UNIVERSITY OF SOUTHAMPTON

The Development of Pre-Clinical Models to Study and Identify Novel Biomarkers in Muscle Invasive Bladder Cancer

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Abstract

Bladder cancer is the 4th commonest malignancy in the United Kingdom and worldwide there are nearly 400,000 new cases every year with over 150,000 deaths. The gold standard treatment for muscle invasive bladder cancer is radical cystectomy with neo-adjuvant chemotherapy. Despite this the overall survival at 5 years is only around 50%.

To improve outcomes new pre-clinical models of greater physiological relevance are needed and the ability to translate research from the laboratory to clinical practice needs to be improved. The tyrosine kinase HER2 is an attractive therapeutic target in bladder cancer and has the potential to be used in clinical practice. The hypothesis of this thesis was that HER2 would be a prognostic biomarker in patients with bladder cancer requiring radical cystectomy and that it has a critical role in bladder cancer cell invasiveness.

To test this the aims were firstly to create a novel three dimensional cell culture to be used as a more physiological method of studying the invasiveness of bladder cancer. Secondly a tissue micro-array and associated database of cystectomy patients was created for biomarker discovery and to investigate the role of HER2 and its family members as biomarkers in patients with bladder cancer treated with cystectomy.

The novel three dimensional organotypic model was successfully optimized and its ability to reproduce invasive characteristics confirmed with primary invasive cancer cells harvested from a cystectomy patient. Lenti-viral knockdown of HER2 failed to affect the invasive nature of the T24 cell line.

The TMA consisted of 226 cystectomy patients treated over a 10-year period with a median follow up of 49 months. The 5-year overall survival was 48.8% with a cancer specific survival of 62.1% and 27.4% of patients received neo-adjuvant chemotherapy. 17% of patients overexpressed HER2 and HER2 was an independent risk factor for worse overall survival with a hazard ratio of 1.66. Other biomarkers screened for included Nrf-2, which this TMA suggests predicts response to cisplatin based chemotherapy, AIMP3 which may predict resistance to radiation when down regulated and β-HCG, which demonstrated a potential role as a marker of recurrence when measured in blood serum.

In conclusion, HER2 appears to be prognostic of poor outcome in this cohort but is not critical for bladder cancer invasion in the organotypic model. The process of testing this has created two valuable models for biomarker discovery that will be used in future research.

Table of Contents

Author's declaration

I James William Robert Douglas, declare that this thesis and the work presented in it are my own and have been generated by me as the result of my own original research.

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself.

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Finally, I would like to thank Brian Yuen for his statistical guidance and teachings on SPSS software.

Abbreviations

AIMP-3	Aminoacyl-tRNA synthetase interacting multifunctional protein 3
BC	Bladder Cancer
BCG	Bacillus Calmette-Guerin
BRCA	BReast CAncer gene
β-HCG	Beta sub unit of human chorionic gonadotrophin
CDK	Cyclin dependent kinase
Chr	Chromosome
CIS	Carcinoma in situ
CISH	Chromogenic in situ hybridisation
CRUK	Cancer research United Kingdom
DBC	Deleted in bladder cancer gene
DNA	Deoxyribonucleic Acid
EGFR	Epidermal Growth Factor Receptor
ERK	Extracelllular signal- related kinase
FGFR3	Fibroblast growth factor receptor
FISH	Fluorescence in situ hybridisation
HER-2	Human epidermal growth factor receptor
HERCEP	Immunohistochemical scoring syste for HER-2
HRAS	Harvey rat sarcoma
IHC	Immunohistochemistry
Ki-67	Antigen associated with cell proliferation
LOH	Loss of heterozygosity

MAPK	Mitogen activated protein kinase
MIBC	Muscle invasive bladder cancer
mTOR	Mammalian target of rapamycin
MYC	Myelocytomatosis oncogene
Nrf-2	Nuclear factor erythroid 2-related factor 2
OS	Overall survival
p53	Tumour protein 53
PDK	Phosphoinositide-dependent kinase
PI3K	Phosphoinositide 3-kinase
PSA	Prostate specific antigen
PTCH	Protein patched homolog 1 protein
Rb	Retinoblastoma
REMARK	REporting recommendations for tumour MARKer prognostic studies
RFS	Recurrence free survival
TMA	Tissue microarray
TNM	Tissue Nodes Metastasis scoring system
TORC	Transducer of regulated CREB-binding proteins
TSC1	Tumour sclerosis gene 1
VEGF	Vascular endothelial growth factor

1. Introduction

1.1 Background

Cancer of the bladder is the fifth most common malignancy in the United Kingdom (4th most common in men and 11th in women being more than 2.5 times as common in men) with over 10,000 new cancers diagnosed each year. [1-3] Worldwide, there were an estimated 386,000 cases and 150,000 deaths from bladder cancer in 2008[4] and there is an increasing incidence with increasing industrialization and exposure to environmental carcinogens, although in the developed countries the incidence is marginally falling due to removal of the work place carcinogens. In the western world bladder cancer (BC) is a disease of middle to late age with over 90% of patients being aged over 55 years. Smoking is the major risk factor with around 50% of cases being linked to cigarette use and other factors including exposure to aromatic amines, polycyclic aromatic hydrocarbons and aniline dyes.[5] These carcinogens seem to exert their effect via alterations in cell cycle regulation.[6,7] Other risk factors such as hair dyes influence the control of the cell cycle and alter the regulation of gene expression and/or signal transduction.[6,8] Finally, chronic irritation from recurrent urinary tract bacterial infections increases the risk of bladder cancer, in particular the squamous cell carcinoma sub-type. The spinal injuries population are particularly at risk as they often suffer incomplete bladder emptying and/or require catheter use. Chronic irritation and squamous cell carcinoma is also known to arise from Schistosomiasis infection in at risk areas.

Painless visible haematuria is the first symptom the majority of patients present with. In over 90% of cases the bladder cancers arise from the transitional cells lining the bladder (urothelium) and are described as transitional cell carcinomas (TCCs). The remaining

bladder cancer variants are made up of squamous cell carcinomas (5%), adenocarcinomas (1%) and the rarer sub types including lymphoma (<1%), melanoma (<1%), leiomyosarcoma (<1%), small cell carcinoma (<1%) and metastatic tumours.[2,9] The majority of bladder cancers are therefore transitional cell carcinomas and it is also important to highlight that transitional cell carcinomas originate from the urothelium that lines the proximal urethra, bladder, both ureters and the renal pelvis. It is therefore highly possible for visible haematuria to be the first sign of a ureteric or renal pelvis tumour and these are commonly known as upper tract TCC. They share the same molecular pathogenesis as bladder TCC but have different surgical and unclear chemotherapeutic managements and will not be directly covered in this thesis.

Approximately 70% of bladder cancers are staged at initial presentation as non-muscle invasive or superficial disease, which means that the tumour has not invaded deeper than the loose connective tissue beneath the epithelial cells (known as the lamina propria)[10]. These tumours are described as Ta. (figure 1.1) These tumours are not usually associated with the flat tumour carcinoma in situ (CIS) which, although superficial, consists of poorly differentiated cells and is considered to be high risk for recurrence and progression.[11] Poorly differentiated lesions are also referred to as high-grade lesions and conversely low grade for well differentiated lesions. Superficial papillary BC recurs frequently and is often multifocal. However, it does not often progress to muscle invasive disease. When there is progression it is debatable whether this is due to a progression of the superficial tumour or a new invasive tumour arising de-novo.

As well as the stage being important for prognosis, the grade is also a major factor. The grade of the disease refers to how poorly differentiated the cells are. The more poorly

differentiated the cells the worse the prognosis in terms of both recurrence and progression. Traditionally, transitional cell carcinomas were graded 1-3 using the 1973 world health organization (WHO) system, with grade 1 being the least aggressive and grade 3 being the most. This however, has led to some confusion in terms of planning the management of grade 2 lesions as some will behave like a high grade lesion and others more benignly like a grade 1, low grade lesion. For this reason, in 2004, the WHO released a new classification that simplified transitional cell carcinomas into low-grade and high-grade disease. This has only slowly been adopted into routine clinical practice but is now more or less routine.

Superficial bladder cancer is primarily managed with resection using a specialized endoscope called a resectoscope plus or minus the addition of disease modifying agents such as Mitomycin C and BCG (Bacillus Calmette-Guérin). The prognosis of superficial bladder cancer is very good with survival rates of over 90%.[1] Of the 10% who die of bladder cancer it is almost exclusively due to progression and invasion of their superficial disease into muscle invasive disease which can metastasis early and has a poor outcome.

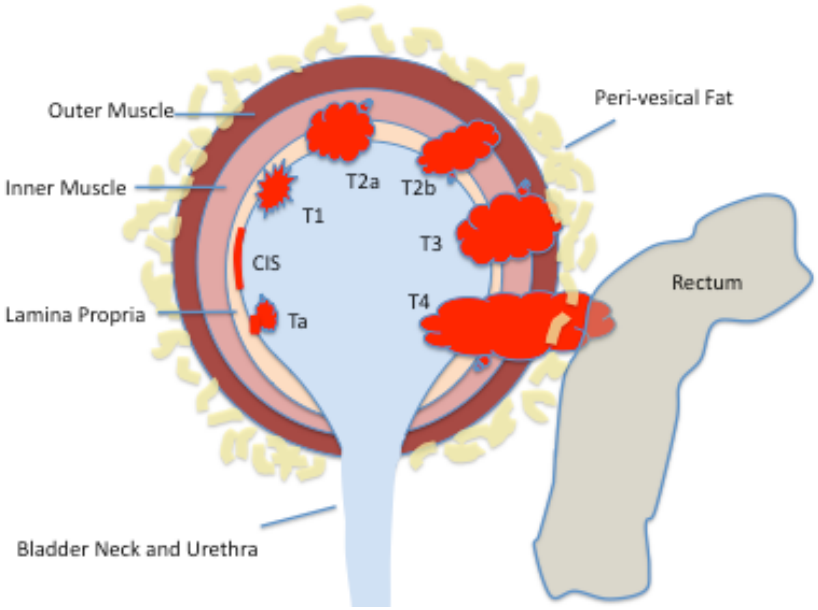


Figure 1.1: Schematic demonstrating the different stages of bladder cancer, the circular object represents the bladder and the red blocks of colour represent tumour of different stages. (please see table 1.1 for classification of invasion) Blue represents urine, and the surrounding organs at risk of invasion, such as the rectum, is illustrated.

Muscle invasive tumours are usually diagnosed in patients with no previous history of papillary tumours. Tumours invading through the lamina propria to the muscle layer are described as T2. Tumours extending as far as peri-vesical fat are T3 and into adjacent structures such as bowel, uterus or prostate referred to as T4. (figure 1.1) In the majority of cases the tumour is poorly differentiated and is often associated with CIS elsewhere in the bladder, which is believed to be the precursor lesion. Tumours that have penetrated through the lamina propria but not as far as the muscle layer are called T1 tumours and represent a difficult group as it is not known whether to treat these as truly superficial cancers or more like muscle invasive cancers. They are normally poorly differentiated with a worse prognosis to Ta tumours and share many genetic alterations in common with muscle invasive disease.[12] Roughly 30% of new bladder cancers present as muscle invasive disease.[13] In this scenario the disease behaves much more aggressively with an overall 5 year survival, after radical therapy, of around 50%.[14]

Thirdly, transitional cell carcinoma of the bladder can present as carcinoma in situ (CIS). This is a non-invasive intraepithelial disease without papillary growth. Anaplasia and a disarranged growth form of the epithelium are histopathological characteristics. Carcinoma in situ can also appear as a secondary disease combined with a papillary tumour or incidentally after transurethral resection of a papillary tumour. CIS is associated with an increased risk of recurrence and progression and is associated with a poorer prognosis. [15,16]

A patient's prognosis and the treatment decision-making processes are determined by the grade and stage of the disease. The accepted method to classify this is to use the staging system endorsed by the American Joint Committee on Cancer (AJCC). This is more commonly known as the TNM staging system where the "T" score represents the depth of

Tumour invasion (stage), "N" score represents the degree of spread of the cancer to lymph **N**odes and the "M" score represents the degree of **M**etastatic disease. (Table 1.1)

As stated above the vast majority of superficial (Ta, T1) bladder cancers can be treated with local resection of the tumour. The patient will be treated with cystoscopic follow up to ensure any recurrences are correctly staged and managed accordingly. In contrast muscle invasive bladder tumours (T2, T3, T4), that are confined to the bladder and have not spread to the lymph nodes nor metastasized to other organs, require much more radical treatment. If left untreated the disease is rapidly fatal. The aggressive natural history of muscle invasive bladder cancer (MIBC) meant that radical cystectomy with pelvic lymphadenectomy was the gold-standard of care with a 5-year survival of 33%-73% when selected for stage.[17] In 2003 level 1 evidence demonstrated a 5% absolute survival benefit with neo-adjuvant chemotherapy[18] which has now become the standard of care prior to radical cystectomy in suitable patients. Radical cystectomy is a very morbid procedure and as such bladder preservation techniques are also an option for selected patients. External beam radiation has had limited success as a single modality but when combined with appropriate chemotherapy the 5 year outcomes are reported to be approaching those of surgery, however the optimal chemo-radiation protocol is yet to be agreed and a very aggressive trans-urethral resection and endoscopic follow surveillance is paramount.[19] To date there are no randomized trials comparing surgery to radiotherapy that have managed to recruit in large enough numbers

Tx	Primary tumor cannot be assessed.
T0	No evidence of primary tumor.
Ta	No evidence of primary tumor.
Tis (CIS)	Carcinoma *in situ*: "flat tumor."
T1	Tumor invades subepithelial connective tissue (lamina propria)
T2a	Tumor invades superficial muscularis propria (inner half).
T2b	Tumor invades deep muscularis propria (outer half).
T3a	Tumor invades perivesical tissue microscopically.
T3b	Tumor invades perivesical tissue macroscopically
T4a	Tumor invades prostatic stroma, uterus, vagina.
T4b	Tumor invades pelvic wall, abdominal wall, bowel.
Nx	Lymph nodes cannot be assessed.
N0	No lymph node metastasis.
N1	Single regional lymph node metastasis in the true pelvis (hypogastric, obturator, external iliac, or presacral lymph node).
N2	Multiple regional lymph node metastases in the true pelvis (hypogastric, obturator, external iliac, or presacral lymph node).
N3	Lymph node metastases to the common iliac lymph nodes.
M0	No distant metastasis.
M1	Cancer has spread to distant parts of the body

Table 1.1: Table demonstrating the latest TNM staging criteria for bladder cancer and endorsed by the American joint Committee on Caner (AJCC). 'T' refers to tumor stage, 'N' the nodal involvement and 'M' the presence of metastasis.

to be able to answer the question and surgery remains the first choice in patients who are fit enough.

When data from the neo-adjuvant studies is analysed further, other prognostic factors have been shown. Unsurprisingly if a bladder removed at cystectomy is downgraded to pT0, due to an absence of residual tumour, the patient has a much better prognosis than someone with residual tumour. In this pT0 group the 5 year survival rate is approaching 90%.[18] The reason for a lack of tumour in the resected specimen is presumed to be due to a complete endoscopic resection prior to cystectomy in patients who have not received neo-adjuvant chemotherapy and occurs in approximately 10-15% of cases.[18] In the group who have received neo-adjuvant chemotherapy this increases to 38%[18] implying that some chemo-sensitive tumours are being "cured" by chemotherapy. This is a key area for future research. To identify which patients have sensitive tumours and to potentially increase the numbers of pT0 cases with targeted therapies would dramatically improve the survival for bladder cancer patients. Appropriate pre-clinical models are crucial for this to allow drug development prior to clinical studies.

Overall the survival for MIBC is still disappointingly poor. Unlike other solid tumours, such as in breast cancer, we have no prognostic or predictive biomarkers or targeted therapies that are used in the clinical setting to better stratify patients into different personalized treatments. Currently tumour suppressor genes such as P53[20,21] and Retinoblastoma gene (Rb)[21,22], cluster of differentiation genes such as Fibroblast Growth Factor Receptor – 3 (FGFR3)[23,24] gene and oncogenes such as HRAS[24] (Harvey rat sarcoma viral oncogene homolog) have been shown to be overexpressed and to have prognostic significance in BC. Although clearly promising their routine use has failed to be adopted into the clinical management of patients due partly to the results of in-vitro laboratory working failing to be reproduced in the clinical

environment and partly to a lack of effective targeted therapies. To improve the reproducibility of results there is a need for a more stringent methodology to reduce the variations in quality and technique between different research groups, as will be discussed later with regards the REMARK guidelines. We must also optimise and improve our pre-clinical models to allow effective translation of results from the laboratory to the clinic.

One possible way to improve survival in patients with MIBC is to develop new predictive biomarkers and novel therapies aimed at specific targets. To do this we need better in-vitro techniques that are able to more accurately predict in-vivo success and also a way to rapidly assess the clinical value of new potential biomarkers. The epidermal growth factor receptor (EGFR) superfamily and particularly human epidermal growth factor receptor 2 (HER2) are receptor tyrosine kinases that have clinically available targeted therapies that are used in the personalised treatment of some breast cancers. EGFR and HER2 have been shown to be overexpressed and associated with poor prognosis in a significant subset of bladder cancer patients (chapter 1.3) and at present are an encouraging area for further research.

