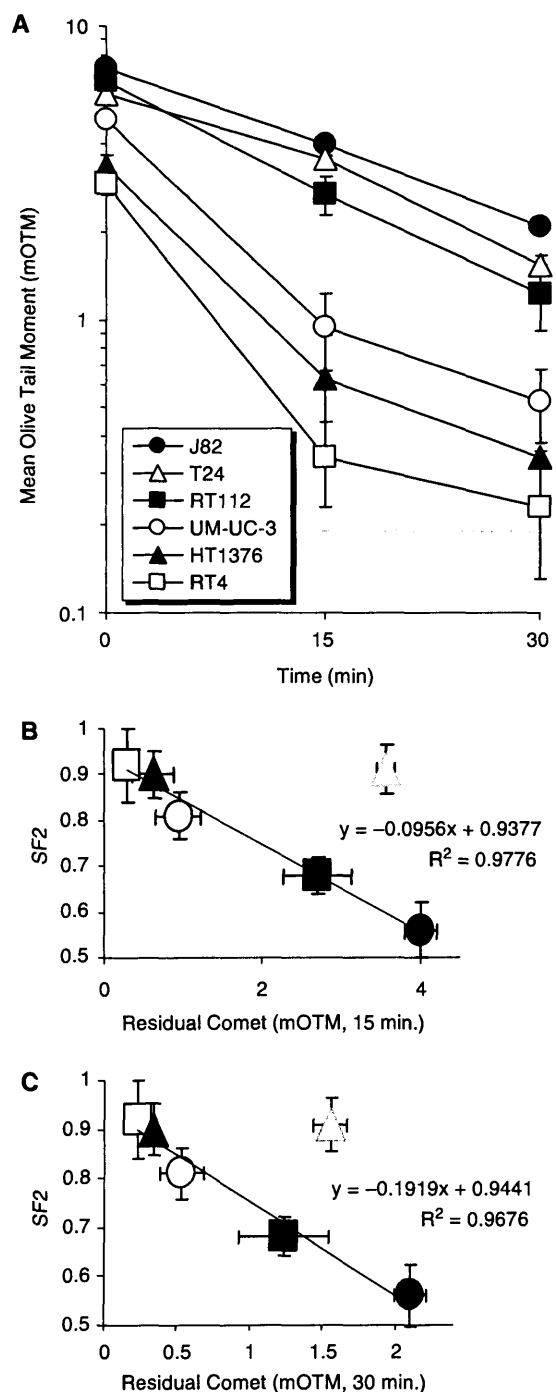
The relationship between mean OTM for initial comet formation and clonogenic cell survival is shown in Figure 1C. The high degree of correlation between the collated measures for all six cell lines ( $R^2 = 0.9656$ ) indicates that the measures of initial comet formation accurately reflects clonogenic survival for these six cell lines over the dose range studied. For each individual cell line, the determined slopes and correlation coefficients ( $R^2$ ) for comet formation vs survival are as follows: RT4 slope,  $-0.118$  ( $R^2$  0.9575); T24 slope,  $-0.1226$  ( $R^2$  0.9785); HT1376 slope,  $-0.1074$  ( $R^2$  0.9485); UM-UC-3 slope,  $-0.0999$  ( $R^2$  0.9785); RT112 slope,  $-0.1023$  ( $R^2$  0.9977); J82 slope,  $-0.1275$  ( $R^2$  0.9867), with the values of the derived individual slopes varying by no more than ~15% of the collated value ( $-0.1203$ ).

Figure 2A illustrates the ACA assessment of damage repair as determined by measurement of residual comets after incubation (37°C) of the 'cell-slides' (see Materials and Methods) for either 15 or 30 min, after 2.5 Gy irradiation. For the most radioresistant cell lines (RT4 and HT1376), the majority of repair occurs by 15 min, while for the more radiosensitive cell lines (J82 and RT112), a significant ACA measure of residual comet remains after 30 min. Figure 2B and C show the relationship between the measures of residual comets at the two repair incubation time points (15 and 30 min), as determined by ACA, and the determined SF2 values for the five cell lines J82, RT112, UM-UC-3, HT1376 and RT4. For these five cell lines, the measures of residual comet after 15 and 30 min correlate with SF2 with  $R^2$  values of 0.9776 and 0.9676, respectively. However, the inclusion of the data for the T24 cell line (light grey open triangle) significantly worsened the correlations, yielding  $R^2$  values of 0.39934 and 0.5097 for 15 and 30 min repair, respectively; for the T24 cells, there is a higher ACA measure of residual comet manifest during repair incubation.

Figure 3 compares the ACA measures of initial comet formation in a pilot study of epithelial cells prepared from biopsies of human invasive bladder tumours ( $n = 8$ ), over a dose range of 0 to 6 Gy. The plots of the mean OTM reveal clear dose–response curves, with a >4-fold difference in the initial comet formation being noted between the samples with the highest and lowest measures of comet formation.

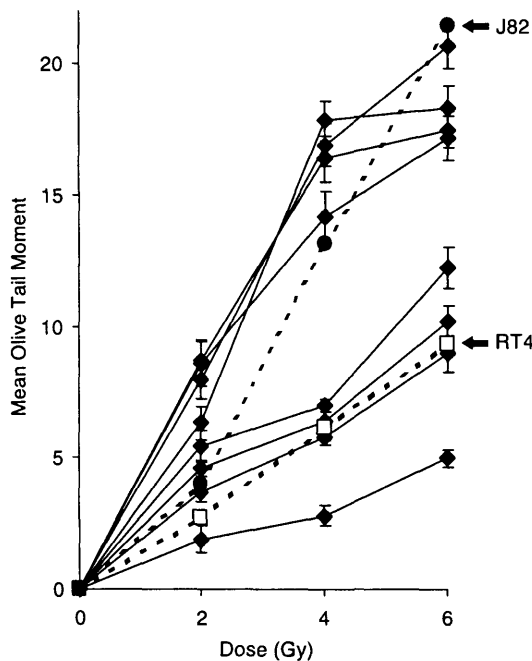
## DISCUSSION

The development of rapid and valid predictive tests of cell radiosensitivity has important implications for clinical practice; allowing for the rational choice and tailoring of treatments to each individual patient, based on that individual's tissue–cell radiation sensitivity (Peters and McKay, 2001). The comet assay is a straightforward and highly sensitive method for assessing DNA damage formation and repair at the individual cell level (Singh et al,



**Figure 2** (A) The extent of damage repair as determined by ACA, after irradiation with 2.5 Gy of X-rays. Each data point is the mean or three independent experiments  $\pm$  s.e. The dashed line represents the mean 'background' level for unirradiated/control cells. (B and C) The correlation between the measures of residual damage at (B) 15 and (C) 30 min, as determined by ACA, and the determined SF2 values for the five cell lines J82, RT112, UM-UC-3, HT1376 and RT4. Data points for T24 (open grey triangles) were excluded from the analysis.

1988; Singh, 1996), and is particularly suitable for the measurement of radiogenic damage (Olive, 1999). The assay is inexpensive, data are available within 24 h and only low numbers of cells are



**Figure 3** Extent of initial comet formation, as measured by mean OTM, for epithelial cells prepared from invasive bladder tumour biopsies, over a dose range of 0–6 Gy, as determined by ACA. Each data point is the mean of duplicate or triplicate irradiations  $\pm$  s.e. Also shown is the extent of initial comet formation for the bladder cancer cell lines J82 and RT4.

required, allowing results to be generated from small clinical biopsies. This technique is therefore ideal for clinical application.

In the present study, we demonstrate ACA to be capable of predicting radiation cell survival for a panel of six bladder cancer cell lines. For initial measures of comet formation, there is a strong inverse correlation with clonogenic survival for all six cell lines, with the most radiation-sensitive cell lines displaying the highest measures and the most radioresistant cell lines the lowest. Furthermore, for one cell line, T24, the notable relative change in survival response, from being relatively radiation resistant to relatively radiation sensitive with increasing dose, was mirrored in the ACA response. For the studies of repair, which entailed measures of residual comets at various repair time points, there was good correlation with survival (SF2) for five of the six cell lines investigated, with the apparent extent of repair being greater in the radioresistant cell lines. However, the superior correlation of clonogenic radiation survival with the extent of immediate comet formation, for *all* six cell lines (including the anomalous behaviour of T24), signifies that it is immediate comet formation that best predicts survival for these cell lines. Virtually identical findings are reported in the two accompanying articles (Dunne *et al*, 2003; McKeown *et al*, 2003).

For each individual cell line, the measures of mean OTM increase with dose due to the dose dependency of radiation-induced DNA damage formation. However, a conclusion that it is the differing levels of immediate radiogenic DNA damage noted between cells, which is responsible for their differing radiosensitivities, conflicts with previous reports that fail to detect such damage (SSB or DSB) differences/correlations using comet or other assays (Olive *et al*, 1991; Evans *et al*, 1993; Woudstra *et al*, 1996). This may reflect the fact that studies that fail to correlate initial damage or repair with cell survival tend to have compared cell lines of different origins (Olive *et al*, 1991, 1994; Woudstra *et al*, 1996) which may have different DNA conformations and therefore

may exhibit considerable differences in radiogenic DNA damage assays. However, studies using cell lines of the same origin at least show correlations between survival and repair/residual damage (Marples *et al*, 1998; Eastham *et al*, 1999). A further factor that may influence the extent of comet formation is the nature of the DNA anchoring matrix-associated regions (MARs). MARs allow the maintenance of contiguous looped regions of nuclear DNA of varying superhelical densities. Consequently, differences in the character of MARs may result in weaker DNA–matrix associations, thereby allowing additional release of the adjacent/contiguous loops of DNA resulting from the presence of radiation-induced DNA damage. Indeed, studies of radiation-sensitive and -resistant mutants have revealed differences in IRS to correlate with DNA stability in the presence of DNA damage as measured by DNA loop rewinding ability, and with changes in nucleoid protein composition (Malyapa *et al*, 1994, 1996).