This project aimed to address the need to identify predictive and prognostic biomarkers as well as optimizing the in-vitro techniques in two ways. Firstly, to create a cystectomy tissue micro-array (TMA) and associated clinical database to enable the screening of promising biomarkers, including HER2, and assess their clinical significance. Secondly, to evolve a promising new three dimensional in-vitro technique into a reproducible bladder cancer model aimed to more closely recapitulate the in-vivo environment and use it to study targeted drug therapies such as those used against HER2.

1.2 Biology of bladder cancer

Normal urothelial cells that line the bladder and urinary tract undergo a series of changes that evolve progressively to a neoplastic state in an almost Darwinian fashion. It is necessary for the cells to develop various mechanisms to allow; evasion of growth suppressors, invasion and metastasis, replicative immortality, angiogenesis, resist cell death and to sustain proliferative signaling.[25] These six hallmarks of cancer, known as the Hanahan and Weinberg principles hold true for bladder cancers but it is important to also acknowledge the key role of the tissue micro-environment and hosts immune system.

It is appropriate to think of two types of genetically distinct bladder cancers. Low grade non-muscle invasive disease has a completely different natural history compared to high grade muscle invasive disease. The low grade non-muscle invasive cancers are unlikely to kill the host and have a different molecular biology to that of invasive disease.[26] The high grade non-muscle invasive lesion such as G3pTa and CIS share many of the genetic alterations found in muscle invasive bladder cancer and as such are felt to be the pre-cursor lesions.[27]

Advances in molecular biology have identified many genetic alterations in bladder cancer. The two predominant chromosomes involved with the development and progression are chromosomes 9[28-30] and 17[31]. Chromosomal analysis has demonstrated that allelic loss only on chromosome 9 is found exclusively in low grade, early stage disease, compared to more advanced disease where other genetic changes are frequently observed. Loss of heterozygosity (LOH) of chromosome 9 is considered to be the earliest event of bladder cancer initiation. LOH of chromosome 17 is found in around 40% of bladder cancers and virtually exclusively in high stage and grade tumours.[26] Also a great deal of attention has

been given to the oncogene fibroblast growth factor receptor 3 (FGFR3), which maps to chromosome 4p16.3. It appears to have a crucial role in non-muscle invasive disease. [23,32]

There are two main categories of gene that are responsible for the malignant transformation and these are called oncogenes and tumour suppressor genes. Proto-oncogenes may be transformed into oncogenes by a mutation, overexpression, gene amplification or insertion of viral material into the human DNA. Oncogenes then exert their effect by derangement of cell cycle control. Tumour suppressor genes have two alleles which are recessive in function and both must be inactivated to induce tumourigenesis, as popularized by Knudson's two hit hypothesis. The proposed mechanisms for this event are via LOH, point mutations or inactivation of the sequence of the remaining allele.

1.2.1 Low-grade non-muscle invasive bladder cancer

Low-grade non-muscle invasive tumours, unsurprisingly, are the most similar to normal urothelial cells demonstrating few molecular alterations apart from deletions involving chromosome (Chr) 9 and mutations of the oncogene FGFR3.[33,34] The loss of Chr 9 is by far the most common finding and similarly loss of heterozygosity (LOH) of Chr 9 has been found frequently in these tumours.[35]

Low-grade superficial tumours are relatively genetically 'stable'. Multiple tumours sampled from the same patient generally show a striking similarity in the genetic alterations found.[36] LOH of Chr 9 is the least divergent event, indicating that this is likely to be an early evolutionary change. It has also been observed in histopathologically normal tissue surrounding a bladder tumour[37] further highlighting its likely role in tumourigenesis. Apart

from FGFR3 and Chr 9 abnormalities there has been a relative lack of common events discovered in superficial tumours and as such much research using genomic microarray technology is under way.

1.2.1.1 Chromosome 9

The cytogenetic observation that many bladder cancers had monosomy of Chr 9 led to the discovery of Chr 9 LOH in over half of all BCs irrespective of grade and stage.[29,31] In fact many BCs demonstrate a LOH of the entire chromosome, suggesting loss of function of tumour suppressor genes on both arms. As a result, much work has gone into identifying the genes involved on Chr 9 to try and understand the pathogenesis of the disease. The critical regions of LOH have been mapped on both 9p and 9q. In small primary tumours, the frequency of small deletions appears higher, suggesting that initially small regions of LOH may develop and coalesce during tumour development.[28,38]

The areas of loss on Chr 9 that are currently best characterized are mapped to 9p at 9p21 and on 9q at 9q22, 9q32-33 and 9q34).[38-40] The candidate genes at the 9p21 locus are CDKN2A (cyclin-dependent kinase inhibitor 2A), also known as p16 and p14ARF (alternate reading frame), and CDKN2B (p15).[41] At the 9q22 locus is the PTCH (protein patched homolog) gene[39] that is found in Gorlin Syndrome and at 9q32-33 the DBC1 (deleted in bladder cancer 1) protein[42]. At the 9q34 locus is the TSC1 (tuberous sclerosis syndrome 1) gene.[43]

The CDKN2A locus on 9p21 encodes for two key proteins, p16 and p14ARF, which are key regulators of the cell cycle. These genes share a coding region in exon 2 but have distinct exons 1. The protein products are translated in different reading frames to create two entirely

different proteins. The p16 protein is a negative regulator of the Rb pathway and p14ARF is a negative regulator of the p53 pathway (figure 1.2). Both of these genes are commonly found inactivated in BC by homozygous co-deletion. A reduced copy number or a LOH of 9p21 is observed in about 45% of both superficial and muscle invasive bladder cancers[44] and it is still unclear whether 9p21 deletion is related to BC stage and grade.

On the Chr 9q region there are three genes with tumour suppressor functions. PTCH (the Gorlin syndrome gene) is found as a small area of deletion at 9q22. Reduction in its mRNA expression is a common finding. Mutations of the gene are infrequent however, it is felt that PTCH may be haploinsufficient in the urothelium and have a role in BC. At 9q33 a novel gene has been identified called DBC1 and is the only gene within the common region of deletion. DBC1 has been affected by homozygous deletion in a subset of bladder cancers,[45] however, its precise role is not fully understood. That being said it may mediate cell death by delaying the G1 phase of the cell cycle.[46] Thirdly, at 9q34, TSC1 is found mutated in about 13% of bladder cancers.[43] The gene product of TSC1 is hamartin. Hamartin acts in complex with tuberin (TSC2 gene product) to negatively regulate mTOR (mammalian target of rapamycin) in the PI3K pathway (phosphoinositide 3-kinase). (figure 1.3)

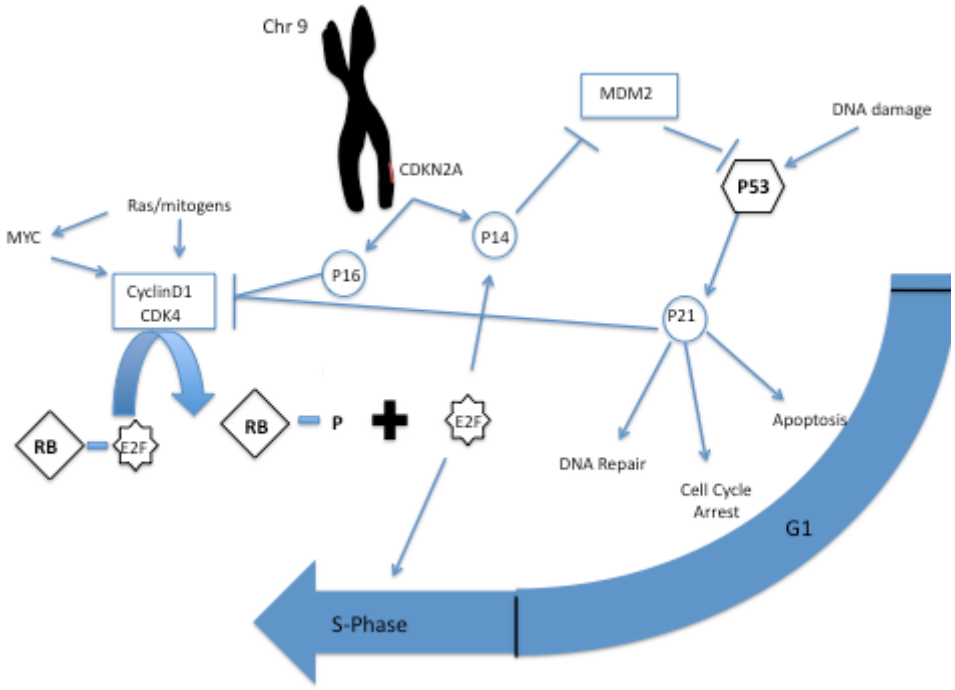


Figure 1.2: RB and P53 pathways are essential for tumour suppression via the mechanisms of cell cycle arrest and apoptosis. The CDKN2A locus on the long arm of Chr 9p encodes for both P14 and P16. P14 is a negative regulator of the P53 pathway and P16 is a negative regulator of the RB pathway.

RB pathway: The CyclinD1/CDK4 complex (stimulated by mitogens) phosphorylates the RB-E2F complex to release E2F. E2F then induces the expression of genes required for entry to S-Phase. This process is negatively regulated by P16 and also P21 (downstream of P53) which interacts with CDK4.

P53 pathway: Cell stress such as DNA damage activates the P53 pathway and increases levels of P21. This leads to cell cycle arrest via inhibition of CDK4 or initiation of apoptosis. MDM2 and P53 regulate each other by means of a negative feedback loop.

The two pathways are linked by P14, which sequesters MDM2 in the nucleus and is up regulated by E2Fs in response to mitogenic signalling. Overexpression of E2Fs and oncogenes such as MYC can result in cell cycle arrest via P14.

Variations in the expression of the above proteins can be altered in bladder cancers.

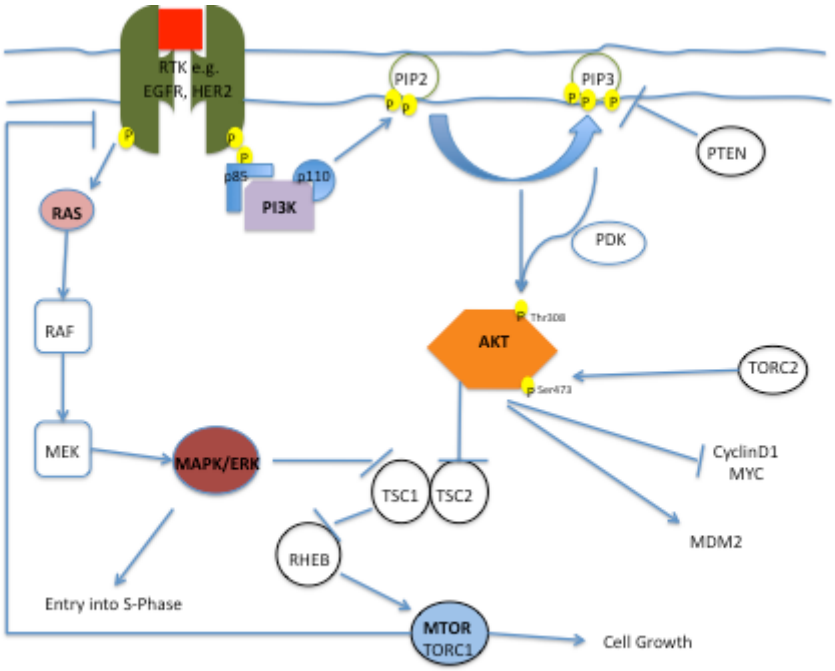


Figure 1.3: The MAPK (ERK) and PI3K pathways. These pathways are stimulated either by growth factors or mutational activation of RAS oncogenes.

The membrane receptor (tyrosine kinase) is activated by an extra-cellular ligand. This leads to phosphorylation of the cytoplasmic domain of the receptor leading to activation of RAS. This in turn activates RAF kinase which phosphorylates MEK. MEK then phosphorylates and activates MAPK which leads to various translational and transcriptional affects.

PI3K activation allows phosphorylation of PIP2 to PIP3. This activates AKT and localises it the plasma membrane. It is an important pathway of cell cycle regulation and is negatively regulated by factors including PTEN.

1.2.1.2 Fibroblast growth factor receptor 3 (FGFR3)

In addition to the Chr 9 abnormalities described FGFR3 has been identified as an oncogene that is activated by mutation in BC. Mutation of FGFR3 is strongly associated with a low tumour grade and stage with up to 80% of low grade pTa tumours demonstrating the mutation.[47] Furthermore mutations in FGFR3 have been shown to be associated with a lower risk of recurrence giving it a potential role as a prognostic biomarker. Interestingly, despite many studies of different tumour types high levels of FGFR3 mutations only appear to occur in BCs. It appears that activation of FGFR3 in the urothelium leads to activation of the MAPK (mitogen activated protein kinase) and PI3K pathways. A mutation of either a RAS gene or FGFR3 occurs in 82% of all superficial BCs indicating a common biological mechanism.[24] Interestingly mutations in the RAS genes and FGFR3 mutations appear to never occur in the same tumour, suggesting biological equivalence in non-muscle invasive disease.

Similarly FGFR3 and P53 mutations are rarely seen together and are largely mutually exclusive.[26] Mutations in the tumour suppressor gene P53 are predominantly associated with high grade, muscle invasive tumours and as such it has been hypothesized that a FGFR3/RAS pathway may illustrate low grade non-muscle invasive disease and a P53 driven pathway define the high grade, muscle invasive cases. (figure 1.4).

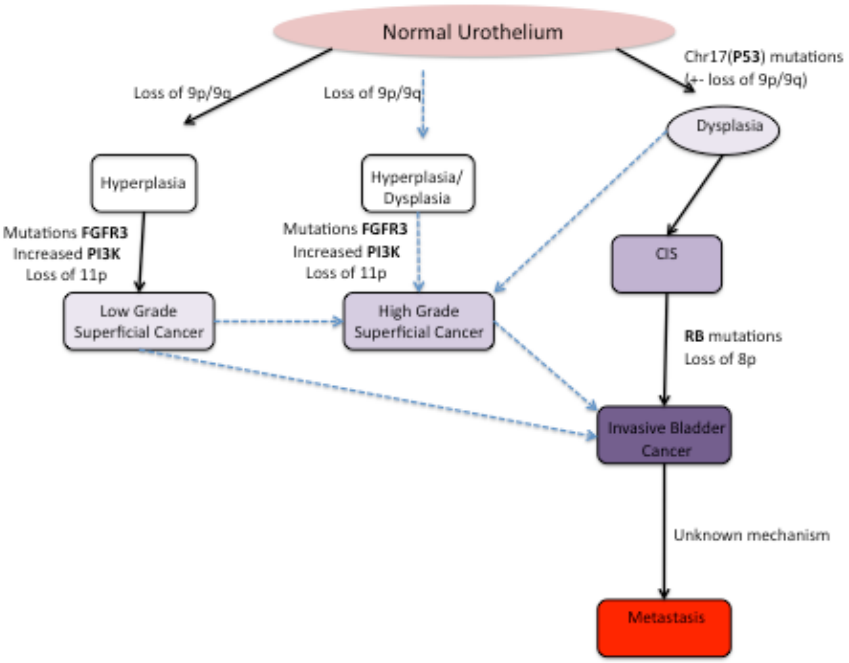


Figure 1.4: Different Molecular pathways proposed for tumourigenesis in bladder cancer. The solid arrows indicate likely pathways and the dotted arrows indicate uncertain relationships. There appears to be two main pathways involved. The first pathway involves Chr 9 deletions and high levels of FGFR3 and PI3K that result in superficial bladder cancers. The second has deletions of Chr 9 as an infrequent finding and favours more mutations in P53 and RB resulting in muscle invasive tumours. (modified from Knowles MA et al., Cancer Metastasis Rev (2009) 28:305–316)

1.2.2 Molecular changes in muscle invasive bladder cancer

Muscle invasive bladder cancer and high grade non-muscle invasive bladder cancer share many of the same genetic alterations and tumour biology and as such high grade non-muscle invasive tumours (G3pTa and CIS) are felt to be precursors to muscle invasive disease. Compared to low grade non-muscle invasive bladder cancer there are a high number of genetic alterations and accordingly the cells display a poorly differentiated growth pattern and exhibit the properties of invasion and early metastasis. These tumours are responsible for a dismal overall survival for a patient and represent an area where the development of targeted therapies in paramount. The diversity of genetic alterations leads to significant intra-tumour heterogeneity that will provide a complex puzzle for future studies designed to personalize targeted therapies based on underlying genetic alterations.[48]

1.2.2.1 Oncogenes

A gene that acts in a dominant way to contribute towards a cancer cell's phenotype is termed an oncogene. The cellular counterparts to these genes are termed the proto-oncogene and can be activated by overexpression of the normal gene (gene amplification or chromosomal polysomy), mutations of the gene or via translocations of the genes. They will predominantly exert their effect in muscle invasive BC (MIBC) via activation of the MAPK or PI3K pathways.

Two important proto-oncogenes reside within the epidermal growth factor receptor family. This consists of four members (EGFR, HER2-4) with EGFR (epidermal growth factor receptor) and HER 2 (human epidermal growth factor receptor 2) being the most appreciated.

HER2 is a receptor tyrosine kinase that is amplified in 5-59% and overexpressed in 9-89% of muscle invasive bladder cancers. [49-52] [53,54] Clinically there are available drugs that target HER2 and as such is a key target for bladder cancer research. Similarly EGFR is overexpressed in 30-50% of invasive tumours and this is associated with a poor prognosis[55] and again only a very small percentage has gene amplification. The underlying mechanism behind this disparity is yet to be elucidated. To date very little is known about the roles of HER3 and HER4 other than as dimerising partners of the other family members.

The rat sarcoma (RAS) family members are a class of protein called small GTPases and are involved in cell signalings that ultimately lead to cell growth, differentiation and survival. Harvey RAS (HRAS) is the only member of the RAS gene family that has been shown to be mutated in some invasive BCs however, there is no obvious delineation between invasive and superficial disease. In vitro experiments on human tumour cells indicate that HRAS can up regulate EGFR expression and induce an invasive phenotype. [56] However, further work in transgenic mouse models engineered to express mutant HRAS failed to demonstrate an invasive phenotype with only papillary, non-invasive tumours developing.[57]

MYC (myelocytomatosis oncogene) is up regulated in many bladder tumours although the mechanism for this is unclear.[58] MYC is found on Chr 8 and although high levels of amplification of 8q are found in some MIBCs (figure 1.4) an associated high protein level of MYC is not always apparent. Perhaps MYC may be transcriptionally activated by other molecular events in MIBC.

1.2.2.2 Tumour suppressor genes

Several tumour suppressor genes have been implicated in MIBC. These genes are also well described in other human cancers and include P53, Rb1, CDKN2A and PTEN (phosphatase and tensin homolog). The interconnecting pathways controlled by P53 and Rb that regulate cell cycle progression and responses to cell stress are commonly altered in MIBC.

The P53 protein is the 53kDa product of the p53 gene located at 17p.13.1 and has a key role in regulation of the cell cycle, apoptosis and DNA synthesis and repair. Mutation of p53 is found in many MIBCs[59,60] and mutations in p53 are felt to be central to carcinogenesis in bladder cancer (figure 1.4).[61] When p53 is mutated the P53 protein product has an increased half life leading to an accumulation and expression in the cell nuclei which can then be imaged using traditional immunohistochemical (IHC) staining techniques. Both high levels of nuclear P53 and p53 mutations have been associated with increased stage and grade of bladder cancer as well as poor clinical outcome.[62-64] Despite this a meta-analysis in the year 2000 of 3,764 patient tumours found only a borderline significance between positive P53 staining (by immunohistochemistry) and poor prognosis making the role of p53 as a prognostic marker unclear.[63] However, the validity of this meta-analysis is limited, as acknowledged by the authors themselves, due to the multiple techniques used in staining as well as different cut offs for significance. Reduced expression of p21 which acts down-stream of p53, is associated with disease progression.[65]

The RB gene product was the first tumour suppressor gene to be identified in human cancer. The RB protein is encoded for by the RB gene mapped to 13q4. It also plays a critical role in many cellular processes, including apoptosis and cell cycle regulation by exerting control

over the transition from G1 to S phase of the cell cycle (figure 1.2).[61] Deletions and dysfunctional mutations of RB often via LOH of 13q, with mutation in the remaining allele, are associated with increasing grade and stage and poor prognosis in bladder cancer.[61,65,66]

The CDKN2A locus on 9p21 which codes for p16 and p14 is commonly deleted in BC of all grades and stages. These proteins interact with and link the RB and P53 pathways. (figure 1.2) Due to the multiple regulatory feedback mechanisms that operate in these pathways it is likely that inactivation of both of these will provide greater freedom from the G1 checkpoint than by inactivation of P53 or RB alone. Altered p21 expression both alone and in combination with other events also represents a significant risk factor.[65]

PTEN is encoded at 10q23 which is a known region of loss of heterozygosity (LOH) in BC of high grade and stage.[67] PTEN is a negative regulator of the PI3K pathway and thus exerts its effects on the cell phenotype by affecting proliferation, apoptosis and cell migration.[68] (figure 1.3) Loss of PTEN leads to PI3K pathway activation with high levels of phosphorylated AKT. As mentioned above, the TSC1 product hamartin also acts in the PI3K pathway, possibly providing an alternative mechanism for activation of the pathway. When P53 and PTEN are both inactivated in a mouse urothelial bladder model, tumourigenesis is promoted in human bladder cells.[69]

1.2.3 Signaling pathways in bladder cancer

It appears that superficial and invasive tumours may utilise separate signaling pathways. (figure 1.4) The finding of FGFR3 and Ras gene mutations in the vast majority of superficial tumours suggests that these tumours share changes in pathway activation. Similarly, the common inactivation of RB and P53 pathways only in invasive tumours as well as the low frequency of FGFR3 mutations in this group may indicate a distinct signaling route.

The oncogenes and alterations in the tumour suppressor genes exert their effect via intra-cellular signaling pathways. As illustrated in figures 2 and 3 several known alterations could potentially activate both the MAPK and PI3K pathways and inactivate the RB and P53 pathways. Ras, Raf, MEK and ERK make up the MAPK cascade to be discussed here and are all mitogen-activated kinases. The initial process in the MAPK pathway is the binding of an extracellular mitogen to the membrane ligand. This enables Ras (p21) to swap its GDP for a GTP. Ras can then activate Raf (MAP3K), which can go onto to activate MEK (MAP2K) and ultimately MAPK, also known as ERK (extracellular signal-regulated kinase) (figure 1.3). MAPK can then activate a transcription factor such as MYC with the desired effect.[70] ERK was the first mitogen activated kinase described and was later renamed MAPK. The role of the MAPK pathway is not so clear as it has been shown to not be highly activated in a study using cell lines from muscle invasive tumours and in contrast high levels of MAPK/ERK phosphorylation are seen in normal urothelial cells in culture.[71] This study would suggest an alternative signaling pathway for muscle invasive disease but more research is needed in this area.

PI3K is a heterodimer consisting of a regulatory subunit (p85) and a catalytic subunit (p110). Once activated, a receptor tyrosine kinase recruits PI3K via the p85 subunit and moves it to the membrane. This process activates p110 which in turn phosphorylates phosphatidylinositol-,4,5-biphosphate (PIP2) to PIP3. The reverse reaction is catalysed by PTEN. PIP3 then recruits protein dependent kinase (PDK1) and AKT to the cell membrane. AKT is phosphorylated by PDK at Thr308 and again by mTOR complex 2 (TORC2) at Ser473. (figure 1.3) AKT is then the key regulator in the PI3K pathway leading to a number of cellular processes including activation of mTOR complex 1 (TORC1), which integrates signals from nutrients, energy status and growth factors to regulate many processes, including autophagy, ribosome biogenesis and metabolism. It has been shown that there can be activation of the PI3K pathway in tumour samples via the mutation of PTEN and TSC1.[72] In bladder cancer mutations have been discovered in the p110 subunit of PIK3A, AKT, TSC1 and PTEN[67,73,74], which have been associated with higher stage and grade of the disease and it is expected that further work with these proteins will provide insight into the molecular mechanisms involved in bladder cancer. [58, 64, 65]

1.3 Biology of HER2

HER2 is a promising target in MIBC and has demonstrated its clinical use in breast cancer where it is also overexpressed in a subset of patients. It is routinely screened for and treatment personalised using a selection of widely available monoclonal antibodies (Trastuzumab) or tyrosine kinase inhibitors (Lapatinib) targeting HER2. For this reason, HER2 was chosen as a biomarker of interest for this thesis and will now be looked at in detail.

HER2 is a 185 KD transmembrane phosphoglycoprotein with associated tyrosine kinase. The HER2 protein is encoded for by a proto-oncogene that is located at chromosome 17q21.[75] The HER family (type 1 tyrosine kinases) has four members: epidermal growth factor receptor (EGFR, ErbB1), HER2, HER3 (ErbB3), and HER4 (ErbB4). They are all similar in structure; containing an extracellular domain that has two cysteine sequences, a transmembrane domain, and a cytoplasmic domain that contains a tyrosine kinase and to a variable extent a carboxy terminus.[76]

Terminology can become confusing with HER2 being referred to by many names ranging from neu to ErbB2 to c-erbB-2. It is worth noting the rationale behind this terminology to both prevent confusion and to correctly interpret papers on the subject. The terminology refers to the species (eg. human or rodent) being studied. "Neu" is the rodent homologue to "HER2" and they were both discovered independently in the 1980s. In 1981 Shih et al.[77] described a transforming oncogene in a rat brain tumour model that they named neu. In the mid-80s, Schechter and colleagues[78] then found neu to be homologous to the v-erbB (viral avian erythroblastosis) oncogene and the epidermal growth factor receptor (EGFR) gene. At

about the same time, King et al.[79] discovered a gene, which he named HER2, that was structurally related to EGFR and was amplified in human breast cancer. Research on tumours derived from both mouse and human cancer cell lines has discovered numerous variants and mutants of HER2 and neu. "ErbB2" is used to refer to the gene across both human and rodent species, "HER2" in reference to the human gene and gene product/protein and "neu" when referring to the rodent species.

1.3.1 The biology of HER2 in normal tissues

The HER2 protein is not commonly present in normal adult tissues except for the renal tubules[80], and the mammary gland at various levels of development and during pregnancy[81,82]. It has been shown to be expressed in the fetus at high levels in the urothelium, proximal and distal renal tubules, gastrointestinal epithelium, bronchiolar epithelium[80], the heart, and nervous system.[83]

Activation of HER2 initiates a complex signalling network that transforms the cell and plays a critical role in the regulation of tissue development, growth and differentiation. As mentioned above it appears that HER2 has an important function in the mammary gland during the various periods of proliferation experienced during puberty, pregnancy, lactation and indeed in the luteal phase of the menstrual cycle.[82] In the heart, both HER2 and HER4 are expressed in the myocardium, including the developing atria and ventricles.[83,84] Mutant embryos, deficient in HER2, fail to develop the essential cardiac trabeculae resulting in death.[83] There is also a suggestion that HER2, HER4 and neuregulins (ligand to HER3 and HER4) are involved in the survival and growth of adult cardiac myocytes and may play a role in cardiac adaptation to physiological stress.[85]

As well as the defects in cardiac development associated with HER2/neu- and neuregulin-deficient mice, abnormalities in neural development have also been observed. Both neuregulins (ligand to HER3/HER4) deficient and HER2/neu deficient mice embryos lack neural crest-derived cells of sensory ganglia, as well as the corresponding axonal connections to the mid brain. This ultimately leads to a severe reduction in size of the cranial nerves' ganglia for: V, VII, VIII and IX.[83,86] HER2 also plays an important role in Schwann cell differentiation. The interest in HER2's role in this area occurred after the observation of an over-expression of a mutant HER2 gene in schwannomas and neuroblastomas.[87] Subsequently it was shown over-expressed by Schwann cells in the developing peripheral nerves.[88]

1.3.1.1 Molecular biology of HER2

The molecular biology to be discussed below is based upon work done in cell line models, predominantly in breast and neural cell lines. Very little has been studied in bladder cells.

When a ligand binds with the extracellular domain of a HER family member, the protein undergoes dimerization and transphosphorylation of it's intracellular domains. The phosphorylated tyrosine kinase interacts with numerous intracellular signalling molecules leading to activation of a plethora of downstream pathways and crosstalk with other transmembrane signalling pathways leading to diverse biological effects.[89] (figure 1.5)

The extracellular domains of the HER family members can exist in either a closed (inactive) or an open (active) conformation. When a ligand binds, the extracellular domain conforms to

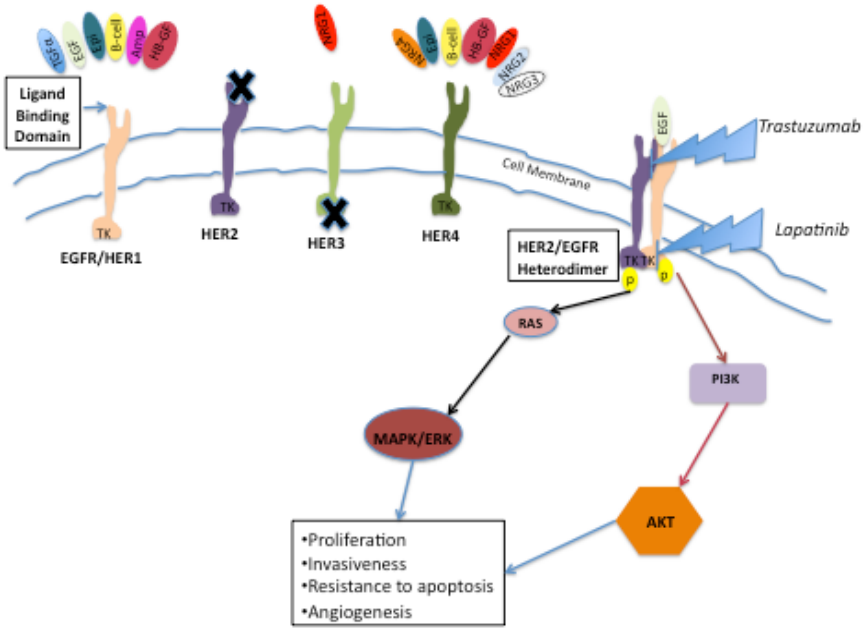


Figure 1.5: Figure to represent the four HER family members and their respective ligands. When a ligand binds with the extracellular domain of a HER family member, the protein undergoes dimerization and transphosphorylation of it's intracellular domains. The phosphorylated tyrosine kinase interacts with numerous intracellular signalling molecules leading to activation of a plethora of downstream pathways and crosstalk with other transmembrane signalling pathways leading to diverse biological effects.

The crosses represent a lack of ligand for the binding site on HER2 and a lack of tyrosine kinase on HER3. The lightning arrows show the points of action of the mono-clonal antibody Herceptin and the dual tyrosine kinase inhibitor Lapatinib.

(HER family ligands: TGFα - transforming growth factor α, EGF – epidermal growth factor, Epi – epiregulin, β-Cell - β cellulin, Amp – amphiregulin, HB-GF – heparin binding growth factor, NRG 1-4 – neuregulin 1-4)

the active state and promotes the active dimerization and ultimate transphosphorylation.[90] Unlike the other family members, HER2 does not change between the active and closed state and remains constantly in the open (activated) conformation.[91]

The HER family members differ in their methods of activation and patterns of dimerization. Epidermal growth factor receptor (EGFR) is activated by epidermal growth factor (EGF), transforming growth factor-α and amphiregulin, whereas betacellulin, epiregulin and the heparin binding growth factor bind both EGFR and HER4.[92,93] The neuregulins (or heregulins) are the ligands for HER3 and HER4.[94] In contrast HER2 is considered an orphan receptor, as there is no known ligand. Interestingly, despite the lack of a ligand, overexpression of HER2 results in cell transformation whereas EGFR will not induce transformation without activation by a ligand, even when highly overexpressed.[95]

The activation of HER2, therefore, is dependent upon its ability to dimerise in either a homo- or hetero-dimerising fashion. It is known that HER2 can act by dimerising with other HER family members, including itself. HER2 is the preferential dimerising partner for all HER family members. Although it has been shown that EGFR can form heterodimers with HER3 and HER4 in cells expressing only these receptor pairs, these receptors will preferentially dimerise with HER2 when it is present.[96] This is not more so than for HER3 as HER2 has no ligand and HER3 has no active tyrosine kinase. Thus, when HER3 binds an activating ligand, it cannot transmit a signal without associating with an active tyrosine kinase. As a result, HER2 is the natural dimerising partner and is often associated with HER3. The dimerisation appears to occur on a three amino-acid sequence immediately C-terminal to the kinase domain that is common to all EGFR, HER3 and HER4 proteins.[97] In fact the

HER2:HER3 complex has a higher affinity for neuregulin than HER3 alone.[98] This is also true for the EGFR:HER2 complex and its ligand.[99]

1.3.1.2 HER2 signaling pathways

The majority of knowledge regarding the downstream signaling pathways of HER2 again comes from breast and neural cell line work with the exact pattern of pathway activation yet to be clearly defined in bladder cancer.

How the HER family dimerise appears to be of crucial importance on the activation of various cytosolic proteins. Their dimerisation affects their downstream consequences and ultimately leads to cell transformation. Increases in HER2 homo- and hetero-dimers can deregulate cell polarity and adhesion by disrupting apical-basal polarity through interaction with components of the Par polarity complex such as PAR6 (partition protein 6) and aPKC (atypical protein kinase C).[100]

Receptor dimerisation may even effect the HER family proteins' affinity for various ligands and also their own recycling. For instance, it has been shown that HER3 and HER4 cannot transform cells unless co-expressed with HER2 or EGFR.[95]. Activation by heregulins leads to phosphorylation of HER3 and the activation of MAPK and PI3K pathways.[101]

The PI3K/Akt pathway is critically important for cancer cells. It functions at a crossroads of multiple signal transduction pathways that regulate numerous cellular functions such as cell survival, cell proliferation, glucose metabolism, cell invasiveness and angiogenesis.[102] The interaction between HER2 and HER3 in this scenario appears to by synergistic. HER2 does

not have the binding sites for the p85 subunit of PI3K, however, there are seven binding sites for the p85 subunit on HER3.[103] This is re-enforced in clinical studies showing the frequent activation of AKT in HER2 over-expressing tumours.