In the repair studies, it was noted that residual damage (as assessed by measures of residual comets) correlate well with SF2 for five of the six cell lines, with repair being greater in the radioresistant cell lines. The high degree of correlation between the measures of residual comet and SF2 may reflect the inclusion and contribution of the SSB components of DSBs (and other complex lesions) in the ACA assessment of damage at the time points studied. Many studies have shown that a greater abundance of residual DSBs, after a period of repair, is associated with a higher cellular radiosensitivity, for example (Zhou *et al*, 1998). However, for one cell line (T24), measures of residual comet did not correlate with SF2, there being a greater than expected level of residual damage manifest during repair incubation for this cell line. This may be due to the relative change in the survival of this cell line, from being relatively radioresistant to relatively radiosensitive, at doses  $>2$  Gy; at higher doses, the T24 cells may suffer proportionally greater levels of damage. Alternatively, the relatively slow repair of damage (SSB and ALS) may reflect a possible deficiency in the base excision repair for T24 cells. Obviously, this scenario would seriously confound attempts to predict radiosensitivity from estimates of repair determined by ACA. However, the fact that the unusual survival curve response of T24 was also reflected in the ACA response strongly supports the ability of one assay (ACA) to predict the other (survival). Furthermore, this relative change in survival highlights the benefit of conducting predictive tests using clinically relevant doses of radiation. In this particular system, the use of higher test doses ( $>2$  Gy) would yield a false impression of radiosensitivity for T24 at a low dose (2 Gy). However, this does not totally invalidate high-dose experiments.

In a preliminary pilot study, epithelial cells isolated from invasive bladder cancer biopsies also reveal a range of radio responses, as determined by ACA, encompassing the responses noted for the most radiation-sensitive and -resistant cell lines (J82 and RT4, respectively). Most importantly, this demonstrates that the comet assay is possible on cells from biopsy material. From our investigations with cultured bladder cancer cell lines, we speculate that the  $>4$ -fold difference in immediate comet formation in the biopsy-derived epithelial cells potentially reflects similar variations in tumour cell radiosensitivity, but the validity of this remains to be substantiated. However, the observation of differing tumour cell radioresponses, in particular the observation of high degrees of possible radioresistance, may be significant as a contributing factor to the current high level of invasive bladder cancer RT failure (Shipley *et al*, 1985; Duncan and Quilty, 1986).

## CONCLUSIONS

In the present study, we have shown that the initial levels of comet formation, as determined by ACA, best predict bladder cancer cell radiosensitivity at low clinically relevant doses of radiation. The measures of repair correlate with cell survival (SF2), but for only

five of the six cell lines studied. In a pilot study, epithelial cells isolated from human bladder cancer biopsies reveal a potential range of predicted radiosensitivities as determined by ACA at clinically relevant radiation doses, and we speculate that this variation in radiosensitivity may reflect the widely differing response of bladder tumours to RT *in vivo*. Overall, our studies demonstrate ACA to be a good predictive measure of bladder cancer cell radiosensitivity. Further studies are required to evaluate the ACA method fully as a valid predictive test of bladder tumour cell radiosensitivity in the clinical treatment of invasive disease.

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## 2 Original Article

# 3 Concomitant Chemoradiotherapy for Muscle-invasive 4 Bladder Cancer: The Way Forward for Bladder 5 Preservation?

6 B. T. Sherwood, G. D. D. Jones, J. K. Mellon, R. C. Kockelbergh, W. P. Steward, R. P. Symonds

7 *Department of Cancer Studies and Molecular Medicine, University of Leicester, Leicester, UK*

### 13 ABSTRACT:

14 Muscle-invasive bladder cancer is a common malignancy with a high mortality rate. Despite ongoing debates about the optimal primary  
15 intervention, radical cystectomy remains the cornerstone of first-line therapy in many institutions. Over the past decade, bladder-preserving  
16 strategies involving transurethral resection (TUR), chemotherapy and radiotherapy have evolved. However, the advantage of these  
17 approaches over radiation treatment as monotherapy has yet to be fully evaluated. In other tumour models, most notably cervical and anal  
18 cancer, radiation and chemotherapy delivered concomitantly have resulted in significant survival advantages. Here, we consider the  
19 potential value of this approach in the treatment of invasive bladder cancer. Concomitant chemoradiotherapy is currently the mainstay of  
20 several bladder-preserving programmes reported in the medical literature. Overall, local control and survival rates compare favourably with  
21 contemporary cystectomy series; however, difficulties in drawing valid conclusions are highlighted. Concomitant chemoradiotherapy may  
22 have a role in the management of certain patient subgroups, and the debate should remain open. Further large-scale randomised trials are  
23 needed, and information regarding bladder function and quality of life after treatment is deficient at present. The importance of close  
24 follow-up and prompt salvage cystectomy is emphasised. Sherwood, B. T. *et al.* (2004). *Clinical Oncology* ■■■, ■■■■

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26 **Key words:** Bladder cancer, bladder preservation, chemotherapy, radiotherapy

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### 28 Introduction

29 Muscle-invasive transitional cell carcinoma of the bladder  
30 is a common urological malignancy with a relatively poor  
31 prognosis. In many institutions, particularly in the USA,  
32 radical cystectomy remains the preferred primary treatment  
33 modality for organ-confined (T2–4/N0/M0) disease. Chemoradiotherapy, as an alternative strategy aimed at bladder  
34 preservation, is a controversial topic in the field of  
35 urological oncology for several reasons. Practitioners may  
36 regard the extent and duration of treatment needed as  
37 excessive, or feel concerned that the opportunity for  
38 a curative cystectomy may be missed after a failed course  
39 of treatment. This is compounded by the belief that  
40 recurrence in a preserved bladder increases the risk of  
41 death from cancer [1]. Cystectomy and continent diversions  
42 may certainly be more difficult after chemoradiation [2].

43 About 25% of bladder tumours are muscle invasive at  
44 presentation, a feature that is associated with significant

46 risk of metastasis (30–60%). In contemporary radical  
47 cystectomy series, 5-year survival rates vary with tumour  
48 stage, being 75–83% for T1 tumours, 63–89% for T2  
49 tumours, 31–62% for T3 tumours and 21–50% for T4  
50 tumours [3,4]. Where external beam radiation is used as  
51 monotherapy, survival rates are 61% for T1 tumours, 40–  
52 45% for T2 tumours, 26–45% for T3 tumours and 9–12%  
53 for T4 tumours [5,6].

54 Arguments in favour of bladder preservation, based upon  
55 the complications of cystectomy and the impact on quality  
56 of life, are countered by ongoing improvements in urinary  
57 diversion techniques and improved rehabilitation after neo-  
58 bladder construction or nerve-sparing cystectomies. Fur-  
59 thermore, radiation therapy is associated with its own  
60 significant morbidity (about 5% [7]), arising from bowel  
61 and bladder being included in the radiation field. Un-  
62 surprisingly, introducing chemotherapy as a second mo-  
63 dality potentially increases normal tissue toxicity, and  
64 successful concomitant chemoradiotherapy must therefore  
65 strive to improve disease control without compromising  
66 normal tissue tolerance.

67 We have conducted a comprehensive literature review to  
68 determine the current status of concomitant chemoradio-  
69 therapy in the management of muscle-invasive bladder

Author for correspondence: Dr R. P. Symonds, Department of  
Oncology, Osborne Building, Leicester Royal Infirmary, Leicester LE1  
5WW, UK. Tel: +44-116-258-6296; Fax: +44-116-258-5631; E-mail:  
paul.symonds@uhl-tr.nhs.uk



cancer. Relevant clinical studies over a 10-year period (1993–2003) were identified from searches of Medline, ISI Web of Science and Cochrane databases, using the terms 'bladder cancer', 'chemotherapy', 'radiotherapy' and 'bladder-preservation'. Studies were included that involved at least 14 patients, and in which local response rates or bladder preservation rates were available in addition to survival data. References from papers obtained from the search were also reviewed. The literature review was carried out by BTS.

### Mechanisms in Combined Radiotherapy and Chemotherapy

The concept of combined modality therapy in cancer treatment, using chemotherapy and radiotherapy, is designed to reduce the need for surgery by maximising the interaction between the radiation and the chemotherapeutic agent. Combining chemotherapy and radiotherapy is, of course, not a new concept, but despite its widespread application, the mechanisms underlying chemotherapy and radiotherapy interactions are incompletely understood.

When discussing the ways in which multimodality therapy might be more effective than chemotherapy or radiotherapy alone, it is important to appreciate the distinction between additive processes, whereby each treatment exerts its own independent effect and synergy, whereby one modality effectively modifies the response of the other. Initially, it was assumed that concomitant chemotherapy and radiotherapy interacted via simple spatial co-operation, whereby ionising radiation was responsible for local control and chemotherapy targeted sub-clinical metastases at more distant sites. In fact, radiotherapy and chemotherapy can interact much more closely. Adding a chemotherapeutic agent may cause radiosensitisation, giving rise to an alteration in the shape of the cell-survival curve after irradiation. This may be due to direct tumour cell cytotoxicity, or inhibition of sub-lethal or potentially lethal radiation-induced damage repair [8], leading to an enhanced response. Chemotherapy may also act synergistically with radiotherapy by targeting cells in radioresistant phases of the growth cycle, leaving a population of synchronised, more radiosensitive ( $G_2$  and M phase) cells. Unfortunately, although attractive in theory, this effect is not readily exploitable for therapeutic gain. The biological basis of chemotherapy and radiotherapy interactions is outlined in Table 1.

In concomitant chemoradiotherapy, radiotherapy and chemotherapy agents are given, as the term implies, simultaneously. Neo-adjuvant chemoradiotherapy involves a course of chemotherapy followed by radiotherapy, with the two treatments being separated by a variable time interval.

### Neo-adjuvant Chemoradiotherapy

In neo-adjuvant chemoradiotherapy, initial cycles of chemotherapy are aimed at debulking the primary tumour, thereby rendering the reduced number of cells more susceptible to cell death by ionising radiation. Such

**Table 1 – The biological basis of chemoradiotherapy interactions**

Spatial co-operation
Prevention of emergence of resistant clones
Cell-cycle synchronisation
Hypoxic cell sensitisation
Hypoxic cell killing by chemotherapy
Inhibition of potentially lethal and sub-lethal radiation-induced damage repair
Inhibition of cellular repopulation during fractionated radiotherapy

a process may also enhance the radiosensitivity of the residual tumour by making it less hypoxic. It is also suggested that sequential treatment may be less toxic than when given concurrently. Although this approach makes intuitive sense, the results of such strategies are variable in clinical practice. Indeed, in some solid tumours, it has been suggested that initial chemotherapy causes accelerated repopulation of resistant clones, compromising the ability of radiation therapy to achieve control [9]: the so-called 'leaner, meaner' tumours.

In bladder cancer, the Advanced Bladder Cancer meta-analysis collaboration provided the most comprehensive assessment of neo-adjuvant chemotherapy in this disease to date [10]. The analysis included data from 2688 patients from 10 randomised trials of neo-adjuvant regimens, in which patients received definitive local treatment with or without neo-adjuvant systemic chemotherapy. Platinum-based combination chemotherapy showed a significant beneficial effect on overall survival, with a 5% improvement in survival at 5 years (from 45% to 50%). This effect was observed when definitive local therapy involved either radiotherapy or radical cystectomy. The meta-analysis was insufficiently powered to allow valid evaluation of individual regimens, or to reliably determine the effect of single-agent cisplatin on survival. Unfortunately, the implications of this meta-analysis for clinical practice are further limited by the fact that few of the included trials allowed meaningful conclusions to be drawn about toxicity and quality of life, both of which are of paramount importance when discussing primary treatment options.

### Concomitant Chemoradiotherapy

By delivering radiotherapy and chemotherapy simultaneously, the opportunity for cross-resistant tumour cells to become established is minimised. In addition, the delivery of potentially curative radiation therapy is not delayed.

Cisplatin is one of the agents most closely investigated for its interactions with ionising radiation. Like several other drugs, it has been shown to inhibit the repair of radiation damage. Specifically, cisplatin exerts its cytotoxic effects by chelating guanine residues, yielding monofunctional adducts and intra-strand or inter-strand cross-links. If a cisplatin adduct and a radiation-induced single strand break arise simultaneously within close proximity, the result is mutual inhibition of effective repair [11,12].



The presence of cisplatin therefore causes an increase in the number of radiation-induced DNA strand breaks. DNA double strand breaks are the lesions most closely associated with radiation-induced tumour-cell death. Inhibition of repair or conversion of single to double strand breaks has the effect of steepening the radiation survival curve, leading to an enhanced response.

This observation of a lack of overlapping toxicity of cisplatin and radiotherapy and report of synergism between radiation and cisplatin in animal cell lines, mouse models and human xenografts [13–15] stimulated interest in the use of concurrent cisplatin and radiotherapy. In their pre-clinical studies with animal tumour models and human bladder carcinoma cell lines, Mothersill *et al.* [16] demonstrated synergistic cytotoxicity when cisplatin was combined with concurrent radiotherapy.

### Concomitant Chemoradiotherapy in Other Tumour Types

The use of concomitant chemoradiotherapy has been evaluated extensively in other tumour models, most notably cervical cancer. Historically, radiotherapy alone has been the mainstay of treatment in locally advanced disease. Unfortunately, the dose of radiation required to control large tumours in many cases exceeds that tolerated by the normal pelvic tissues. This has prompted the search for alternative regimens. In a recent meta-analysis [17], randomised controlled trials comparing concomitant chemoradiotherapy with radiotherapy alone were examined. A total of 19 trials, undertaken before 2001, were included. This study strongly suggested that concomitant chemoradiotherapy improved both survival and progression-free survival in this disease (by 12% and 16%, respectively). However, such benefit is not echoed in the use of neo-adjuvant chemoradiotherapy for cervical carcinoma, where trials have been universally disappointing [18].

Elsewhere, randomised trials have confirmed the superiority of concomitant chemoradiotherapy over radiotherapy alone in anal cancer [19,20], which is now treated predominantly non-surgically [21]. In oesophageal cancer, early randomised trials of synchronous combination chemotherapy and radiotherapy vs radiotherapy alone showed no difference in survival; however, more recent studies have shown more encouraging results. Cooper *et al.* [22] conducted a prospective, randomised trial (RTOG 85-01) of chemoradiotherapy using cisplatin and 5-fluorouracil with radiotherapy. Combined therapy significantly increased overall survival compared with radiotherapy alone (5-year survival: 26% vs 0% in the radiotherapy group). However, the incidence of life-threatening toxicity reactions was 10% in the combined therapy group compared with 2% in the radiotherapy group. Chemoradiotherapy could only be given as planned to 68% of patients.

### Concomitant Chemoradiotherapy in Bladder cancer: Phase II Studies

The first phase II study of concurrent cisplatin and radiotherapy in bladder cancer was reported in 1982 [23],

and, during the past 10 years particularly, a growing number of phase II studies have evaluated the use of concomitant chemotherapy after transurethral resection in the treatment of muscle-invasive bladder cancer. Shipley *et al.* [24] treated a total of 190 patients with concurrent cisplatin-based chemotherapy and radiotherapy after rigorous transurethral resection of the primary tumour. Response was assessed after 40 Gy by biopsies and cytology. Patients with an incomplete initial response or recurrent tumours after radiotherapy had prompt salvage cystectomy. Using this approach, the investigators achieved overall survival rates for 5 and 10 years of 54% and 36%, respectively, with disease-specific survival rates of 63% and 59%, respectively (stage T2 disease 74% and 66%; stage T3–4a 53% and 52%). The similarity of these results with those of contemporary cystectomy series may have been due to the prompt use of salvage cystectomy where indicated (35% of patients entered in the series). This clearly illustrates the importance of meticulous follow-up in patients entered for bladder preservation.

Rodel *et al.* [25] reported the long-term results of their selective bladder preservation programme in 2002, in which 289 patients were treated with chemoradiotherapy. Response was evaluated by restaging/transurethral resection, 6 weeks after completing treatment. Once again, salvage cystectomy was carried out for residual or recurrent disease. Ten-year disease-specific survival was 42%, with some 80% of survivors retaining their native bladder. Compared with non-randomised controls receiving radiotherapy alone ( $n = 126$ ), chemoradiotherapy was deemed to be more effective in terms of survival and achieving local control.

In their final report of a bladder-preserving programme, Arias *et al.* [26] assessed outcome in 50 patients with T2–4 operable disease treated with methotrexate, vinblastine, adriamycin and cisplatin (M-VAC) after TUR, followed by chemoradiotherapy with cisplatin. Patients were evaluated cystoscopically after 45 Gy. Complete initial response was achieved in 68% of cases. Overall 5-year survival was 73% for T2 disease, 50% for T3 and 25% for T4. Although complications such as anaemia and leucopenia were reported, severe toxicity was uncommon, and there were no treatment-related deaths.

The Radiation Therapy Oncology Group phase II trial 88-02 [27] assessed a combined modality programme incorporating TUR, two courses of methotrexate, cisplatin and vinblastine (MCV regimen) followed by 39.6 Gy of radiotherapy with concurrent cisplatin. Patients with complete local response received consolidation chemoradiotherapy. Of the 91 patients included in this series, 37 (40%) underwent salvage surgery for incomplete initial response or recurrence. The overall 4-year survival was 62%. The 4-year actuarial survival rate with an intact bladder was 44%. The efficacy of this approach compared with concomitant cisplatin and radiotherapy only was assessed in a phase III trial conducted by the Radiation Therapy Oncology Group 89-03 [28]. Two cycles of MCV neo-adjuvant chemotherapy were not shown to increase the rate of complete local control achieved with their standard

Table 2 – Selected phase I–II trials of concomitant chemoradiotherapy

Investigators	Regimen	Number	Local response rate (%)	Bladder preservation	Survival
Arias <i>et al.</i> [26]	TUR, M-VAC + RT with concomitant cisplatin	50	47 (5 years)		48% at 5-year survival
Danesi <i>et al.</i> [54]	TUR, C + 5-FU	25	87.5	71% alive with native bladder	75% actuarial at 4-year survival
Rodel <i>et al.</i> [25]	RT +/- C/carboplatin	289	72	80% of 10-year survivors	42% at 10-year disease-specific survival
Einstein <i>et al.</i> [30]	TUR, MCV, RT	27	55.9		18% at 3-year survival
Fellin <i>et al.</i> [31]	TUR, CMV, RT	56	50	41%	59% at 5-year disease-specific survival
Hussain <i>et al.</i> [55]	MMC, 5-FU, RT	31	74		65% overall at 1-year survival
Housset <i>et al.</i> [33]	TUR, C + 5-FU, RT	54	74		59% overall at 3-year survival
Kaufman <i>et al.</i> [56]	TUR, C + 5-FU, RT	42	66	58% at 4-year median	48% actuarial at 5-year survival
Kachnic <i>et al.</i> [34]	TUR, MCV, RT	106	70	43% at 5 years	60% at 5-year disease-specific survival
Orsatti <i>et al.</i> [57]	TUR, C + 5-FU, RT	76	81	47% at 4 years	42% actuarial at 6-year survival
Shipley <i>et al.</i> [24]	TUR, C, RT	190		73%	63% at 5-year disease-specific survival; 59% at 10-year disease-specific survival
Tester <i>et al.</i> [27]	TUR, MCV, RT	91	80	44% at 4-year survival with intact bladder	62% at 4-year survival
Vikram <i>et al.</i> [58]	TUR, MVC, deoxyrubicin, RT	21	89	84% at median 2 years	60% at 3-year survival
Zietman <i>et al.</i> [59]	TUR, C + 5-FU, RT	18	78		83% at 3-year actuarial survival

C, chemotherapy; 5-FU, 5-fluorouracil; MMC, mitomycin C; M-VAC, methotrexate, vinblastine, adriamycin, cisplatin; RT, radiotherapy; TUR, transurethral resection.

chemoradiotherapy regimen, nor did it increase freedom from metastatic disease. No effect on 5-year survival was demonstrated.