It could also be postulated that the altered affinity of a dimerised HER family protein for a ligand may contribute to an alternative signalling programme. However, there are other theories as to how the signalling pathway could be altered. One such possibility is based on the observation that activation of EGFR by EGF stimulates Src kinase activity. This is necessary for ligand-induced endocytosis.[104] EGFR is unique amongst the HER family as it undergoes endocytic degradation after activation whereas the other members undergo endocytic recycling. [105]In contrast, when there is co-amplification and dimerization of HER2 with EGFR, there is inhibition of the down-regulation of both EGFR and HER2[106], implying that the heterodimerisation of HER2:EGFR may alter the signals for endocytosis. This is further supported by the down-regulation of homodimeric EGFR being significantly different to heterodimeric EGFR:HER2 or EGFR: HER3. In these situations homodimeric EGFR is degraded, whereas heterodimeric EGFR dissociates at the early endosome and is recycled to the cell surface, resulting in enhanced signalling.[107] Therefore, overexpression of HER2 will lead to increased membrane expression and activity of EGFR.

Complexes formed between HER2 and HER3 or EGFR can activate the MAPK and PI3K/AKT pathways in response to either heregulins or EGF.[108,109] The combination of these HER2 complexes and the possibility of abnormalities within the proteins will lead to altered downstream effects. This is demonstrated in the observation that when HER2 dimerises with a kinase deficient EGFR it will still activate MAPK and SHC (SRC homology 2 domain). However, downstream effectors such as the proteins, tyrosine kinases and signal transducers,

GAP, p62, JAK1 and STAT, that are normally triggered by wild-type EGFR, are not activated. This implies that HER2 is probably responsible for the activation of MAPK and SHC, whereas the other signalling proteins are dependent on the kinase activity of EGFR.[110]

1.3.2 Molecular biology of HER2 in bladder cancer

1.3.2.1 HER2 expression and dimerisation patterns in bladder cancer

How HER2 dimerises with the other members of its family in bladder cancer is not known. Only a handful of studies have looked at the rates of the EGF family co-expression in bladder cancer samples. These have shown some interesting results leading to the conclusion that it is necessary to look at the co-expression of the whole EGF family, rather than individuals, to draw prognostic implications.[111,112]

Memon et al. looked at 88 patients with bladder cancer ranging from superficial to invasive disease. When HER2 protein is overexpressed with associated high levels of HER3 and HER4 survival is significantly improved. Survival is also improved, but to a lesser extent, with overexpression of HER2 in combination with high levels of either HER3 or HER4 alone. They showed that 0% of patients with high HER2 and low HER3 and HER4 survived 30 months. In contrast, over 75% of patients with high levels of HER2, HER3 and HER4 survived 120 months. If EGFR is substituted for HER2 an almost identical picture is presented.[112]

Kassouf et al. created a tissue micro array of 248 bladder cancer specimens, which again included all stages and grades, and found that a high expression of EGFR associated with a low expression of HER4 was associated with high-grade invasive tumours with a shorter survival. In this series, however, there was no association found for the expression levels of HER2 or HER3.[111] It is worth noting that this tissue micro array, like most published series, includes a mix of bladder cancers of different stage and grade. As superficial and muscle invasive bladder cancers behave in very different fashions, probably due to fundamental genetic differences, this mixing may dilute patterns that may be seen if the cancer types had been studied individually.

These studies have, unlike other studies, been unable to show that EGFR and HER2 are able to predict overall survival. However, when looked at in combination with HER3 and HER4 they may provide predictive information. Both studies conclude that HER3 and HER4 provide a protective or regulatory role that may negate their sibling's effect.

1.3.2.2 Correlation between HER2 protein overexpression and gene amplification

In breast cancer it is believed that there is a causative relationship between HER2 gene amplification and HER2 protein overexpression, with over 90% of breast cancer cases with HER2 overexpression also demonstrating HER2 gene amplification.[113] As the HER2 gene is located on chromosome 17 it would follow that not only gene amplification but also increased copy number via polysomy of this chromosome would lead to a proportionate increase in gene copy number and therefore may lead to an overexpression of the encoded protein. However, Wang et al studied polysomy of Chromosome 17 in breast cancer

specimens and found no relationship with protein overexpression and Chromosome 17 polysomy.[113]

In contrast bladder cancer specimens have failed to mirror these results. Although bladder cancer samples that possess HER2 gene amplification appear to inevitably over express the protein,[114,115] HER2 gene amplification, quantified using FISH, only appears to occur in about 7-13% of cases. Furthermore, Simonetti et al. have found polysomy of Chromosome 17 to be present in over 90% of their HER2 over-expressing bladder cancers whereas they only observed gene amplification in 6.3%. They went on to observe a significant correlation between polysomy of chromosome 17 with stage and grade with no polysomy observed in G1 tumours, polysomy in 28% of G2 tumours and 100% of G3 tumours.[116]

About 8% of bladder cancers demonstrate amplification of the HER2 gene but a far greater proportion of bladder cancers demonstrate HER2 protein overexpression. This implies that, in contrast to what is known in breast cancer, there must be other, as yet unknown mechanisms that are responsible for the overexpression of the HER2 protein.

1.3.2.3 HER2 co-expression with other oncogenes

It has been shown in breast cancer that the co-amplification of HER2 with other oncogenes such as MYC and TOP2A may impact tumour growth and influence response to HER2 directed therapies.[117,118] In bladder cancer, Hansel et al. found that MYC was co-amplified with HER2 in a subset of metastatic cases.[53] Eltze et al. found, again in a metastatic subset of cases, that HER2 was co-amplified with Cox-2.[119] This was only with a borderline statistical significance and others have not been able to replicate this. [120]

Looking at the co-expression of HER2 with other oncogenes will not only aid attempts to try and personalise treatments for individual patients but will be useful in understanding the mechanisms by which HER2 is involved in cell transformation. This is currently an area where little work has been performed.

1.3.3 Prevalence of HER2 overexpression in bladder cancer

An overexpression of HER2 occurs in bladder cancers but the exact proportion is a contentious issue. The overexpression rates vary widely between series, demonstrating rates of between 9 and 71% with an average between series of about 40% (table 1.2). Unlike breast cancer, where there seems to be a high concordance between protein overexpression and HER2 gene amplification, bladder cancer appears to demonstrate gene amplification in a much smaller group; 5-59% with an average of 18% between series and only 9% if only the studies using FISH to identify cases are considered (table 1.3). The reason for the disparity between results is probably due to different laboratory techniques and antibodies used between departments. It has been highlighted by various authors that there is a need for consistency among centres to allow a proper comparison between papers. Some studies look at bladder cancers of all stages and grades and others identify specific subsets. It is now becoming apparent from recent literature that using the HercepTest^{R} may be the most frequently used procedure. The HercepTest^{R} is standard practice in breast cancer medicine and has a specific IHC scoring for HER2 protein expression and incorporates FISH for identifying gene amplification.

Antibody	Total no. of specimens	% overexpression	Number of muscle invasive specimens	% overexpression in muscle invasive group	Author, year, ref
21N monoclonal	83	26	36	33	Sato 1992
Rabbit polyclonal	54	17	9	89	Moriyama 1991
Rabbit polyclonal	236	22	22	27	Underwood 1995
CB11 monoclonal	36	61	36	61	Caner 2008
CB11 monoclonal	1005	9	1005	9	Lae 2010
HercepTest	138	41	138	41	Kruger 2002
CB11 monoclonal	90	56	90	56	Kolla 2008
Rabbit monoclonal (4B5)	53	36	52	37	Hansel 2008
Hercep & modified HercepTest	90	45-55	90	45-55	Gardmark 2005
Mouse monoclonal	39	71	39	71	Gandor-Edwards 2002
HercepTest	149	7.4	149	7.4	Fleischmann 2011
Rabbit polyclonal	80	28	80	28	Jimenez 2001
CB11 monoclonal	75	57	75	57	Latif 2004

Table 1.2: Table summarising HER2 protein overexpression rates in bladder cancer patients between series. There is a difference in the type of anti-body used and scoring system between studies. Over-expression levels range from 7.4- 71% for all bladder cancers and 7.4-89% for just muscle invasive cancers.

Method	Total no. of specimens	% amplified	Number of muscle invasive specimens	% amplified in muscle invasive group	Author, year
Differential PCR	256	9	22	54.5	Underwood 1995
Quantitative PCR	57	32	24	59	Miyamoto 2000
FISH	141	7.1	67	13.4	Sauter 1993
FISH	36	11	36	11	Caner 2008
FISH	1005	5	1005	5	Lae 2010
FISH	25	8	25	8	Latif 2003
FISH	75	7	75	7	Latif 2004
FISH	147	8.8	147	8.8	Fleischmann 2011
FISH	50	10	50	10	Hansel 2008

Table 1.3: Table summarising HER2 gene amplification rates in bladder cancer patients between series. Different methods to determine gene amplification were used, using differential and quantitative PCR (polymerase chain reaction) as well as FISH (fluorescence in situ hybridisation). The amplification rates range from 5-32% for all bladder cancers and 5-59% for muscle invasive cancers.

1.3.4 Association between HER2 and grade and stage of bladder cancer

Although there is some conflicting data in the literature, it is now generally accepted that tumours over-expressing HER2 are associated with a higher stage and grade as well as a poorer prognosis when compared to those that do not. [121, 49,50,122-124]

In a relatively small study of 25 patients, Latif et al.,[124] showed that 52% of patients with pre-invasive disease overexpressed HER2 compared with 76% of those with invasive disease. In a bigger study of 90 patients with invasive disease who underwent radical cystectomy, Kolla et al.[121] found significant decrease in disease free survival in the HER2 positive group of 30 months compared to 60 months in the HER2 negative group. They were unable to show significant difference between groups for overall survival but the trend was strong, with an overall survival of 48 months in the HER2 positive group compared to 61 months in the HER2 negative group. This compares with the earlier study by Sato et al.[123] of 88 patients with bladder cancer covering all stages and grades, where they demonstrated significant differences of 48.5% vs 9.7% for 5 years disease free survival and 65.5% vs 41.8% 5yr overall survival for HER2 negative and HER2 positive disease respectively.

When looking at Kolla and Sato's results for stage and grade, HER2 was overexpressed in 16% of G1, 21-32% of G2 and 43-71% of G3 tumours. There was a similar trend for stage with 16.7-33% of T2, 33-69.2% of T3 and 38-75% of T4 tumours over expressing HER2.

Kruger et al.[125] were able to show an increase in tumour grade with HER2 positivity but were unable to show any differences between groups with respect to stage or lymph node metastasis. In contrast, Underwood was unable to find any correlation between HER2

overexpression and progressive disease but did find that amplification of HER2 at the gene level was associated with progressive disease. [126]

Although they were unable to show HER2 overexpression to be predictive of stage and grade in their sample of patients with metastatic disease, Jimenez et al. found that the overexpression rates were increased in distant metastasis when compared to lymph nodes and compared to the primary bladder cancer at 86%, 63% and 37% respectively.[50] This implies that an overexpression of HER2 may be a primary event in metastatic lesions or, as the authors admit, limitations of their study may have meant that they did not sample representative tumour from the primary sample. In contrast, Gardmark et al. found, using the agreed scoring system as used in breast cancer, that HER2 was overexpressed in 45% of their primary tumours and 37% of the associated metastatic lymph nodes with 100% of concordance between HER2 positive primary and HER2 positive lymph nodes although 5% of the HER2 positive lymph nodes came from HER2 negative primaries. [127]

A recent large study of 150 patients who underwent cystectomy and were found to have lymph node metastases, went further to look at not only HER2 overexpression but also HER2 amplification as assessed using FISH (fluorescence in situ hybridization). They found 100% concordance in HER2 amplification between the primary sample and the lymph node with a higher incidence of HER2 amplification in the lymph node (15.3%) compared to the primary sample (8.7%). They also found that HER2 amplification was an independent predictor for a reduced overall survival. When looking at HER2 protein overexpression, they were unable to find a correlation with survival or concordance in expression levels between the primary sample and the lymph nodes.[51]

1.4 Biomarkers in Bladder Cancer

In general terms a biomarker is anything that can be used as an indicator of a particular disease state or other physiological state of an organism and can range from simple measurements such as temperature and blood pressure to antibodies and molecular proteins. In recent years, in medicine, the term biomarker has become a synonym for molecular biomarker, such as is the case with prostate specific antigen (PSA) in prostate cancer.

There are many ways to classify biomarkers but with relevance to this thesis molecular biomarkers are generally termed 'diagnostic', 'prognostic' or 'predictive'. Biomarkers, such as PSA, have a diagnostic as well as prognostic role in screening populations for a particular disease state. Prognostic biomarkers, such as P53, provide information on the likely cause of a disease if left untreated but now the definition also includes the outcome of the disease despite treatments such as surgery. Finally, predictive biomarkers are probably the most valuable as they identify sub-populations of tumours which are likely to respond to a given treatment. This is the aim of precision medicine. For example, overexpression of HER2 in breast cancer predicts response to Herceptin as well as being prognostic.

Diagnostic biomarkers therefore provide an opportunity to diagnose tumours earlier and with greater accuracy. Prognostic biomarkers can identify patients who are at a higher risk of tumour recurrence and predictive biomarkers aim to pick out patients who will respond to a particular therapy. In this thesis the interest will be with both prognostic and predictive molecular biomarkers in patients with invasive bladder cancer.

1.4.1 REMARK guidelines

Numerous potential biomarkers have been discovered and published with respect to cancer however very few have made it into everyday clinical use.[128] One reason is the difficulty in validation between centers. This can potentially lead to truly useful biomarkers being disregarded and conversely much time and effort spent on fundamentally flawed markers. In many cases a biomarker that is predictive initially in one study fails to be validated or often there is no attempt at validation. A variety of problems have been identified as possible cause for these discrepancies. Very often general differences in methodology such as non-standardized assays and scoring systems, poor study design and inappropriate statistical size can be identified as reasons for the lack of consistency.

As a result of many tumour marker studies not being reported in a rigorous fashion[129] the development of guidelines for the reporting of tumour marker studies was a recommendation of the National Cancer Institute – European Organisation for Research and Treatment of Cancer in a convention in Denmark in 2000. On the back of this a collaborative group consisting of clinicians, statisticians and scientists the REMARK guidelines were developed to try and help produce high quality and reproducible biomarker research. (table 1.4) REMARK stands for REporting recommendations for tumour MARKer prognostic studies. The REMARK guidelines were published in Nature in 2005 and are strongly endorsed by the Program for the Assessment of Clinical Cancer Tests (PACCT) strategy group of the United States national cancer institute.[129] Table 2 shows the twelve points of the REMARK guidelines. In summary, these guidelines attempt to provide a structured approach for biomarker research by setting out study objectives, identifying an appropriate study population, which assay method to be used and taking into account which statistical test will

be appropriate. It then advises on how to comment on the study population and recommends ways of presenting results before discussion of the findings.

REporting recommendations for tumor MARKer prognostic studies (REMARK).
Introduction 1. State the marker examined, the study objectives, and any pre-specified hypotheses.
Materials and methods Patients 2. Describe the characteristics (e.g. disease stage or comorbidities) of the study patients, including their source and inclusion and exclusion criteria. 3. Describe treatments received and how chosen (e.g. randomized or rule-based). Specimen characteristics 4. Describe type of biological material used (including control samples) and methods of preservation and storage. Assay methods 5. Specify the assay method used and provide (or reference) a detailed protocol, including specific reagents or kits used, quality control procedures, reproducibility assessments,quantitation methods, and scoring and reporting protocols. Specify whether and how assays were performed blinded to the study endpoint. Study design 6. State the method of case selection, including whether prospective or retrospective and whether stratification or matching (e.g. by stage of disease or age) was used. Specify the time period from which cases were taken, the end of the follow-up period, and the median follow-up time. 7. Precisely define all clinical endpoints examined. 8. List all candidate variables initially examined or considered for inclusion in models. 9. Give rationale for sample size; if the study was designed to detect a specified effect size, give the target power and effect size. Statistical analysis methods 10. Specify all statistical methods, including details of any variable selection procedures and other model-building issues, how model assumptions were verified, and how missing data were handled. 11. Clarify how marker values were handled in the analyses; if relevant, describe methods used for cutpoint determination.
Results Data 12. Describe the flow of patients through the study, including the number of patients included in each stage of the analysis (a diagram may be helpful) and reasons for dropout. Specifically, both overall and for each subgroup extensively examined report the numbers of patients and the number of events. 13. Report distributions of basic demographic characteristics (at least age and sex), standard (disease specific) prognostic variables, and tumor marker, including numbers of missing values. Analysis and presentation 14. Show the relation of the marker to standard prognostic variables. 15. Present uni-variate analyses showing the relation between the marker and outcome, with the estimated effect (e.g. hazard ratio and survival probability). Preferably provide similar analyses for all other variables being analyzed. For the effect of a tumor marker on a time-to-event outcome, a Kaplan–Meier plot is recommended. 16. For key multivariable analyses, report estimated effects (e.g. hazard ratio) with confidence intervals for the marker and, at least for the final model, all other variables in the model. 17. Among reported results, provide estimated effects with confidence intervals from an analysis in which the marker and standard prognostic variables are included, regardless of their statistical significance. 18. If done, report results of further investigations, such as checking assumptions, sensitivity analyses, and internal validation.
Discussion 19. Interpret the results in the context of the pre-specified hypotheses and other relevant studies; include a discussion of limitations of the study. 20. Discuss implications for future research and clinical value.