In summary, a number of published series have shown that complete clinical response, as determined by check cystoscopy, is achievable in 70–80% of patients receiving concomitant chemoradiotherapy, with acceptable toxicity. Table 2 shows selected phase I–II trials of chemoradiotherapy regimens and a summary of outcome data. Overall, survival at 5 years is about 50%, and compares favourably with radical cystectomy for patients with stage T2–3a disease. Five-year survival with an intact bladder is in the order of 40–45%.

### Phase III Trials

Although such studies suggest that TUR or partial cystectomy combined with radiotherapy and chemotherapy achieves bladder preservation more consistently than monotherapy [24,29–40], such findings should be approached with caution, as they are exposed to inherent selection bias, and patient numbers are small in many series. Unlike neo-adjuvant chemotherapy, concomitant chemoradiotherapy in bladder cancer has yet to be sufficiently evaluated in randomised controlled studies. It is, therefore, somewhat surprising that concomitant chemoradiotherapy is applied as 'standard care' in many institutions. Undertaking randomised controlled trials of chemoradiotherapy in advanced bladder cancer is, of course, not straightforward. Recruitment is difficult and complicated by the advanced age, co-morbidity and impaired renal function of many patients, rendering them unsuitable candidates for nephrotoxic chemotherapeutic agents, such as cisplatin.

In the only prospective, randomised trial involving concomitant chemoradiotherapy in bladder cancer to date, Coppin *et al.* [41] randomised participants with muscle-invasive disease to receive radiotherapy with or without concurrent cisplatin, before definitive local treatment. Local response was assessed by check cystoscopy at 3 months. Those with complete response had consolidation radiation therapy, whereas those with persistent disease underwent salvage cystectomy (Fig. 1). At 5 years, 40% of patients in the cisplatin arm had suffered pelvic recurrence, compared with 59% in the control arm ( $P = 0.036$ ). Significance was established after correcting for imbalances in tumour grade and stage (hazards ratio, 0.50; 90% CI, 0.29 to 0.86). Although 3-year survival was 47% and 33% in the cisplatin and non-cisplatin arms, respectively, this difference was not statistically significant owing to the small size of the trial ( $n = 99$ ) [41]. Furthermore, as response was assessed after sub-maximal doses of radiation, this study fails to provide a complete evaluation of local control rates or survival after chemoradiotherapy alone.

### Novel Targets for Chemoradiotherapy Interactions

Systemic chemotherapy as part of any bladder-preserving strategy is of course not without risk. Indeed, as many as 20% of patients in published series do not complete planned therapy [35]. Problems of toxicity associated with systemic chemotherapy have prompted the search for more specific molecular targets for chemoradiotherapy interactions. Novel molecular agents in bladder cancer are currently under evaluation. Epidermal growth factor receptor (EGFR) is the product of oncogene c-erb B-1. When activated, phosphorylation of tyrosine kinase receptors in the intracellular component occurs. This leads to activation

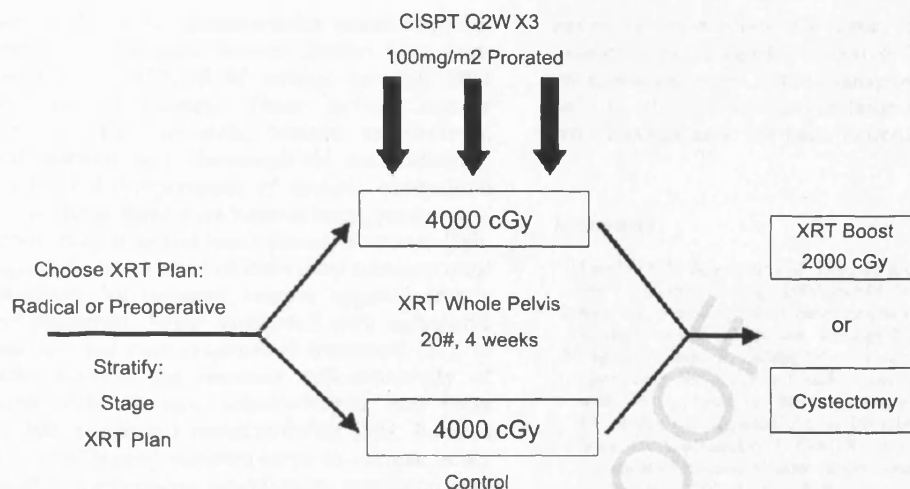


Fig. 1 – Patient flowchart from the Coppin *et al.* [41] trial. Participants with muscle-invasive disease were randomised to receive radiotherapy with or without concurrent cisplatin, before definitive local treatment. Those with complete response had consolidation radiation therapy, whereas those with persistent disease underwent salvage cystectomy.

of a signalling cascade, ultimately stimulating cellular proliferation. In bladder tumours, EGFR positivity is associated with high tumour stage, tumour progression and poor clinical outcome [42,43]. Lammering *et al.* [44] demonstrated the radiosensitising effect of disrupting EGFR function *in vitro* using an adenovirus vector to transfect human malignant glioma cells with inactive EGFR. Further work is currently under way to assess the clinical potential of agents that block EGFR during radiotherapy treatment. Tarceva (TM) and ZD1839 (Iressa(TM)), two small molecule epidermal growth factor receptor-selective tyrosine kinase inhibitors, are currently in development as single agents or in combination with conventional therapies, such as radiotherapy or chemotherapy [45].

## Discussion

Despite the successful application of concomitant chemoradiotherapy in the management of other cancers, trials of concomitant chemoradiotherapy in bladder cancer are few and patient numbers in reported series are relatively small. In comparison, 19 trials of concomitant chemoradiotherapy in cervical carcinoma were conducted before 2001. However, at phase II level at least, such combination therapy seems to offer improved local response rates in muscle-invasive bladder cancer compared with monotherapy, and may therefore exploit synergy between chemotherapy and radiation.

Unfortunately, comparing the results of any non-randomised trial of chemoradiation therapy with contemporary cystectomy series [4,46] is fraught with difficulty. Differences in survival are explained in part by inherent selection bias. Patients recruited for bladder-preserving treatment tend to be older, with significant co-morbidity, compared with those selected for radical surgery. Further-

more, outcome is confounded by discrepancies between clinical (bladder-preserving regimens) and pathological (cystectomy) staging. It has been shown that clinical staging is more likely to under stage disease with regard to invasion into the lamina propria or beyond than is pathological staging [47]. Any outcome bias is therefore in favour of the radical cystectomy series. The variety of treatment protocols and chemotherapeutic regimens being used further limit comparison of outcomes between chemoradiotherapy series.

Other end points in addition to local control and survival rates are equally important. Further studies of bladder-preserving therapies should include assessment of bladder capacity and function. It should be remembered that patients with muscle-invasive bladder cancer are often elderly, with significant co-morbidity. It is, therefore, important that quality of life and actuarial disease-specific survival be taken into account when management strategies are being considered.

The extent and duration of treatment needed are a source of concern for many practitioners opposed to bladder-sparing treatments, together with the significant potential complications. The implications of recurrence after course of treatment are unclear, as is the effect of delaying cystectomy in patients who turn out to have refractory disease. One potential drawback with concomitant chemoradiotherapy, as with any bladder-sparing regimen, is the meticulous follow-up required to identify the significant numbers of patients with chemoresistant, radioresistant or aggressive invasive disease who would still benefit from salvage cystectomy, as well as those who suffer more superficial recurrence and therefore require further therapy. Prompt salvage cystectomy, where indicated, should be considered an integral part of a bladder-sparing approach.

The fact that some patients do remarkably well with bladder-preserving therapies raises the question of how to

identify patient and tumour characteristics associated with greatest response to treatment. Several factors have been associated with the likelihood of patient survival after bladder-preserving radiotherapy. These include tumour differentiation, vascular invasion, tumour morphology, DNA content, tumour size, haemoglobin concentration, serum creatinine and the presence of ureteric obstruction [6,7,48,49]. Additional factors are tumour stage, patient age, extent of tumour resection and local clinical response [50].

The biological characteristics of individual tumours must also be considered. For instance, tumour hypoxia greatly affects cancer prognosis, being associated with aggressive growth, metastasis and poor response to treatment [51]. In addition, differences in the intrinsic radiosensitivity of human tumour cells are now acknowledged, and these differences relate to clinical radiocurability [52]. Patients with very low proliferating tumours seem to achieve better local control after fractionated radiotherapy compared with other patients [53]. Assays providing predictive information about the sensitivities of individual tumours to chemoradiotherapy may hold the key to improving patient selection for treatment using bladder-preserving approaches.

Further evaluation of concomitant chemoradiotherapy as a bladder-sparing alternative will require large-scale randomised phase III trials, and should include longer-term outcome measurements of bladder function and quality-of-life assessment. A randomised phase III study comparing standard vs reduced volume radiotherapy with and without concomitant 5-fluorouracil and mitomycin C has been launched (UKCCR) and is currently recruiting. In addition to determining the effects of reduction in the volume of bladder irradiated, the efficacy and toxicity of synchronous chemoradiotherapy will be compared to those of radiotherapy alone. End points will include assessment of bladder capacity and quality of life, and this study will no doubt help to determine the value of concomitant chemoradiotherapy in the management of muscle-invasive bladder cancer.

## Conclusions

Bladder-preserving chemoradiotherapy has yet to gain widespread acceptance as an alternative to cystectomy, as primary intervention for muscle-invasive bladder cancer. Compared with neo-adjuvant chemoradiotherapy, concomitant chemoradiotherapy remains under-represented in the world literature. Despite the growing body of research in this area, interpretation of the studies to date is hampered by differing protocols, wide-ranging inclusion criteria and indications for salvage cystectomy. Although at phase II level, local control and survival rates compare well with cystectomy in certain patient subgroups, data relating to preserved bladder capacity and function and quality of life are lacking. Together with treatment toxicity, these are vitally important considerations when a bladder-preserving approach is being considered. As with any bladder-preserving strategy, concomitant chemoradiotherapy will

never be appropriate for every patient, and such programmes should remain selective. The role of concomitant chemoradiotherapy in the management of this disease will only be clarified by further large-scale randomised trials, which as yet have not been undertaken.

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# **Carbonic anhydrase IX (CA IX) expression and outcome after radiotherapy for muscle-invasive bladder cancer**

Sherwood, B.T., Richardson, D., Jones, G.D.D., Colquhoun, A.J., O'Byrne K.J., Kockelbergh, R.C., Symonds R.P. and Mellon, J.K.

Department of Cancer Studies and Molecular Medicine, University of Leicester, Leicester, LE1 9HN, UK

**Corresponding author:** Professor JK Mellon MD FRCS(Urol)  
Clinical Sciences Unit  
Leicester General Hospital  
Gwendolen Road  
Leicester LE5 4PW  
Tel/Fax: +44 166 2584617  
Email [jkm7@le.ac.uk](mailto:jkm7@le.ac.uk)

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## **Running title:**

CA IX expression in muscle-invasive bladder cancer.

**ABSTRACT**

**Purpose:** Carbonic anhydrase IX (CA IX) expression has been described as an endogenous marker of hypoxia in solid neoplasms. In other tumour types, CA IX expression has been associated with an aggressive phenotype and resistance to radiotherapy. Here we assess the prognostic significance of CA IX expression in patients with muscle-invasive bladder cancer treated with radiotherapy.

**Methods and Materials:** A standard immunohistochemistry technique was used to demonstrate CAIX expression in 110 muscle-invasive bladder tumours treated with radiotherapy. Clinico-pathological data was obtained from medical casenotes.

**Results:** CAIX immunostaining was detected in 89 (80.9%) patients. Staining was predominantly membranous, with areas of concurrent cytoplasmic staining and was abundant in luminal and perinecrotic areas. No significant correlation was demonstrated between CAIX status and initial response to RT, time to local recurrence or five-year disease-specific survival.

**Conclusions:** The distribution of CAIX expression in paraffin-embedded tissue sections seen in this series is consistent with previous studies in bladder cancer but does not provide significant prognostic information with respect to local control and survival following radical radiotherapy.

## INTRODUCTION

Approximately 30% of bladder tumours are muscle-invasive at presentation and are therefore associated with a significant risk of metastasis and a poor prognosis. Radical radiotherapy (RT) is the cornerstone of treatment regimens aimed at bladder preservation; however, complete local response is seen in only 50% of cases. Predictive information regarding the likely response of a bladder tumour to RT would be of enormous benefit in enhancing patient selection for bladder preservation.

An association between tumour hypoxia and resistance to treatment with ionising radiation has long been recognised. In other solid tumours, polarographic needle measurements of hypoxia correlate with increased metastatic potential, resistance to radiotherapy and an adverse prognosis [1-3]. However, bladder carcinomas are not readily accessible to microelectrodes and alternative strategies aimed at hypoxia measurement need to be assessed.

The transcriptional complex hypoxia-inducible factor-1 (HIF-1) is recognised as a key mediator of gene expression in hypoxic tumours. Hypoxic induction of the carbonic anhydrase genes CA9, CA12 and corresponding proteins (CA IX, CA XII), has been shown to be HIF-1-dependent [4]. In tumour cells, these enzymes are pivotal in maintaining the intracellular pH at physiological levels. The overall effect of CA activity is the relative acidification of the extracellular space. This has important ramifications in promoting further tumour growth and invasion. CA IX expression has been reported as an endogenous surrogate marker of hypoxia in solid neoplasms. In cervical carcinoma, for example, CA IX expression correlates well with



polarographic measurements of tumour oxygen tension [5]. More recently, CA IX expression has been associated with poor prognosis in non-small cell lung cancer [6] and has been associated with poor response to chemoradiotherapy in head and neck cancer [7].

In bladder cancer, CA IX immunostaining is attractive as a marker of hypoxia, since it is non-invasive and does not require systemic administration, compared with polarographic needle measurements and pimonidazole respectively. Significant correlations have been observed between CA IX expression and pimonidazole levels in bladder cancer [4]; however, there is no clear consensus as to the prognostic value of CA IX immunostaining in this disease. Turner *et al.* [8] studied the distribution of vascular endothelial growth factor (VEGF) mRNA (by in situ hybridisation) and CA IX expression in 22 bladder cancer of varied pathological stages. Co-localisation of VEGF and CA IX expression was observed, with both being expressed predominantly on luminal surfaces and in perinecrotic areas. Expression of both factors was greater in superficial compared with invasive disease. The authors went further, to study the relationship between expression of CA IX and clinical outcome in 49 patients with superficial bladder cancer. CA IX expression was not predictive of clinical outcome. Hoskin *et al.* [9] investigated GLUT1 and CA IX as endogenous markers of hypoxia and their relationship to outcome in a retrospective series of 64 patients treated with radical radiotherapy with carbogen and nicotinamide (ARCON). GLUT1 and CA IX staining were found to be independent predictors for overall and disease-specific survival, but not for local control or metastasis-free survival. A prospective study was also reported in which pimonidazole, GLUT1 and CA IX staining was compared in 21 patients with bladder cancer. A good correlation was reported between CA

IX/GLUT1 expression and pimonidazole staining. More recently, CA IX expression has been studied in 57 patients with superficial or invasive disease [10]. Again, significantly more superficial bladder cancers expressed CA IX strongly. No significant association between CA IX staining and survival was established in either superficial or invasive disease.

In the present study, we evaluate CA IX expression in invasive bladder cancer using standard immunohistochemistry. We determine the prognostic significance of tumour CA IX expression in patients treated with RT.

## METHODS AND MATERIALS

### *Study Population*

Ethical approval was obtained for the study of archival paraffin-embedded tissue sections from 110 patients with pathological stage T2-T3 bladder cancer. All patients were treated with RT (mean total dose = 57.9Gy) with curative intent. Of the stained specimens, 91 (82.7%) were from male and 19 (17.3%) were from female patients. Staging was based on biopsy reports from the initial transurethral resection of the bladder tumour (TURBT). The clinico-pathological data is summarised in table 1. Hospital notes were reviewed to determine clinical outcomes (table 2). Response to radiotherapy was determined by check-cystoscopy after completing treatment. A positive response was defined as disease regression or remission. A negative response was defined as persistence or progression.

## MATERIALS

The tumour specimens evaluated were routinely processed, formalin-fixed and paraffin-embedded. Tissue sections of 4µm thickness were cut onto glass slides that were previously treated with 2% 3-aminopropylethoxysilane (in methanol) and dried overnight at 37°C to promote section-to-slide adhesion.

The murine monoclonal antibody M75, recognising the *N*-terminal domain of MN/CA IX protein, has been previously reported by Pastorekova et al [11]. The specificity of the monoclonal antibody M75 for CA IX has been previously reported using Western blots and immunostaining of COS-7 cells transfected with CA IX cDNA [12]. The secondary antibody was polymer-conjugated rabbit antimouse from the Envision kit (Dako, Ely, UK).

### *Immunohistochemistry*

No antigen retrieval was required. Slides were dewaxed in xylene before rehydration by passage through graded alcohols. Endogenous peroxidase was blocked by applying 0.03% hydrogen peroxide containing sodium azide from the Envision kit (Dako) for 10 minutes. Non-specific staining was prevented by the application of 100µl of 10% human serum for 15 minutes. M75 working solution (stock solution diluted 1:50 (vol/vol) in 5% human serum) was added for 30 minutes. Polymer-conjugated rabbit anti-mouse secondary antibody from the Envision kit (Dako) was then added for 30 minutes. Diaminobenzidine substrate (DAB, applied for 8 minutes) was used to visualise CA IX. Sections were washed in Tris-buffered saline for 5

minutes in between incubations. After DAB staining, slides were immersed in running tap water for 5 minutes and counterstained with haematoxylin. Slides were dehydrated by reverse passage through graded alcohols and mounted using DPX (BDH Chemicals Ltd.).

### *Interpretation of CAIX staining*

Tissue sections from a Non-Small Cell Lung tumour were used as positive controls. Negative controls consisted of bladder tumour sections processed without the use of the primary antibody. Tissue sections were evaluated blind using light microscopy by two independent observers. Consensus was then reached using a conference microscope. Sections were classified as positive or negative for CA IX expression and in positive cases, scored according to the percentage of tumour cells expressing CA IX. In addition, the type of staining (i.e. membranous, cytoplasmic or nuclear) and the presence or absence of necrosis was documented. For further analysis, slides showing CA IX positivity were sub-classified according to the percentage of tumour cell population stained (0  $\leq$ 5%, 1 = 6-25%, 2 = 26-50%, 3 = 51-75%, 4  $\geq$ 76 %).