Table 1.4; REMARK Guidelines (modified from McShane et al. Nature 2005 2(8), p419) Each biomarker study should aim to fulfill the above criteria in order to enable accurate and relevant comparisons between studies.

1.4.2 Biomarkers in bladder cancer

There are no bladder cancer biomarkers that are used in routine clinical use. In breast cancer, by comparison, there are numerous biomarkers such as the BRCA1 (breast cancer susceptibility protein 1) and BRCA2 genes that give useful prediction on tumour occurrence[130]. Moreover, overexpression of the tyrosine kinase HER2 (human epidermal growth factor receptor 2) acts as a predictive and prognostic biomarker and allows the clinician to initiate target directed therapy with the administration of Trastuzumab (a monoclonal antibody directed against HER2). These biomarkers allow personalization of treatments for breast cancer patients with the associated improvement in survival.[131]

The lack of biomarkers in bladder cancer in everyday clinical use is mainly due to no single marker being specific or sensitive enough to justify a change to treatment but also due to a lack of effective targeted therapies to be used. Ultimately it is unlikely that a single biomarker will hold the key and studying combinations of biomarkers is more likely to predict outcome in cancer. Some of the most researched biomarkers in bladder cancer are P53, RB, FGFR3, EGFR, Ki-67 and VEGF. Most of these have been highlighted in the biology section of this thesis but will be briefly summarized again here along with other promising examples such as β-HCG, Nrf2, AIMP3 and HER2 that are worthy of further interest. (Table: 1.5)

Name of Biomarker	Gene	Associated outcome	Gene mutation (%)	Protein overexpression (%)	References
P53	17p13	Tumour progression, higher stage and grade with shorter overall survival	HGIBC – (48.8 – 53) LGNIBC – (0-8%)	HGIBC – (39-71) LGNIBC – (11.1-37)	132-138
RB	13q14	Higher stage and grade with shorter overall survival	HGIBC – (13-36) LGNIBC – (21)	HGIBC – (34-81) LGNIBC – (10-43)	21, 137-139
FGFR3	4p16	Predominantly low-grade and stage	HGIBC – (14.5-16%) LGNIBC – (74-80)	HGIBC – (42-50) LGNIBC – (50-70)	23,133,139, 140
EGFR	7p12	Higher stage and grade with shorter overall survival	HGIBC - (7.6-9) LGNIBC – (0-36)	HGIBC –(35-86) LGNIBC – (12-14)	133,141,142
Ki-67	10q26	Tumour progression, higher stage and grade with shorter overall survival	HGIBC – (2.2 -7.6)	HGIBC – (42.5 – 75) LGNIBC (27.8 – 32.5)	133,143,144
VEGF	6p12	Higher stage and poor prognsosis	HGIBC – (2-4-4.6)	HGIBC – (86-92)	133,145,146
β-HCG	19q13	Poor prognosis	Not known	HGIBC – (15-65) LGNIBC –(0-24)	147,148
HER2	17q21	Higher stage and grade with shorter overall survival	HGIBC – (5-59)	HGIBC – (7.4-89)	49,52,125,126

Table 1.5: Table highlighting some of the common biomarkers being studied in bladder cancer (HGIBC – high grade invasive bladder cancer, LGNIBC – low grade non-invasive bladder cancer) The outcome associated with the biomarker is highlighted and the gene expression rates and protein overexpression ranges are illustrated in the appropriate columns.

Aminoacyl-tRNA synthetase interacting multifunctional protein 3 (AIMP3)

AIMP3 is a novel tumour suppressor that was identified from a global gene expression dataset. It mediates protein transcription and has been implicated as an upstream activator of P53. Recently published work, from collaboration between this author and University College London, found AIMP3 expression to be predictive of overall survival in patients receiving radical radiotherapy in bladder cancer.[149]

Nuclear factor erythroid 2-related factor 2 (Nrf2)

Nrf2 is a critical transcription factor that regulates the cellular protective response against toxic chemicals and has generally been considered a "good" transcription factor. It has however, been shown to be involved in resistance of cancer cells against chemotherapeutic agents.[150] Recently published work in our research group, as part of this thesis, has demonstrated increased levels of Nrf2 in bladder cancer cell lines that have been made resistant to cisplatin and overexpression of Nrf2 predicting reduced survival in cystectomy patients who received neo-adjuvant chemotherapy.[151] As a result Nrf2 may be crucial in bladder cancers resistance mechanisms against cisplatin chemotherapeutic agents and further research is warranted.

β-HCG

Although it cannot be called a new or emerging biomarker it is not clear why interest in this subunit of Human Chorionic Gonadotrophin (HCG) waned at the end of the twentieth century. HCG is a heterodimeric glycoprotein comprising two non-covalently bound subunits

named ‘alpha’ and ‘beta’. The alpha subunit comprises a 92 amino acid sequence[152] encoded by a single gene located on chromosome 6q21.1-q23, and is common to all members of the glycoprotein family including thyroid stimulating hormone (TSH), follicle stimulating hormone (FSH) and luteinising hormone (LH). The beta subunit however, is unique to HCG and comprises a 145 amino acid sequence. It can be encoded for by any one of six non-allelic genes; ß1,2,3,5,7 and 8, present on chromosome 19q13.32.[152]

HCG is perhaps best known for its physiological role in pregnancy where it is initially secreted by the developing embryo and later by syncytiotrophoblasts of the placenta. In pregnancy it has a number of described roles such as promoting angiogenesis of the uterine vasculature, stimulating foetal testosterone production and enhancing corticosteroid production and maternal immunosuppression to prevent rejection of the foetus and placenta.[153]

Serum concentrations of ß-hCG in patients with bladder cancer have been shown to be raised, but there is a wide variation in the positivity observed. In 1994, Smith et al.[154] performed a prospective study of 163 patients being managed cystoscopically for disease across all stages and grades. They found that 10% of the patients had high levels of serum ßhCG but this had no correlation with survival, stage or grade.

In contrast, Dobrowlski[155] and Iles[156] have both observed increased levels of serum ßhCG with increasing stage and grade of the disease. Here, Iles showed that 2/64 patients with locally confined bladder cancer had a raised ßhCG compared with 16/21 patients with metastatic disease. Similarly, a more recent study by Venyo et al.[157] looked at 120 patients with bladder cancer being treated cystoscopically and measured their serum ßhCG levels.

They included two control groups: group A consisting of 30 patients with benign conditions (none of whom had raised ßhCG levels), and group B that consisted of 70 patients with a history of TCC bladder but were currently disease free cystoscopically (of these only one had a raised ßhCG). In the study group, 30% (36/120) of the patients had a raised serum ßhCG, and the levels increased through stage and grade, with 23% of superficial tumours and 47% of muscle invasive tumours being associated with a raised serum ßhCG.[157]

Human Epidermal Growth Factor Receptor 2 (HER2)

HER2 is a transmembrane phosphoglycoprotein with an intracellular tyrosine kinase. It is a member of the EGFR family and its activation results in a complex signalling network that transforms the cell and plays a critical role in the regulation of tissue development, growth and differentiation. As mentioned above, the profound success and adoption of HER2 into routine clinical use in breast cancer treatment makes it an obvious biomarker to be studied in bladder cancer. Published studies vary in their findings with some authors finding HER2 to be overexpressed and indicative of a poor prognosis in bladder cancer[49,121,125] and others failing to find a correlation.[50] There are often vast differences in methodology between groups and the scoring technique of IHC stains may be one of the greatest sources of error. Despite this the overall evidence supporting HER2 as a biomarker in bladder cancer as well as the availability of drugs such as Lapatinib (dual EGFR/HER2 tyrosine kinase inhibitor) and Trastuzumb (monoclonal antibody against HER2) in the clinical setting means that clinical trials are underway to assess the efficacy of some of these agents in the metastatic setting.[158]

In this thesis the role of HER2 in muscle invasive bladder cancer patients, treated by cystectomy, will be investigated. HER2 has no known ligand and as a result has to either homo- or hetero-dimerize with other members of the EGFR family. The expression of HER2 with its family members will be studied with respect to survival and recurrence in this cohort.

1.5 Pre-clinical models in bladder cancer

In order to introduce new and effective clinical treatments and strategies for patients it is necessary to target drug development on relevant genes, proteins and signaling pathways. These are identified by utilizing effective and physiologically relevant pre-clinical models.

1.5.1 Biomarker screening and validation

Biomarker research has three main areas of interest. The first is to identify a biomarker that is prognostic of outcome or predictive of treatment, the second is to understand the molecular biology of the biomarker and thirdly to develop therapeutic agents targeted at the biomarker of interest. Comparing the expression of a molecular biomarker with clinical outcome can be an expensive and time-consuming process. One method to overcome this problem is the creation of Tissue Microarrays. These allow hundreds and possibly thousands of patients' tissue to be studied in a single test. Once the discovery has then been made screening for the biomarker in an external TMA can allow validation of the studies findings. Genomics is the other field which, as the cost falls, is becoming more popular as a way to rapidly identify biomarkers. (table 1.6)

There is a significant time lag between the identification of a potential biomarker and validation of its clinical usefulness as marker of prognosis or response to treatment. To do this requires measuring the expression levels of a particular marker in hundreds of tissue samples. This can be extremely time consuming and prohibitively expensive. To try and overcome this hurdle Tissue Microarrays (TMAs) have been developed allowing the possibility of hundreds of patients' tissue samples to be analysed at once on a single slide, saving on the cost of expensive antibodies, reagents and time.

In 1986 the "sausage block" method was described by Hector Battifora.[159] In this technique he wrapped 1milimetre (mm) thick rods of tissue in sheets of small intestine. This was then embedded with up to 100 different samples in a paraffin tissue block. He evolved this technique into his checkerboard arrangement[160] to try and improve identification of the individual tissue samples, however, the inability to robustly confirm the tissue samples limited any meaningful interpretation. In 1998 Kononen was the first to describe[161] what we now consider to be a TMA.

The modern TMA is constructed in a normal paraffin tissue block. The tumour blocks from particular patients with the disease process of choice are identified and representative cores of tissue are removed using a specialized coring machine from the "donor" block and transferred to the new "host" block. The length and diameter of the cores can vary depending on the size of the donor block and how many samples are desired to fit in the host block. The diameter can vary from 0.6mm, 1.0mm, to 2.0mm. Once the block is constructed it can then be cut onto slides for Haematoxylin and Eosin (H&E) stain, IHC or even Fluorescence Insitu Hybridization (FISH).

Pre-clinical model	Advantage	Disadvantage
TMA	Analysis of hundreds of tumour samples on one slide. Cost effective.	Tumour heterogeneity hard to account for. Loss of tumour cores. Difficult to score and validate rates of expression.
Whole genome sequencing	Most complete picture of entire genome. Identifies non-coding elements that may be functional in disease.	Prohibitively expensive but getting cheaper. Complex bio-informatics to interpret results. Tumour heterogeneity means that significant driver mutations may be lost in the 'noise' if only relatively small numbers. Ethical dilemmas as to who 'owns' results.
Whole exome sequencing	Cheaper and quicker than whole genome sequencing. Availability of array chips. Reduced cost allows higher sample numbers	Expensive. Does not look at 99% of the genome and may miss relevant structural and non-coding gene anomalies. Often requires PCR. Complex bio-informatics to interpret results. Ethical dilemmas as to who 'owns' results.
Gene expression profiling	Identifies key 'active' genes involved in biological process. Sensitive indicator of biological activity.	Expensive. Not as precise as proteomic techniques. Ethical dilemmas as to who 'owns' results.
In-vitro techniques		
2D cell culture	Cheap. Relatively quick and accessible.	Fails to take into account cell-cell interactions and crucial role of micro-environment. No immune system.
3D cell culture/organotypic	More closely recreates the in-vivo model. Allows interaction with micro-environment. Accurate model for invasion.	No immune system. Time consuming. Still lacks many physiological constituents such as blood, urine, lymph etc.
In-vivo techniques		
Genetically engineered mice	Physiologically relevant model. Can test specific genetic alteration. More likely to be translated into human studies.	Ethical issues. Animal welfare paramount. Expensive. Different trans-species phenotype of tumour might be expressed. May be an immune compromised host.
Carcinogen induced mouse model	Mimics initiation steps of some cancers. Can study early events. Can study prevention.	Ethical issues. Animal welfare paramount. Expensive. Different trans-species phenotype of tumour might be expressed. Variability of tumour progression. May need large numbers.
Patient derived Xenografts	Human cancer cells. Closest model to human.	Ethical issues. Animal welfare paramount. Expensive. Immune compromised host. Difficulty in growing Xenografts. Different trans-species phenotype of tumour might be expressed. Artificial location and altered vasculature.

Table 1.6: Table to show the most commonly used pre-clinical models in cancer research. The advantages and disadvantages are described.

Many significant advantages have been reported from the large-scale analysis of human tissues, made possible by TMAs. As an example, if a TMA containing 1,000 cores is cut 200 times, as many as 200,000 individual assays can be produced from a single block.[162] TMA also has the added advantage that all specimens are processed at the same time using identical conditions, including antigen retrieval, reagent concentration and incubation times. Furthermore, a TMA contains hundreds of tissue samples and as such positive and negative controls are invariably present on each slide.

There are of course potential problems with TMAs. A core of tissue taken from a tissue block may contain tumour at the surface but as progressively deeper cuts are taken tumour may finish and normal tissue continue. Also within a tumour there can be great heterogeneity with one part of a tumour expressing a particular marker and another area not. It can be difficult to know how to interpret this on whole sections and in TMAs there is a risk of under scoring a marker if a representative core has been missed. Another area of difficulty is the adhesion of the cores to the slide when cutting the TMA block and also when performing antigen retrieval techniques. Five percent of cores can be lost with 2mm cores[163] and up to twenty percent with 0.6mm cores.[164]

Despite the pitfalls TMAs do appear to be reliable and reproduce the results obtained from whole tissue sections adequately. Variables that may affect results include the diameter of the core as well as the number of cores taken from each donor block. Lee et al. used four 2mm cores in their TMA and compared the expression of MUC2 in 51 gastric cancers. The results were then compared to the original whole tissue blocks. They found a good correlation with a kappa value of 0.87.[165] Camp et al. looked at the question of how many cores to take per sample. They compared the staining performed on between two to ten per specimen and the

original tissue block. They showed that even two cores of tumour were comparable to the whole tissue section in more than 95% of cases and that this increased to 99.5% if five cores were taken.[166] Finally, Hoos et al. looked at the expression of Ki-67, P53 and RB from 59 fibroblastic tumours using one, two and three 0.6mm cores. They found that three cores gave the best concordance with the full sections with 96%, 98% and 91% respectively for Ki-67, P53 and RB staining.[167]

Although TMAs are useful and can even look at particular gene mutations using FISH techniques,[52] they are limited by their ability to only look at one potential biomarker at a time. The patterns or signatures of genetic mutations or protein expression that may truly relay the biology of the cancer may be missed. The field of genomics holds the potential to overcome this hurdle and as the cost of the technology falls may well become the technique of choice for true precision medicine.

The area of genomics in cancer encompasses many areas including genome sequencing, exome sequencing, gene expression profiling and methylation. Whole genome sequencing provides the most comprehensive collection of an individual's genetic variation and looks at the entire genome which is made up of about $3x10^9$ bases which accounts for around 20,000 encoding genes, pseudogenes and noncoding genes. Due to alternate splicing from overlapping of read frames and post-translational modification this allows for the potential of over 1,000,000 distinct proteins. This technique looks at the template and tells us what the cell could possibly do. The limiting factor traditionally has been the cost, with one of the main companies, Illumina, charging $48,000 to sequence one genome in 2009, however the cost has fallen dramatically with Ilumina announcing its first $1,000 genome in 2014. Furthermore, complex computer based networking is required, for all 'omics' techniques, to

interpret the huge amount of data generated. Whole genome sequencing however, allows for non-hypothesis led discovery of predictive signatures by comparing normal tissue to cancer tissue and identifying abnormalities. This can then be used to target research into the underlying biology or target available drugs.

It is estimated that approximately 85% of the disease causing mutations are made up in coding and functional regions of the genome, known as the exome. The human exome is made up of about $3x10^7$ bases, or about 1% of the entire genome. For this reason, sequencing of the entire exome has the potential to uncover the predisposing variants in cancer and has the advantage that it is significantly cheaper than sequencing the entire genome. When there are areas that are well understood, such as the androgen receptor in prostate cancer, it is possible to simply sequence the known targetable mutations to allow personalization of therapies as mutations develop. At the same time, an obvious limitation is that 99% of the human genome is not sequenced and will miss the structural and non-coding elements that may be crucial in disease.