### *Statistics*

The SPSS software system (SPSS for Windows, version 9.0; SPSS Inc., Chicago, IL) was used to perform statistical analysis. Survival curves were plotted using the Kaplan-Meier method and statistical significance was assessed using the log-rank test. Multivariate analysis was performed using Cox's regression to determine any potential independent prognostic factors for diminished bladder-cancer specific

survival. In the first step of the model, univariate analysis was used to identify potential prognostic factors. Factors which predicted diminished survival at a significance level of  $<0.10$  were retained for further stepwise conditional log rank analysis.

## RESULTS

### *CAIX Tumour Cell Expression*

Concordance between the two observers with respect to positivity of immunostaining in this series was 97%. For subclassification by percentage of tumour cells staining positive, concordance was 91%.

Tumour cell CA IX immunostaining was detected in 80.9% (89) of patients. Staining was predominantly membranous, with areas of concurrent cytoplasmic staining ( $n = 42$ ) (figure 1). CA IX expression was abundant in luminal and perinecrotic areas (figures 1 & 2). In a small number of cases ( $n = 25$ ), areas of nuclear staining (figure 3) were observed along with membranous/cytoplasmic patterns. Where staining was sub-classified by category, the majority of slides (56.4%) were assigned to category 1 (5-25%). Only 1 slide (0.9%) was assigned to category 4 ( $\geq 76\%$ ).

### *CAIX Expression and Initial Response to RT*

87 patients (79%) underwent check cystoscopy at 3-6 months post RT, to determine response to treatment. 60 (69%) had a positive response, 27 (31%) had a negative

response. No significant correlation was demonstrated between CA IX status and initial response to RT ( $p = 0.561$ ).

#### *CAIX Expression and Local Recurrence*

55 (50%) patients suffered local recurrence of their bladder cancer. No significant association between CA IX status and time (days) to local recurrence was demonstrated ( $p = 0.4679$ ).

#### *CAIX Expression and Survival Following RT*

Overall 5-year survival was 23.6% in this series. 5-year bladder cancer-specific survival was 40.9%. No significant association was observed between CA IX status and disease-specific survival following RT treatment ( $p = 0.9014$ , figure 4). No significant survival differences were demonstrated when patients were stratified by staining category (0-4). In the final model of multivariate analysis 4 factors retained independent prognostic significance for reduced survival; pre-treatment ureteric obstruction, lack of response to radiation therapy at three-month cystoscopy, local recurrence and metastatic spread ( $p=0.005$ ,  $p=0.009$ ,  $p=0.001$ ,  $p=0.04$  respectively). Nuclear CA IX expression was not found to be of prognostic significance with respect to initial response to RT, local recurrence or disease specific survival.

## DISCUSSION

CAIX immunostaining has attracted widespread attention as a surrogate marker of hypoxia in many tumour models. However, previous studies of CA IX as a marker of hypoxia in bladder cancer have been undertaken using relatively small numbers of tumours, which are often heterogeneous with respect to tumour stage and treatment modality. Collectively, they provide no clear consensus as to the prognostic value, or clinical utility of CA IX immunostaining in major patient groups. No previous studies have examined the relationship between CA IX expression and outcome after radical radiotherapy (as monotherapy) which is widely used in the treatment of muscle-invasive disease. There is a well recognised need for predictive information regarding tumour radiosensitivity, to improve patient selection for RT. The present study, involving a large series of 110 patients, provides a definitive assessment of the prognostic value of CA IX immunostaining in this important group of patients.

The abundance of CA IX immunostaining in luminal and perinecrotic areas of the tumour is consistent with previous studies in bladder cancer [8-10] and supports the notion that CAIX localises to areas of tumour hypoxia. The observation that CA IX immunostaining localised to the nucleus in 25 cases is interesting and not reported elsewhere. Although nuclear staining was not found to be of prognostic significance using the outcome measurements employed in this series, further work is required to determine whether this subgroup represents a distinct phenotype.

In our series, CA IX immunostaining was not found to be of prognostic significance with respect to response to radiotherapy in patients with muscle-invasive bladder

cancer. In addition, no association with local recurrence was observed. Pre-treatment ureteric obstruction, lack of response to RT at three-month cystoscopy, local recurrence and metastatic spread were all identified as adverse prognostic indicators ( $p=0.005$ ,  $p=0.009$ ,  $p=0.001$ ,  $p=0.04$  respectively). This is in keeping with previous studies [13-15] and provides validation of this series.

The lack of an association between CA IX expression and outcome after RT is to be contrasted with reports of endogenous markers (for example GLUT-1 and HIF-1 $\alpha$ ) [5-7] in other tumours, where expression has been associated with adverse prognosis and radioresistance. This association has not been observed universally, however. In a recent study of HIF-1 $\alpha$  expression in locally advanced cervical cancer [16], no correlation was observed between HIF-1 $\alpha$  expression and outcome after radiotherapy, indeed high HIF-1 $\alpha$  expression tended to be associated with good outcome in larger tumours.

The present findings may reflect the importance of mechanisms other than hypoxia in dictating outcome after radiotherapy in this disease. For example, differences in the intrinsic radiosensitivity of human tumour cells are well recognised and these differences relate to clinical radiocurability [17]. One mechanism proposed to explain this variation is that the amount and distribution of damage induced in critical targets may vary between tumour cells of different radiosensitivity; a view that is supported by previous work with human bladder and breast cancer cell lines [18, 19]. The importance of intrinsic radiosensitivity as a cause of radioresistance in bladder cancer is highlighted by recent studies using the alkaline comet assay, in which comet measurements of damage formation and repair correlated with cell survival as



determined by clonogenic analysis [20, 21]. Differences in tumour cell repopulation rates may represent a further confounding variable. In most centres, radiation therapy for bladder cancer (60-70 Gy) is usually delivered over a period of 6-8 weeks. Using Ki67 immunostaining, Lara *et al.* [22] observed that patients with very low proliferating tumours seemed to achieve better local control after fractionated radiotherapy compared with other patients.

Another explanation for the lack of association between CA IX expression and tumour response to radiotherapy may arise from mounting evidence that different intracellular pathways may modify HIF-1 $\alpha$  expression and expression of its target proteins. This may limit the specificity of CA IX expression as a surrogate marker of radiobiological hypoxia. The phosphatidylinositol 3-kinase (PI3K), mitogen associated protein kinase (MAPK) [23, 24] and Smad pathways are known to influence HIF-1 $\alpha$  expression independently of hypoxia and these pathways are overactive in a wide range of tumour models. Epidermal growth factor receptor (EGFR) is a member of the c-erb family of tyrosine kinase receptors and is commonly over expressed in bladder cancer cells [25]. Several studies indicate that EGFR activation enhances the cellular response to hypoxia. In prostate cancer cell lines, EGF stimulation induces HIF-1 $\alpha$  via the PI3K pathway independently and additively to hypoxia [26]. The effect of interactions between the hypoxia response pathway and other cell signalling pathways should be borne in mind when immunohistochemical studies using so-called surrogate markers of hypoxia are undertaken.

A further explanation may arise from growing evidence that superficial and invasive bladder cancer exhibit significant biological differences, particularly in the expression

of hypoxia-inducible genes. There is differential regulation of VEGF in non-invasive compared with invasive cell lines. The expression of VEGF mRNA has been shown to be greater in superficial compared with invasive bladder cancer [27]. In addition, CA IX expression has been shown to be greater in superficial than invasive disease [8]. Paradoxically, these differences in HIF-1-induced gene expression between superficial and invasive tumours suggest relatively less activation of hypoxia regulated transcription pathways in invasive tumours.

This investigation, like any other immunohistochemical study of bladder cancer, may be hindered by the fact that tumours are often extensive, multifocal and heterogeneous, containing numerous biologically different populations within a single neoplasm. This can give rise to tumour heterogeneity with respect to cellular morphology, karyotype, ploidy, cell cycle and cell proliferation kinetics, clonogenic potential, receptor expression and metastatic and tumorigenic properties [28, 29]. Biological heterogeneity has been shown to cause differing radiosensitivity in human bladder cancer cell lines *in vitro* [30] and could therefore be considered as a cause of differing clinical response to radiation treatment for invasive bladder cancer.

## CONCLUSIONS

The distribution of CA IX expression in paraffin-embedded tissue sections seen in this series is consistent with previous studies in bladder cancer. However, CA IX immunostaining in tissue sections from patients with muscle-invasive bladder cancer does not provide significant prognostic information with respect to local control and

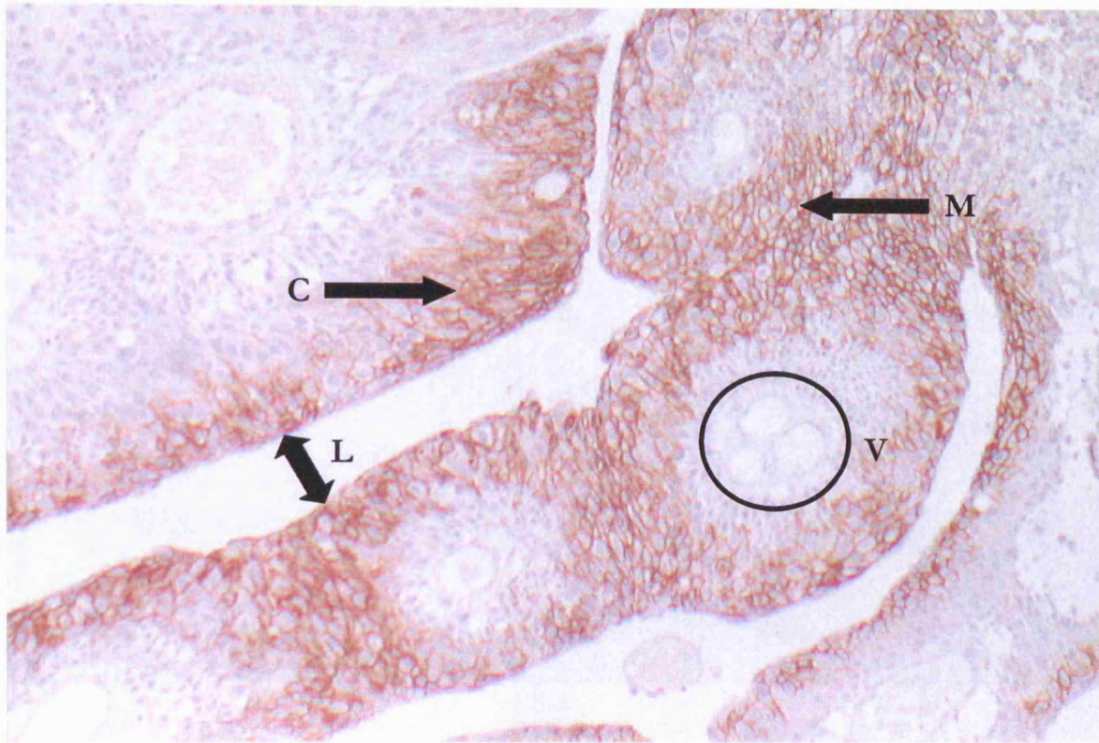
survival following radical radiotherapy. The clinical utility of CA IX immunostaining in this sub-group of patients is therefore limited.

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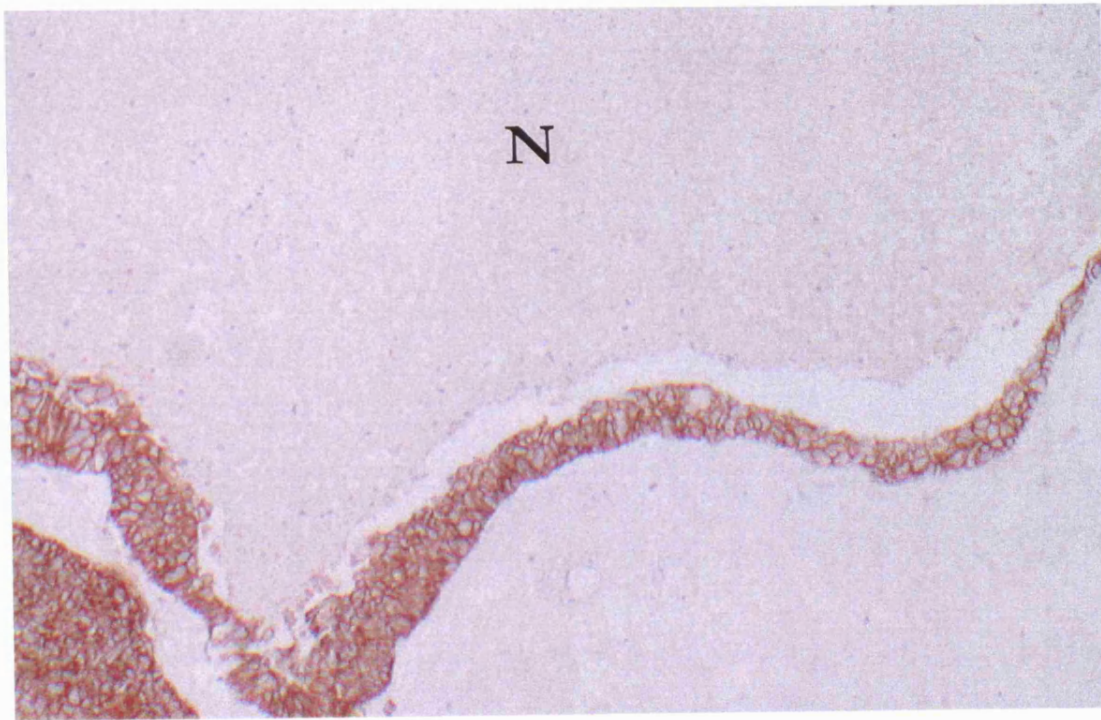
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**Figure 1**



**Figure 2**





**Figure 3**

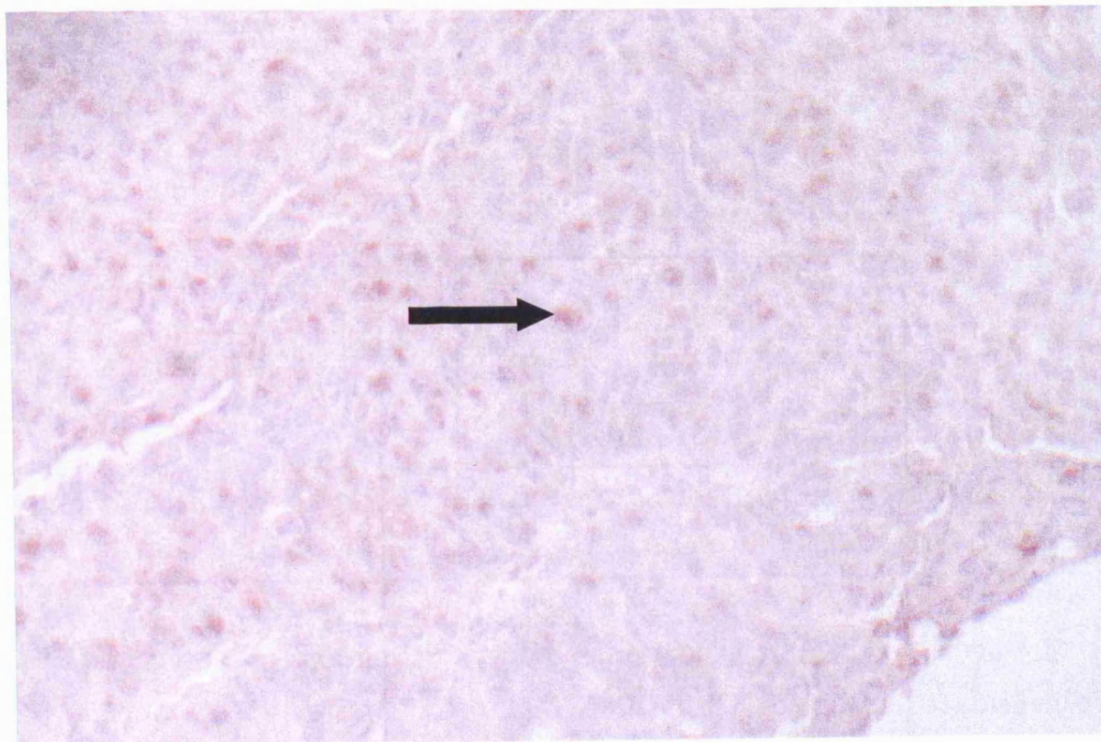
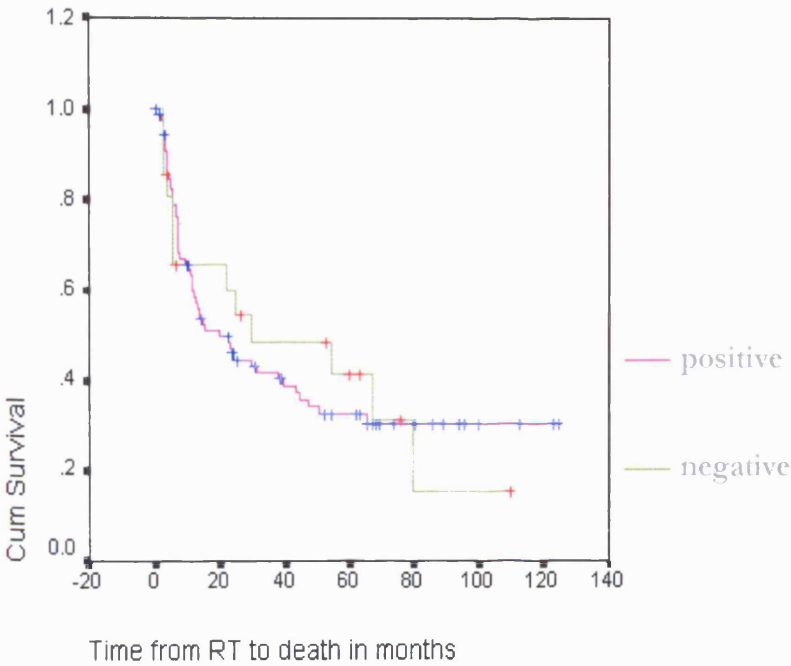


Figure 4





## FIGURE LEGENDS

**Figure 1:** Typical CAIX staining distribution. CAIX expression is predominantly membranous (M) with areas of concurrent cytoplasmic staining (C). Expression was strongest in tumour cells near the luminal surface (L) and increased with distance from the tumour microvasculature (V).

**Figure 2:** Prominent CAIX expression adjacent to a large area of necrosis (N)

**Figure 3:** Nuclear CAIX staining (n = 25)

**Figure 4:** Kaplan-Meier survival curve for disease specific survival following treatment with RT, stratified by CAIX status. No significant difference is demonstrable ( $p = 0.9014$  by log rank).