After sequencing a genome, the next obvious step is expression profiling. This gives information on the activity of genes for a given environment at a given moment in time. Genes contain the instructions for making messenger RNA (mRNA) but mRNA is produced from 'active' genes and only a tiny proportion of genes are used for mRNA production at any moment. Therefore, measuring the amounts of mRNA will indicate which genes are active and measuring the relative amounts of mRNA expressed in two or more experimental conditions will infer which genes are turned 'on' or 'off' to various environmental stimuli. By inference this gives a global picture of the likely proteins that have been translated and

although not as precise as the direct measurement of the proteins, as is possible with mass spectrometry in proteomics, it gives a quicker and immediately complete picture.

The ‘pan-omics’ approach gives an unprecedented global view of all the genetic factors involved and with decreasing costs will be the technique of choice for truly personalising therapies.

1.5.2 In vitro models

Once a particular biomarker has been identified, be it at the gene or protein level it is then necessary to understand the biology behind it. Traditionally this has been done with in-vitro laboratory work in the form 2D cell culture on immortalized cell lines of known cancers. This has many advantages including being relatively cheap, easy and quick to do. A gene of interest can be studied for mutations, amplifications and deletions using various techniques including PCR and FISH. Beyond this protein expression is often quantified with techniques such as western blotting, flow cytometry and immunofluorescence. Likewise, intra-cellular signaling pathways can be studied.

The 2D cell culture is therefore well positioned to look at the effects of environmental factors, such as drugs and growth factors, on the cells in question. By measuring various end points such as cell numbers, markers of proliferation (eg Ki67), mitochondrial function assays and markers of apoptosis it possible to determine the effectiveness of a given drug. Once this has been established the relevant intra-cellular signaling mechanisms and pathways of resistance can be elucidated.

When studying cancer, it is essential to understand the mechanisms behind invasion and metastasis and again these can be imitated by the development of crude models such as the scratch assay and cell exclusion assays. The major problem in the physical process of scratching the confluent cell is the damage to the underlying extracellular matrix and cells resulting in the release of factors that could compromise the results.[168,169] This problem is somewhat addressed by using the Cell Exclusion Zone model however, neither give much information on the cell's invasive characteristics. Trans-membrane Assays allow the measurement of cells through a membrane towards a chemotactic agent under different conditions over a time frame. The number of cells on the underside of the membrane gives an indication of invasion. This technique has the limitations that it can only be performed over a limited time frame as the chemotactic concentration gradient depletes[170] and the cells have to travel through a non-physiological polycarbonate or polypropylene filter.

When studying invasion, the above techniques are overly simplistic. They do not recreate physiological relevance as they fail to take into account the surrounding tissues and cells that are present in vivo. There is now substantial evidence that the peri-tumoural stroma actively communicates with the cancer cells to influence invasion.[171] It appears that one of the constituents of the stroma, the fibroblast, is of particular importance in modulating the cancer cells ability to invade by paracrine communication.[172] There are now new three dimensional (3D) models, encompassing Organotypics, that attempt to recreate the microenvironment around the tumour with various tissue substitutes.

One of the earliest in vitro models to study invasion in as near physiological conditions as possible was the skin organotypic model developed in 1983 by Fusenig.[173] This system studies invasion of squamous cell carcinoma cells through collagen gels embedded with

fibroblasts. After a variable length of culture, the gels can be fixed and processed for histology.

These 3D models where cells are grown in the extracellular matrix can be formed from re-aggregated cells or tissue explants. Typically, organotypics are made by suspending stromal cells (including fibroblasts) in growth media containing type I collagen, which is liquid at 4^0C, to create a tissue substitute. Once the solution has hardened at room temperature or above, epithelial cells are then plated on top of the gel at the required concentration. Once a monolayer is achieved the gels are then raised onto a scaffold to keep then at the surface of the liquid media and grown at the "air-liquid" interface. An alternative to using the collagen containing tissue substitute would be to use animal tissue such as sections of cardiac, neural or myometrial tissues.[174] After a period of incubation any invasive cells will have invaded into the tissue substitute and the degree of invasion measured and visualized using IHC techniques.[175] The ability of 3D organotypic models to mimic the results from animal models makes them an attractive in-vitro technique.

The aim of organotypic cell culture is to recreate in-vivo conditions in an in-vitro model and represents a useful halfway house between 2D cell work and animal models. There are numerous examples where experimental results from 2D cell culture are not recreated in organotypic or animal models. Cells in organotypic culture are able to withstand drugs and toxins[176] better than in normal 2D culture and the morphology of cells can be completely different.[177] For example human embryonic stem cells in 3D culture, when stimulated by external factors to induce hepatic histogenesis, produced cells that more closely resembled true hepatocytes than those in 2D culture.[177]

Organotypic models have yet to be fully utilized in bladder cancer research. A novel organotypic has been used to study the role of PTEN in bladder cancer cell lines. In this model the urothelium was removed from rat bladders and the remaining bladder tissue used as the base onto which the cells of study were plated. They showed that PTEN inhibited invasion in the cell lines in both their organotypic model and also in vivo when the cells were introduced intra-vesically into nude-mice.[178]

There is an abundance of laboratory techniques designed to study the molecular biology of biomarkers. In cancer, it is of critical importance to try and understand the biomarker's role in the mechanisms of cell migration and invasion, as it is these steps that ultimately lead to metastasis and death. Hence methods are needed where the biomarker can be studied in as near physiological conditions as is possible. Animal models have clear advantages when creating a physiologically relevant model however, differences between species have led to false results[179] and ethical dilemmas are paramount. However, the transgenic mouse model has proved to overcome some of the interspecies differences in recent years[57] and is probably to model of choice before moving to human trials. Eventually, combining patient derived Xenografts mouse models with real time gene profiling, before and after targeted treatments, will allow a truly personalised approach to treating a person's cancer.

2. Hypothesis and Aims

It is clear that there has been a lack of success in improving survival in patients with muscle invasive bladder cancer. This is in contrast to many large steps in our understanding of the biological processes behind bladder cancer initiation and progression. This highlights the difficulty in translating pre-clinical research into valuable clinical treatment strategies. Some of the areas of difficulty are creating physiologically relevant pre-clinical models and knowing whether a biological breakthrough in such a scenario is relevant in a significant number of patients rather than in just a cell line model. Further to this there are a lack of available effective disease modifying drugs. HER2, and its family, is one potential biomarker that is worthy of further study. There is increasing evidence in bladder cancer for its role in cancer initiation and propagation. With the availability of HER2 targeted agents, it has the potential to be a useful target in bladder cancer.

The hypothesis of this thesis was that HER2 expression levels are predictive of survival in patients with muscle invasive bladder cancer treated by radical cystectomy and that it will play a key role in invasion. To test this required relevant model systems for investigation of HER2 biology in bladder cancer. The aims therefore were:

1. To optimize a novel bladder cancer organotypic for a physiologically more relevant environment and to assess the role of HER2 on invasion.
2. To create a TMA and associated database of cystectomy patients to determine the expression characteristics and prognostic power of HER2 and its family members in this population.
3. To use the TMA to screen for other potential biomarkers in bladder cancer.

3. Methods

3.1 Ethical approval

All work had UK national research ethics service approval and covered all human sample work including the TMA (ref: 10/H0405/99)

3.2 Media and Solutions

All reagents were obtained from Sigma-Aldrich (Dorset, UK) unless otherwise stated.

Keratinocyte Serum Free media (SFM) – complete

500mls Keratinocyte – SFM (Invitrogen, UK)

25mg Bovine Pituitary Extract (Invitrogen, UK)

2.5µg Epidermal Growth Factor (Invitrogen, UK)

Complete Dulbecco's modified eagle's medium (DMEM)

500ml DMEM

10% FCS

1%/2mM L-glutamine

500µl pen/strep

(for culture of T24, RT112WT, RT112DD, UmUc3, J82, SK-Br-3, MRC5, Hfff2 cell lines)

Complete Roswell Park Memorial Institute (RPMI)

500ml RPMI

10% FCS

1%/2mM L-glutamine

500µl pen/strep

(for culture of RT112 and RT112CP cell lines)

Phosphate Buffered Saline (PBS)

125 mM Sodium Chloride

16 mM Na_2HPO_4

10 mM NaH_2PO_4

HCl to pH 7.2

Tris (Tris[hydroxymethyl]aminomethane)-Buffered Saline (TS)

10 mM Tris-HCl pH 8.0

150 mM Sodium Chloride

RIPA Buffer (Lysis buffer)

790mg Trizma (Tris) base

75ml distilled water

900mg NaCl

HCl adjust to pH 7.4

10ml 10% NP4)

2.5ml 10% Na-deoxycholate

1ml 100mM EDT

Annexin V Buffer (10x)

0.1 M HEPES

1.4 M NaCl

25 mM $CaCl_2$

Formal Saline

10% formaldehyde in PBS

Type 1 rat tail collagen (BD biosciences)

Matrigel (BD biosciences)

10X DMEM

25% Glutaraldehyde

3.3 Primary Cells and Cell Lines

Primary cells were collected from consented patients treated at University Hospital Southampton. The RT-112 cisplatin resistant (RT-112CP) cell line was a kind gift from John Master, University college London. All other cell lines were obtained from Health Protection Agency Culture Collections, UK.

3.3.1 Isolation of Primary Bladder Cell Populations

Primary MIBC populations were isolated from cancerous bladders of known grade and stage taken from patients at the time of radical cystectomy. Approximately 1cm^2 areas of cancer tissue was excised from the bladder lining of the organ that had just been removed from the body and immediately placed into transfer medium. (PBS with 1/1000 Fungizone)

After transferring directly to the laboratory and under sterile conditions the samples were washed three times with transfer medium and the excess stroma was dissected free from the specimen. The resulting tissue was then cut into roughly 2mm sections and placed into a scored 35mm, 6-well plate and immersed in either 2ml of Bronchial Epithelial Growth Medium (BEGM) or 2ml of Dulbecco's Modified Eagle Medium (DMEM) complete (depending on the cell to be isolated) containing 1/1000 fungizone (Gibco Life Technologies, Paisley, UK) and left overnight at 37^{0}C in 5% CO2. Media was discarded and replenished every two days until growing colonies were visible.

Confluent cells were then washed three times with phosphate buffered saline before trypsinisation for 30secs-1min and transferring to a Greiner Culture Flask T25 (25cm^2) plastic culture flask (Sigma-Aldrich, Dorset, UK). Serum containing media was added to inactivate the trypsin and cell passage performed routinely at approximately 75% confluence as described later. (see 3.2.1)

3.3.2 Cell Lines

Human Cell line	Characteristics
T24	G3 Transitional cell carcinoma cell line
RT112	G3 Transitional cell carcinoma cell line
RT112CP	G3 Transitional cell carcinoma cell line, cisplatin resistant
RT112WT	RT112 cells stably transfected with Fra-1 wild type
RT112DD	RT112 cells stably transfected with Fra-1DD
UmUc3	G3 Transitional cell carcinoma cell line
J82	G3pT3 Transitional cell carcinoma cell line
TERT-NHUC	Normal Urothelial cell line
SK-Br-3	Breast adenocarcinoma overexpressing HER2
MRC5	14 weel old human foetal lung fibroblast cell line
Hfff2	14-18 week old human foetal foreskin fibroblast cell line

Table 3.1: Cell Lines and their Characteristics. RT-112CP cells were a kind gift from John Master, University college London. All other cell lines were obtained from Health Protection Agency Culture Collections, UK

3.3.3 Transduction of T24 cell line with lentiviral knockdown of HER2

Immortalized T24 cells lentivirally transduced with viral particles containing a panel of micro-adapted shRNA already cloned into a lentiviral vector (pGIPZ, see figure 6.) to target HER2 (Open Biosystem Library, Thermo Scientific) were kindly prepared and donated by Ms Christiana Kitromilidou and Dr Tom Powles of the Barts Cancer Center, London.

The cells were maintained in puromycin (InVivoGen, California, SA) containing media and checked for HER2 expression by Western Blot analysis and GFP signal.

Vector Element	Utility
CMV Promoter	RNA Polymerase II promoter
cPPT	Central Polypurine tract helps translocation into the nucleus of non-dividing cells
WRE	Enhances the stability and translation of transcripts
turbo GFP	Marker to track shRNAmir expression
Puro^r	Mammalian selectable marker
AMP^r	Ampicillin bacterial selectable marker
5'LTR	5' long terminal repeat
pUC ori	High copy replication and maintenance in *e.coli*
SIN-LTR	3' Self inactivating long terminal repeat
RRE	Rev response element
ZEO^r	Bacterial selectable marker

Figure 3.1: Lenti-viral T24 cell line. shRNA mir was cloned into the pGIPZ lentiviral vector that harbours several elements with different functions as described.

3.4 Tissue Culture

3.4.1 Cell Passage

Cells were routinely passaged at approximately 75% confluence. 8mls of phosphate buffered saline (PBS, CR-UK) was used to rinse off cell debris and discarded to waste. 4-5 mls of trypsin/versene (Sigma-Aldrich, Dorset, UK) was added to the flask and incubated for a minimum of 3 minutes at 37^0C in 5% CO2 to detach cell adhesions. 5mls of serum containing media was then added to the flask to inactivate the trypsin. In the case of cells requiring serum free medium the trypsin was inactivated with 5 mls of 1% Trypsin Inhibitor (Sigma-Aldrich, Dorset, UK) mixed with media before transfer into a universal and centrifuging at 1200rpm for 4 minutes. The supernatant was discarded and the cells resuspended in growth medium. The cells were split typically 1:5 into a new sterile labelled Greiner Culture Flask T75 (75cm^2) (Sigma-Aldrich, Dorset, UK) and made up to 30mls with appropriate media before being incubated at 37^0C/5% CO2.

3.4.2 Thawing Cells for Culture

Cells were quick-thawed in the hand, to maintain cell viability, whilst the cryotube lid was loosened gently. Thawed cells were transferred to a 15ml universal and 5ml PBS or media was added and centrifuged at 1200rpm for 3mins to remove dimethylsulfoxide (DMSO) (Laboratory supplies, Poole, UK). The supernatant was discarded and the pellet re-suspended in 1ml complete media, which was added to 6ml complete media in a labeled Greiner plastic culture flask T25 (25cm^2) (Sigma-Aldrich, Dorset, UK) and incubated at 37^0C/5% CO2.

3.4.3 Freezing Cells for Long Term Storage

Freezing media was prepared by adding 5ml media to 4ml FCS (foetal calf serum) and 1ml DMSO. Cells were trypsinised as described, centrifuged and the pellet re-suspended in 1ml (T25) or 2ml (T75) freezing media. 1ml aliquots were stored in a labeled cryotube and frozen in a polystyrene container at -80^{0}C overnight and transferred to liquid nitrogen for long-term storage.

3.5 Biochemical Techniques

3.5.1 SDS-PAGE and Western Blotting

3.5.1.1 Preparation of Cell Lysates

Cells were grown in a T75 plastic culture flask until approximately 75% confluent. The conditioned media was removed and centrifuge at 1200rpm for 3 minutes, decanted and placed on ice for supernatant preparation. Cells were rinsed twice with cold PBS and then 1ml of 1x Radioimmunoprecipitation Assay (RIPA) buffer plus protease inhibitors (Sigma, UK) was added and the cells were collected in a 1.5ml eppendorf tube.

The cell lysates were left on ice for 15 minutes and centrifuged at 4^{0}C (13000rpm) for 5minutes. The lysate supernatant was removed, aliquoted and stored at -80^{0}C. Protein content of lysates was quantified on a Lambda 25 UV/VIS (Perkin Elmer, Fremont, CA, USA) spectrophotometer at 750nm using Bovine serum albumin (BSA) as the standard with protein assay reagent (Bio-Rad, UK).

3.5.1.2 Western Blotting

Standard Tris-Glycine gels with 1.5mm x 15 wells were made and placed in a Mini-Protean III electrophoresis module (Bio-Rad Laboratories, UK) and surrounded by 500ml 1X Tris-glycine SDS running buffer. 15µl Full range rainbow^{TM} molecular weight marker (GE Healthcare, Amersham, UK) was loaded to the first well. 3X SDS Sample buffer red (Cell Signalling, UK) supplemented with 0.1M Dithiothritol (DTT; Fisher Scientific, UK) was added to protein extracts normalized to 20µl in a final volume of 30µl. The samples were then heated at 95^{0}C for 5mins and added to the subsequent wells. The power pack was connected and run at 200V for 45 minutes. The polyacrylamide gel was then removed from the cassette and placed in a transfer filter paper and membrane "sandwich" consisting of 2 sponges, 2 filter papers and 1 nitrocellulose blotting membrane. This was placed in a mini-trans blot cell (Bio Rad Laboratories, UK) and surrounded in 1X protein running buffer containing 25% (v/v) ethanol and run at 100V for 90 minutes. The membrane was then removed and non-specific protein binding sites blocked with 5% milk (dried semi-skimmed Marvel) made in TBS-Tween for 1 hour at room temperature. The membrane was then incubated with the primary antibody at the appropriate concentration in 3% milk (in TBS-TW or 0.1% BSA) at 4^{0}C overnight on an automatic roller. The membrane was washed for 5 minutes three times with 0.05% TBS-TW and stained with the relevant horseradish peroxidase (HRP) – conjugated secondary antibody (GE Healthcare, UK) for 1 hour at room temperature typically at appropriate dilution in 3% milk/TBS-TW. The membrane was then washed three times before detection of the protein using Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific, UK) on a Fluor-S-MultiImager (Bio-Rad Laboratories, UK) using Quantity One software (Bio-Rad Laboratories, UK).