**Table 1**

<b>Characteristic</b>	<b><i>n</i></b>	<b>%</b>
<b>No. of patients</b>	<b>110</b>	<b>100</b>
<b>Age at diagnosis</b>		
50-59	<b>8</b>	<b>7.3</b>
60-69	<b>25</b>	<b>22.7</b>
70-79	<b>53</b>	<b>48.2</b>
80-89	<b>24</b>	<b>21.8</b>
<b>Sex</b>		
Male	<b>91</b>	<b>82.7</b>
Female	<b>19</b>	<b>17.3</b>
<b>Histological type</b>		
TCC	<b>106</b>	<b>96.4</b>
SCC	<b>4</b>	<b>3.6</b>
<b>Tumor grade</b>		
2	<b>16</b>	<b>14.5</b>
3	<b>93</b>	<b>84.5</b>
Unknown	<b>1</b>	<b>0.9</b>
<b>Tumor stage (clinical)</b>		
T1	<b>3</b>	<b>2.7</b>
T1-2	<b>2</b>	<b>1.8</b>
T2	<b>40</b>	<b>36.4</b>
T2-3	<b>8</b>	<b>7.3</b>
T3	<b>29</b>	<b>26.4</b>
T3-4	<b>5</b>	<b>4.5</b>
T4	<b>7</b>	<b>6.4</b>
Not recorded	<b>16</b>	<b>14.5</b>
<b>Tumor stage (pathological)</b>		
T2 (at least)	<b>106</b>	<b>96.4</b>
T3	<b>3</b>	<b>2.7</b>
Unknown	<b>1</b>	<b>0.9</b>
<b>Pre-treatment ureteral obstruction</b>		
Yes	<b>64</b>	<b>58.2</b>
No	<b>27</b>	<b>24.5</b>
Not assessed	<b>19</b>	<b>17.3</b>
<b>Previous non-muscle-invasive tumor</b>		
Yes	<b>29</b>	<b>26.4</b>
No	<b>81</b>	<b>73.6</b>
<b>Radiation therapy</b>		
Mean total dose	<b>57.9 Gy</b>	
Modal fraction size	<b>2 Gy</b>	
Modal fraction number	<b>30</b>	
Mean duration of treatment		<b>45.1 Days</b>

**Table 2**

<b>Factor</b>	<b><i>p</i></b>
<b>Ureteric obstruction</b>	<b>0.005</b>
<b>Lack of response to RT at 3 months</b>	<b>0.009</b>
<b>Local recurrence</b>	<b>0.001</b>
<b>Metastatic spread</b>	<b>0.04</b>

## TABLE LEDGENDS

**Table 1:** Summary of clinicopathological data

**Table 2:** Independent prognostic variables associated with adverse survival after RT.

**Leicester General Hospital**  
Gwendolen Road  
Leicester  
LE5 4PW

Tel: 0116 2490490  
Fax: 0116 2584666  
Minicom: 0116 2588188

**Consent form for Studies in Bladder Tumours (v.3 Jan 2003)**

This study is being carried out by: Mr Benedict Sherwood MRCS  
Research Registrar in Urology

Mr Roger Kockelbergh DM FRCS(Urol)  
Consultant Urologist

Professor JK Mellon DM FRCS(Urol)  
University Division of Urology

**This form should be read in conjunction with the information leaflet v.2 provided.**

I agree to take part in the above study as described in the patient information sheet.

I agree to donate the tissue samples as detailed below and allow their use in medical research as described in the Patient Information Leaflet.

I understand that I may withdraw my consent to my tissue being used at any time without justifying my decision and without affecting my normal care and medical management.

I understand that members of the research team may wish to view relevant sections of my medical records, but that all the information will be treated as confidential.

I understand that tissue samples and associated clinical data may be transferred to non-commercial research partners of the University Hospitals of Leicester NHS Trust and Leicester University, but that the information will be anonymised prior to transfer.

I understand that tissue samples will not be used to undertake any genetic tests whose results may have adverse consequences on my or my families insurance or employment.

I understand that if research using my tissues produces information, which has immediate clinical relevance to me, I will be informed by my hospital consultant or GP and be given an opportunity to discuss the results.

I understand that the tissue is a gift and that I will not benefit from any intellectual property that results from the use of the tissue.

The samples which I hereby consent to donate are:

**Leicester General Hospital**  
Gwendolen Road  
Leicester  
LE5 4PW

..... Tel: 0116 2490490  
..... Fax: 0116 2584666  
..... Minicom: 0116 2588188  
.....

I do/do not agree to my tissues being used for genetic studies.

I understand medical research is covered for mishaps in the same way as for patients undergoing treatment in the NHS i.e. compensation is only available if negligence occurs.

I have read the patient information leaflet on the above study and have had the opportunity to discuss the details with .....and ask any questions. The nature and the purpose of the tests to be undertaken have been explained to me and I understand what will be required if I take part in the study.

Signature of patient ..... Date.....

(Name in BLOCK LETTERS)

.....

I confirm I have explained the nature of the Trial, as detailed in the Patient Information Leaflet, in terms which in my judgement are suited to the understanding of the patient.

**Signature of Investigator** ..... **Date**.....

(Name in BLOCK LETTERS) .....

**Leicester General Hospital**  
Gwendolen Road  
Leicester  
LE5 4PW

Tel: 0116 2490490  
Fax: 0116 2584666  
Minicom: 0116 2588188

## **Research into Bladder Tumours**

This study is being carried out by: Mr Benedict Sherwood MRCS  
Research Registrar in Urology

Mr Roger Kockelbergh DM FRCS(Urol)  
Consultant Urologist

You may contact them at: Urology Department  
Leicester General Hospital  
Gwendolen Road  
Leicester

Telephone: (0116) 258 8251

Or via the staff on ward 28

## **We invite you to participate**

When you come to our department for your bladder operation we invite you to participate in a study we are carrying out about bladder tumours.

As you know, at your operation, the surgeon will remove some tissue from your bladder for examination in the laboratory. We would like your permission to examine some of that tissue to help us get a better understanding of bladder tumours.

## **Questions you may have about this study**

### **1. What is the purpose of the study?**

We are looking at the growth of new blood vessels in bladder tumours and the way in which the body's immune system fights the disease. By looking at this we hope to gain a better understanding of bladder tumours and it may help us to predict which tumours come back. We are also looking at tumour samples in the laboratory to try and decide which of these will respond to radiation treatment.

**Leicester General Hospital**  
Gwendolen Road  
Leicester  
LE5 4PW

Tel: 0116 2490490  
Fax: 0116 2584666  
Minicom: 0116 2588188

**2. What will be involved if I take part in the study?**

All we require of you is your permission to examine some of the tissue taken from your bladder during the operation and a urine sample when you come to the ward. We also ask your permission to look at your medical notes to follow up your recovery.

**3. Will information obtained in the study be confidential?**

The fact that you are helping us in this study will be recorded in your notes and all information will be treated with the usual degree of confidentiality under the data protection act.

Should any of the results of this study be published in the medical literature, no individual patient will be identified.

**4. Can I be harmed by the study?**

As this study will not change your treatment in any way, it is highly unlikely that this will occur. However medical research is covered for mishaps in the same way as for patients undergoing treatment in the NHS, i.e. compensation is only available if negligence occurs.

**5. What happens if I do not wish to participate in this Study or wish to withdraw from the study?**

If you do not wish to participate in this study or wish to withdraw from the study you may do so without justifying your decision and your future treatment will not be affected.

Thank you very much for your consideration.

Benedict Sherwood  
Research Registrar in Urology

Roger Kockelbergh  
Consultant Urologist



## Appendix III: Peptide Reports for Proteins of Interest (MS/MS Datasets)

### 1. TP2B – DNA Topoisomerase II

Digest 2

HT1376 Fraction 3

#### Peptide Summary Report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
<a href="#">29</a>	564.85	1127.69	1127.67	0.02	0	16	1.6	1	TLAVSGLGVVGR
<a href="#">50</a>	618.38	1234.74	1234.69	0.05	1	7	5.9	1	KYDTVLDILR
<a href="#">67</a>	668.40	1334.78	1334.75	0.03	0	36	0.015	1	FLEEFITPIVK
<a href="#">109</a>	731.40	1460.78	1460.75	0.03	0	15	4.4	1	IFDEILVNAADNK
<a href="#">130</a>	813.95	1625.89	1625.84	0.04	0	18	1.6	1	GFQQISFVNSIATSK
<a href="#">166</a>	964.02	1926.03	1925.98	0.05	0	19	1.2	1	VTIDPENNLISIWNNGK
<a href="#">172</a>	1032.55	2063.08	2063.03	0.05	1	8	19	1	EDLATFIEELEAVEAKEK

Mowse score > 33 indicates identity or extensive homology (p<0.05)

Mass: 182567 Protein score: 120 Peptides matched: 7

### 2. MSH2 - DNA Mismatch Repair Protein MSH2

Digest 10

HT 1376 Fraction 3

#### Peptide Summary Report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
<a href="#">44</a>	646.83	1291.64	1291.65	-0.01	0	59	0.00017	1	NNSFVNEIISR

Mowse score > 32 indicates identity or extensive homology (p<0.05).

Mass: 104677 protein score: 59 Peptides matched: 1

### 3. LAM2 – Lamin B2

Digest 19

J82 Fraction 4

#### Peptide Summary Report

Query	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Score	Expect	Rank	Peptide
<a href="#">19</a>	587.30	1172.59	1172.58	0.01	1	1	94	3	EELRELNDR
<a href="#">71</a>	733.37	1464.73	1464.78	-0.05	0	45	0.003	1	LESLSYQLSGLQK

Mowse score > 32 indicates identity or extensive homology ( $p < 0.05$ ).

Mass: 67647 protein score: 46 Peptides matched: 2

### 4. ROL - Heterogeneous nuclear ribonuclear protein L

Digest 19

J82 Fraction 4

#### Peptide Summary report

Query	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Score	Expect	Rank	Peptide
<a href="#">25</a>	611.78	1221.55	1221.59	-0.03	0	48	0.0011	1	SSSGLLEWESK

Mowse score > 32 indicates identity or extensive homology ( $p < 0.05$ ).

Mass: 60149 Protein score: 48 Peptides matched: 1

### 5. ROH1 - Heterogeneous nuclear ribonuclear protein H

Digest 22

HT1376 Fraction 3

## Peptide Summary Report

Query	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Score	Expect	Rank	Peptide
<u>165</u>	921.41	1840.80	1840.88	-0.08	0	65	1.2e-05	1	STGEAFVQFASQEIAEK

Mowse score > 32 indicates identity or extensive homology ( $p < 0.05$ ).

Mass: 49198 Protein score: 65 Peptides matched: 1