3.5.1.2 Antibodies for Western Blotting

Antibody	Name/ Clone	Supplier	Type	Antigen (human unless specified)	Concentration/Dilution
EGFR	(1005): sc-03-G	Santa Cruz Biotechnology, Inc	Goat polyclonal	Peptide mapping at the C-terminus of EGFR	1/200 3% dried skimmed milk 0.05%TBS-T
HER2	(C-18): sc-284	Santa Cruz Biotechnology, Inc	Rabbit polyclonal	Peptide mapping at the C-terminus of HER2	1/500 3% dried skimmed milk 0.05%TBS-T
HER3	Clone 2F12	Thermo Scientific	Mouse monoclonal	aa1295-1323	1/200 3% dried skimmed milk 0.05%TBS-T
HER4	(C-18): sc-283	Santa Cruz Biotechnology, Inc	Rabbit polyclonal	Peptide mapping at the C-terminus of HER4	1/200 3% dried skimmed milk 0.05%TBS-T
β- Actin	A2066	Sigma	Rabbit polyclonal	Peptide corresponding to the C-terminal 11 residues	1/1000 3% dried skimmed milk 0.05%TBS-T

Table 3.2: Primary antibodies used for Western Blotting. All antibodies were purchased from the institutions named and diluted at the concentrations described above.

3.5.1.3 Densitometry Analysis

Films were photographed on the Fluor-S-MultiImager and stored as TIFF files and densitometry analysis was carried out using Image J density analysis software (http://rsb.info.nih.gov/ij/).

3.6 3D Organotypic Culture

3.6.1 Cell Culture

The required fibroblast line (Hfff2 or MRC-5) was cultured in DMEM complete medium. The bladder cancer cell lines RT112WT, RT112DD, T24, UmUc3, J82 and primary bladder cancer cells were cultured in DMEM complete medium. The bladder cancer cell line RT112CP was cultured in RPMI complete medium and the immortalized normal human urothelial cell line (TERT-NUHC) in Keratinocyte serum free medium. The T24 lentiviral knockdown cell lines were maintained in DMEM complete supplemented with puromycin. Cells were maintained in a humidified atmosphere of 5% CO2 at 37^0C and routinely passaged as described previously. (see 2.2.1)

3.6.2 Organotypic Culture

To prepare collagen gels, 3.5 volumes of collagen type I (BD Biosciences, UK) were mixed on ice with 3.5 volumes of Matrigel (BD Biosciences, UK) 1 volume 10X DMEM, 1 volume FCS, 1 volume DMEM and 1 volume of fibroblasts, suspended in media, so that 1ml of the

resultant mixture contained $5x10^5$ fibroblasts. Then 1ml of the solution was aliquoted into wells of a 24-well plate and allowed to polymerise for 30min at 37^0C.

After polymerization, 1ml of DMEM was added per well and gels were left for 18 hours at 37^0C to equilibrate. At the same time, a fibroblast free collagen/10XDMEM/FCS/DMEMcomplete gel mix (in ratios 7:1:1:1) was used to coat sterile nylon squares (100µm pore size; Tetko Inc, New York, USA) which after polymerization at 37^0C for 10 minutes were fixed in 1% glutaraldehyde for 1 hour, washed 4 times in PBS and once with DMEM complete and stored at 4^0C. The next day medium was aspirated from the wells of the 24-well plates and 1ml of media containing $5x10^5$ urothelial cells from the cell line of interest mixed with $1x10^5$ fibroblasts was added to each well. The following day, gels were removed from the 24-well plate and placed onto individual collagen coated nylon discs resting on steel grids. These steel grids were made from $2.5cm^2$ squares of stainless steel mesh with the edges bent down to form 4-5mm high legs. This initial time point was defined as day 1 of organotypic culture. The steel grids were placed in six-well plates and sufficient media (appropriate to the cell line/experiment) added to reach the undersurface of the grid allowing the urothelial layer to grow at an air-liquid interface. The organotypic was then cultured at 37^0C and 5% CO2. Medium was changed every 2 days.

3.6.3 Histology

Gels were harvested at day 14, unless otherwise stated, and fixed in formal saline overnight. Specimens were bisected and transferred into 70% ethanol for 24 hours. The specimens were then embedded in paraffin wax to form a tissue block. From this 4µm sections were cut and

mounted onto slides and stained with haemotoxylin and eosin (H&E). Slides were then viewed under a 20X light microscope and invasion assessed.

3.7 Creation of a Cystectomy Database

The theatres' logbooks and pathology databases at University Hospital Southampton (UHS) were interrogated to identify every cystectomy that was performed between the 1st January 2001 and 1st January 2011. All cystectomies performed for bladder cancers were included and cystectomies for benign conditions excluded. All patients were anonymised and assigned a study number. Numerous clinical details including survival data and stage and grade of disease were recorded in a linked anonymised fashion (see appendices) onto a Microsoft Access database that was fully password protected and stored in a university computer.

Experimental data corresponding to the individual patient's tissue samples were subsequently added to the database. The data were then exported into SPSS statistics software for analysis of results.

3.8 Creation of Cystectomy Tissue Microarray

3.8.1 Identification of Donor Tissue Blocks

The pathology reports from the hospitals computer archives, for each cystectomy, were used to gather the specimen numbers for the slides and tissue blocks stored on each patient. Where possible the pre-cystectomy biopsy, cystectomy sample and any associated lymph

nodes were identified and included as separate triplicate entities. The appropriate slides where then retrieved from the hospital archives. Dr. Matthew Sommerlad (Pathologist) reviewed all the slides to determine which contained representative tumour. The representative areas were then marked out with a slide pencil. The corresponding tissue block to the slide of interest was then removed from storage in the hospital periodicals. The slide was then held over the tissue block to work out where the tumour resided in the block. This is where triplicate cores were then removed in the creation of the TMA.

3.8.2 TMA Design

The TMA was designed using the Minicore software (Alphelys, UK) installed on the pathology department's computer. The patient's study number along with specimen type, ie, bladder, lymph node or biopsy, was recorded on a Microsoft Excel spreadsheet and imported into the Minicore software to allow the TMA to be designed. Dependent upon the desired diameter of each core, the space between each core and the overall dimensions of the recipient block, the software determined the total number and layout of the cores into the recipient block. Due to the large number of cases ten recipient blocks were needed to make the TMA.

For each array the diameters of the recipient block were entered into the software whilst using a 1mm diameter tissue punch with 0.4mm between cores. The required data set for the TMA was then opened from the Excel datasheet to allow the Minicore software to calculate the placing of the cores into the recipient block. Each array included 3-4 orientation cores consisting of either kidney or liver tissue to ensure that the slides are always orientated

correctly to ensure correct reading. The template for each tissue block including which core was associated with which patient was recorded and saved.

3.8.3 Making the TMA

The TMA was built using the Minicore 3 tissue arrayer (Alphelys, UK). Depending on which tissue specimens were available the arrays were built in one of three subtypes: either cystectomy only samples, cystectomy and biopsy samples, or cystectomy and lymph node plus or minus biopsy samples.

The appropriate TMA file was opened on the Minicore software. The software instructs the user to place the recipient block onto a carrousel in position 1. The first seven donor blocks were then placed into positions 2-8 and the areas to be cored recorded onto the machine via the onboard digital camera. The machine then aligned the punch to allow a core of wax to be removed from the recipient block in the exact position that was previously calculated. The carousel automatically rotated to the marked area of tumour in the first donor block allowing a core of tumour to be removed. The core was then held in the punch whilst the carousel rotated back to the recipient block and positioned the punch directly above the previous hole, allowing the donor core of tissue to be transferred into this space. The machine then moved the recipient block to allow the next core to be removed. This was repeated three times for each tissue sample. The total process repeated until the seven donor blocks had been used. The donor blocks were then removed and replaced with the next seven blocks and the cycle repeated until the array and finally the TMA was complete.

3.9 Immunohistochemistry

Immunohistochemistry was performed on slides made from the organotypic and TMA blocks. 4μm slices were cut and mounted onto polylysine-coated slides. After the paraffin had been removed with xylene, acetone and alcohol the slides were immersed for 30 minutes in absolute methanol containing 0.6% hydrogen peroxide to quench endogenous peroxidase activity. After rehydration through graded alcohol and distilled water, the sections were immersed in citrate buffer (0.1M, pH 6.0) and boiled in a microwave oven (800W) for 30 minutes for antigen retrieval. The slides were cooled at room temperature and washed in TBS-TW for 5 minutes three times. The required antibody was applied at the optimized concentration and incubated at 4^{o}C overnight.

The streptavidin biotin-peroxidase technique was used. Avidin and biotin was applied in turn for 20 minutes with TBS-TW washes in between. DMEM with 10%FCS and BSA were added for a further 20 minutes as a final block to non-specific binding. After a further TBS-TW wash the biotinylated secondary antibodies were applied for 30 min at room temperature. The washed slides then received streptavidin biotin-peoxidase complex for a further 30min. The chromagen was developed with the emmersion of the slides in 3,3'-diamino-benzidine tetrahydrochloride (DAB) for 5 minutes. The slides were counterstained with haematoxylin, dried and mounted in DPX resin. The cell staining was scored simultaneously by myself and a pathologist (Dr M Sommerlad) on dual console high powered microscopes. Any disagreement was discussed and agreed upon.

3.9.1 HercepTest™

The HercepTest™ is a validated scoring system that utilizes immunohistochemistry and FISH to characterize expression of HER2 in breast cancer. In the absence of any validated scoring system for immunohistochemical HER2 expression in bladder cancer the decision was made to adopt this system for HER2 scoring in the TMA. The ability to use FISH as described in the original HercepTest™ was not possible due to a lack of funds. Similarly, there is no accepted validated scoring sytem for EGFR, HER3 or HER4 in bladder cancer and as a result the HercepTest™ was also used to score these potential biomarkers.

The HercepTest™ scoring system is used daily in clinical laboratories when staining breast cancer samples for HER2 and is now emerging as the validated score of choice for quantifying HER2 expression in all cell line research. This scoring system defines a score 0 as absent staining or staining in less than 10% of the tissue, score 1 as weak membrane positivity in more than 10% of the tissue, score 2 as moderate to complete staining in more than 10% of the tissue and score 3 as strong and complete staining in more than 10% of the tissue.

When scoring the TMA triplicates, if there was a lack of concordance between cores the highest score was assumed to the most biologically relevant and the corresponding score recorded as the result for the individual tumour specimen.

3.9.2 Immunofluorescence

Under sterile conditions coverslips placed into a 12-well plate were sterilised with 100% Ethanol for 30 minutes before three washes with PBS. Onto the coverslips $1.5x10^5$ cells were seeded along with 1ml of media and incubated at 37^0C/5% CO2 overnight until the cells were confluent. Unused media was discarded and the coverslips washed three times with PBS. Non-specific staining was blocked with complete media (containing 10% foetal calf serum) before application of the primary antibody overnight at 4^0C. After three washes the secondary antibody was added to the coverslips and left in the dark for 30 minutes. The coverslips were removed from the plate and mounted onto a slide using DAPI (4',6-diamidino-2-phenylindole) nuclear stain mounting agent (Vectashield, Vector Laboratories, UK) and incubated overnight at 4^0C. Fluorescence was assessed and images recorded on an Olympus IX81 inverted fluorescence microscope at 40X magnification using the Xcellence software provided.

3.9.3 Statistics

Statistical analyses were performed using SPSS, version 20 (IBM, Portsmouth, UK). For the TMA overall survival and recurrence free survival were the primary and secondary end points and were estimated using the Kaplan-Meier and log-rank tests. The effect of staining status for various biomarkers, such as HER2, were measured using Cox proportional hazards model and adjusted for important covariates such as age, sex, tumour grade, tumour stage and neo-adjuvant chemotherapy. Hazard ratio (HR) and confidence interval (CI) calculations were based on the cox model; p-values of <0.05 were considered statistically significant.

Chapter 4.

Creation of a Cystectomy Database

4.1 Introduction

One aim of this project was to create a comprehensive clinical database of all patients who had a radical cystectomy in the ten-year period between 2001 and 2011 to be further linked to a tissue micro-array. Clinical information such as the stage and grade of the tumour were expected to be predictive of survival in this cohort of patients. The database was designed to contain surgical, clinical and pathological information as well as detailed follow up in order to obtain insights into the natural history of the disease as well as using it as an adjunct for biomarker research.

Due to the time consuming and labour intensive nature of building a retrospective clinical database great care was taken to ensure accurate data entry to ensure reliability of results. For this database all patients who received a radical cystectomy between the 1st January 2001 and the 1st January 2011 at University Hospital Southampton were included. This hospital is a tertiary referral centre and as a result a significant number of patients were referred from other hospitals as far away as the Falkland Islands and the Isle of Wight.

Overall survival (OS) was calculated from the day of radical cystectomy to the date of death for patients who did not have neo-adjuvant chemotherapy. Taking this time point for patients who received neo-adjuvant chemotherapy, which was an integral part of their "curative" treatment, would have potentially reduced their survival artificially by the length of time they were receiving chemotherapy. The OS for neo-adjuvant chemotherapy patients was therefore taken from the time they started neo-adjuvant-chemotherapy to the date of death. For patients who were still alive, the length of survival was censored to be the last time they were seen in clinic. A recurrence was defined as any measurable mass on specialist imaging likely to

represent recurrent disease either locally or in distant areas. It also included patients who had recurred early and were clearly dying rapidly of their disease whereby imaging was inappropriate. Recurrence free survival (RFS) was calculated from the day of radical cystectomy, or first day of neo-adjuvant chemotherapy, to the day of the imaging that diagnosed the recurrence or the day when it was recorded as clinically obvious. Again patients who are alive without recurrence or had died without recurrence were censored so that the RFS was calculated to either the last time they were seen in clinic or the date of their death. Cancer specific survival (CSS) was calculated from the day of radical cystectomy, or first day of neo-adjuvant chemotherapy, to the date of death from disease recurrence. Patients who were still alive either with or without disease recurrence were censored to last time they were seen in clinic. When accessing the patients records it could be seen that the follow up data was not complete for a small subset of patients who had been lost to follow up. These patients were excluded from the dataset. Similarly, when looking for predictive markers (Chapters 5 and 6) any patients without full data on that variable were excluded.

At University Hospital Southampton all patients receive a standardised radical cystectomy as published in the European association of Urology (EAU) guidelines. This includes a bilateral pelvic lymph node dissection (bordered by the genito-femoral nerve and common iliac bifurcation superiorly, the obturator nerve inferiorly, and the pelvic sidewall laterally) by one of three urological surgeons with a specialist interest and expertise in bladder cancer. (BRB, MCH, RCL)

All significant clinical variables were recorded (see appendices), however, due to incomplete records and difficulty accessing patient records some variables such as length of operation and intra-operative blood loss have not been analysed due to insufficient numbers for

meaningful interpretation. The start point for data collection was the 1st January 2001 and a data lock for final collection being the 1st January 2013. What is now presented is the most meaningful clinical data extracted from the database.

4.2 Results

Over the ten-year period 262 cystectomies were performed and 35 of these were for benign diseases, leaving 227 radical cystectomies for bladder cancer. Although there was record of the operation and histology report, no patient notes could be found in one instance resulting in a database of 226 patients. There was incomplete essential follow up data either for OS or RFS for 15 patients and as such they were excluded leaving a dataset for 211 patients. The median length of follow up was just over 4 years (49 months). The 30-day mortality for radical cystectomy was 1.9%.

The median age of patient was 72 years and the majority were male with a ratio of over 3 to 1. The overall survival was 48.8% with a cancer specific survival of 62.1% and a disease free survival of 55.7%. Only 27.4% of patients received neo-adjuvant chemotherapy but this trend was increasing rapidly towards the end of the dataset. (see 4.1)

The pre-operative characteristics were typical of a cystectomy cohort with the majority of patients with T2 (37.6%) or T3 (35.4%) disease. The remainder of the patients had surgery due to uncontrolled high-risk non-muscle invasive disease or the occasional T4 or node positive patient opting for surgery or being down staged after chemotherapy. Over 90% of cases were Transitional Cell Carcinomas (TCC) with pure Squamous Cell Carcinomas being the next most prevalent. Subsets of TCC with squamous differentiation and other known poor prognostic indicators were recorded but the numbers were too small to decipher prognostic significance in this group. (see Table 4.2)

Variable	Number of patients Total number (percentage)
Age in years Median (range)	 72 (33-87)
Sex Male Female	 172 (76.1) 54 (23.9)
Dead Alive Not known	102 (51.2) 103 (48.8) 21
BC death Non-BC death Not known	80 (37.9) 131 (62.1) 15
Recurrence No Recurrence Not known	98 (44.3) 123 (55.7) 5
Previous BCG	54 (23.9)
Previous RT	21 (9.3)
Neo-Adj Chemo	62 (27.4)
Pall. Chemo	43 (19)
Post-op serum β-HCG rise (>2 IU/L) Yes No Not known	 53 (53.5) 46 (46.5) 127

Table 4.1: Table demonstrating the basic patient characteristics. (BC – bladder cancer; RT – radiotherapy; Neo-Adj Chemo – neo-adjuvant chemotherapy; Pall. Chemo – palliative chemotherapy; Post-op – post operative) Serum β-HCG has been routinely measured in recent years at University hospital Southampton due to the observation of its utility in measuring response to palliative chemotherapy in bladder cancer patients.

Variable	Number of patients Total number (percentage)	
Pre-Op Stage		
Tis	6 (2.7)	
T1	34 (15)	
T2	85 (37.6)	
T3	80 (35.4)	
T4	8 (5.8)	
N+	13 (5.8)	
Cystectomy Stage	**Overall**	**Neo-Adj Group**
T0	33 (14.6)	22 (35.5)
Ta	14 (6.2)	3 (4.8)
Tis	16 (7.1)	3 (4.8)
T1	15 (17.1)	3 (4.8)
T2	36 (15.9)	12 (19.4)
T3	69 (30.5)	9 (14.5)
T4	11 (4.9)	1(1.6)
N+	32 (14.2)	9 (14.5)
Pre-Op Grade		
CIS	6 (2.7)	
G2	12 (5.3)	
G3	208 (92)	
Cystectomy Grade		
T0	33 (14.6)	
CIS	16 (7.1)	
G2	13 (5.8)	
G3	164 (72.6)	
Downstaged at Cystectomy	80 (35.4)	
Upstaged at Cystectomy	51 (22.6)	
No Change in Stage	95 (42)	
Histological Category		
Adenocarcinoma	3 (1.3)	
Sarcoma	2 (0.9)	
Small Cell Carcinoma	2 (0.9)	
Squamous Carcinoma	15 (6.6)	
Transitional Cell Carcinoma	204 (90.3)	

Table 4.2: Table to show the tumour characteristics at the time of the endoscopic operation and scans prior to cystectomy (pre-op) and from the cystectomy specimen. (N+ - node positive patients. In the pre-op group these were patients who had either been given the "benefit of doubt" or had been subsequently down-staged with chemotherapy)

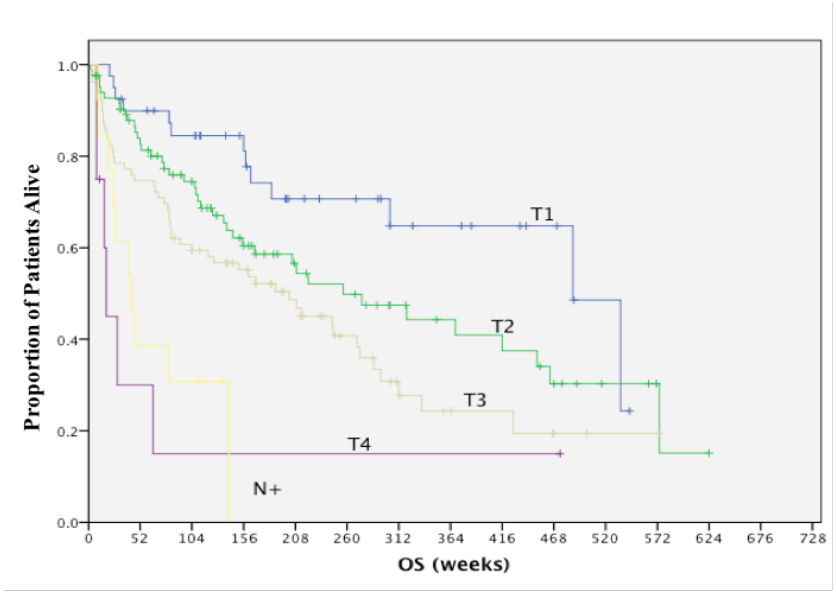
4.2.1 Effect of Stage on Survival and Recurrence

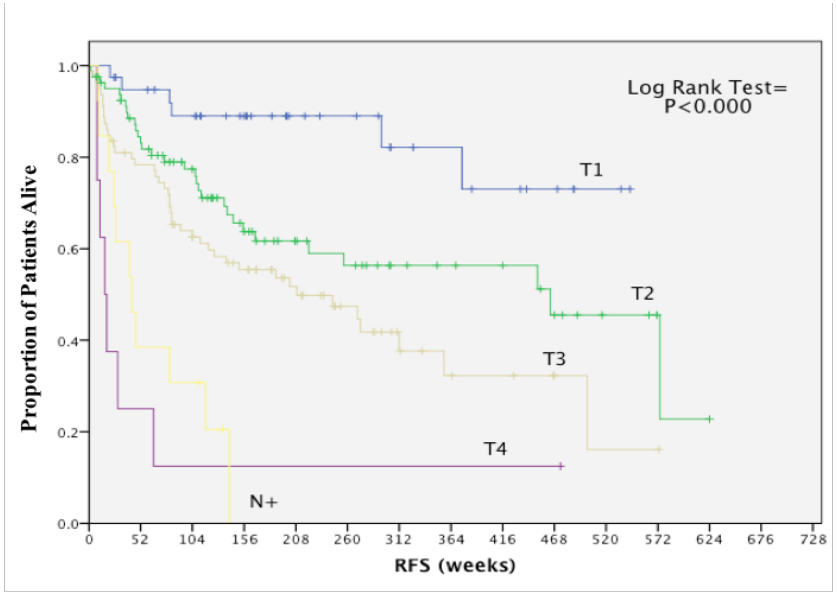
The stage of bladder cancer was calculated at two different time points as there is often a discrepancy between before and after radical cystectomy. Before cystectomy a patient may be incorrectly under or over staged either by incomplete trans-urethral resection or misleading imaging. This, however, is the information used by clinicians to guide treatment and is therefore of crucial importance. Post-operatively (cystectomy stage) a patient's tumour stage may be further changed for the same reasons or from the influence of a neo-adjuvant chemotherapy regime or a previously complete trans-urethral resection. Therefore, the pre-operative stage of the patient was the stage that was calculated as a result of the last tumour resection prior to cystectomy and the associated staging CT (pre-op stage). The cystectomy stage was the post-operative stage as determined from the cystectomy and nodal specimens. All patients with CIS or T1 disease were grouped together and classified as <T2. The data is therefore presented for both staging classifications.

It is known that stage is the most sensitive predictor of survival and it was reassuring to see that this database was broadly in consensus with the published literature.[18] When assessing the survival of the cohort according to the pre-operative stage it can be seen the overall the 5 year overall survival (OS) was 49%. However, no patients with node positive disease lived 5 years compared with 72% of <T2 and 52% of T2 patients. (figure 4.1)

When considering the patients' survival based upon their cystectomy stage there is a new category of pts without any discernable disease left in the specimen. These patients are classified as T0. The 5-year OS for these patients was excellent at 86%. It can also be seen that the survival for each stage is similar but slightly different for the cystectomy stage

patients with the T2 stage having a marginally better 5-year OS at 60% and the <T2 patients having a worse 5 yr overall survival at 58% compared to 72% in the pre-operative stage group. (figure 4.2) This may be indicative of the effect of down-staging from chemotherapy improving the local disease but failing to alter the final outcome of previously undetected micro-metastatic disease.

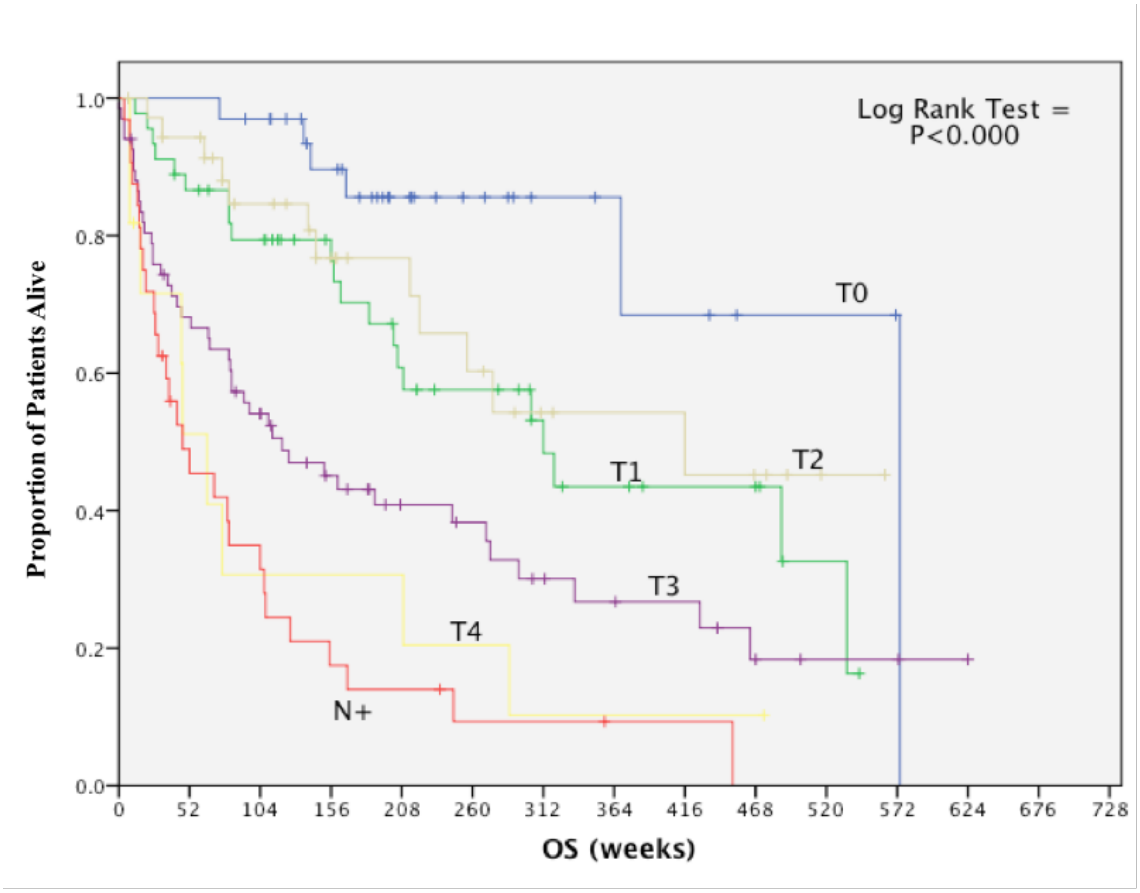




Pre-op Stage	5yr OS (%)	Mean OS (weeks)	95% C.I.	Median OS (weeks)	95% C.I
<T2	72	389	320-458	487	311-663
T2	52	309	250-368	256	137-375
T3	41	234	183-285	202	114-290
T4	18	92	0-214	18	13-23
N+	0	67	37-97	43	20-66
Overall	49	283	247-319	221	150-292

Pre-op Stage	5yr RFS (%)	Mean RFS (weeks)	95% C.I.	Median RFS (weeks)	95% C.I
<T2	90	457	389-520		
T2	58	365	301-430	464	302-626
T3	49	264	207-320	209	79-339
T4	12	79	0-183	16	6.3-26
N+	0	64	36-93	43	20-66
Overall	44	327	287-368	312	163-461

Figure 4.1: Graphs and tables demonstrating overall survival (left) and recurrence free survival (right) for the radical cystectomy patients based upon their pre-cystectomy stage. Both the OS and RFS were shorter in patients with higher stage disease. All patients who had node positive disease died of their disease within three years of surgery.



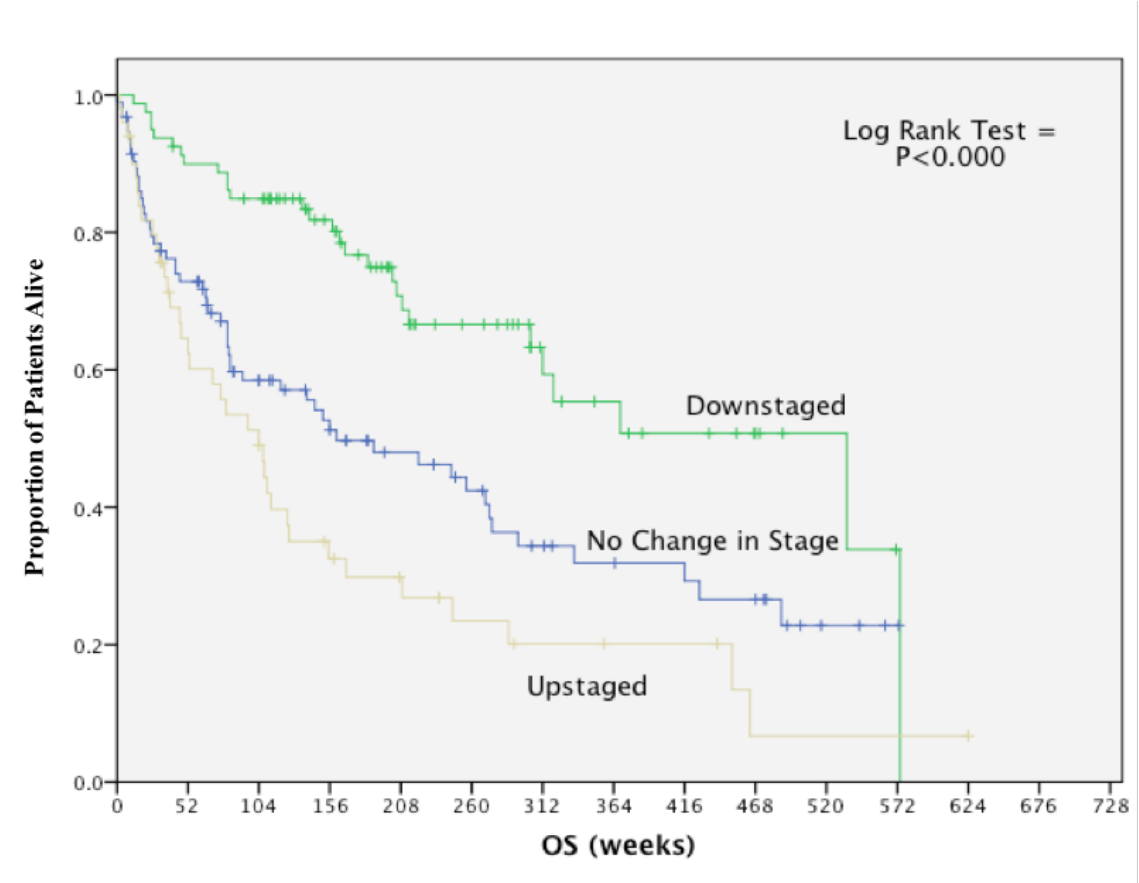
Cystectomy Stage	5yr OS (%)	Mean OS (weeks)	95% C.I.	Median OS (weeks)	95% C.I
T0	86	475	387-564	574	
<T2	58	322	257-388	312	170-454
T2	60	366	284-449		
T3	38	228	167-289	120	52-188
T4	21	126	35-217	65	37-93
N+	10	102	55-149	47	5-89
Overall	49	283	247-319	221	150-292

Figure 4.2: Graph and table demonstrating overall survival for the radical cystectomy patients based upon their final cystectomy stage. Patients rendered T0 had the best survival and this decreased with increasing stage of disease.

4.2.2 The Effect of Down Staging on Survival

Pre-operative staging altered from the cystectomy staging in some patients. Three groups were created according to the staging characteristics: down-staged, upstaged or stayed the same. The survival outcomes were then compared.

The change in overall survival improved to 68% with down staging compared to a worsening in overall survival to only 22% if the patient was up-staged at the time of cystectomy. (figure 4.3)



	5yr OS (%)	Mean OS (weeks)	95% C.I.	Median OS (weeks)	95% C.I
Down-staged	68	377	320-434	535	243-827
No change	43	247	198-298	161	58-264
Up-staged	22	171	113-230	104	63-145
Overall	49	283	247-319	221	150-292

Figure 4.3: Graph and table to demonstrate the effect of being down staged (green curve) or up staged (brown curve) at the time of cystectomy. Patients who were down-staged had a significantly better OS compared to those who were up-staged.

4.2.3 The Effect of Neo-adjuvant Chemotherapy

The patients who were classified as having muscle invasive disease at the pre-operative staging were assessed to look for the effect of neo-adjuvant chemotherapy. Sixty-two patients underwent neo-adjuvant chemotherapy (3 cycles of Gemcitabine/Cisplatin). The median overall survival was 31 months in the neo-adjuvant group compared to 18 months in the no neo-adjuvant chemotherapy group. Similarly, the median recurrence free survival was 47 weeks compared to 30 weeks. (table 4.3, figure 4.4) There was no statistical difference in their overall or recurrence free survival although the trend was apparent.

When looking at the groups of patients who were either upstaged, down staged or stayed the same, at the time of cystectomy, it can be seen that they were more likely to be down staged and less likely to be upstaged if neo-adjuvant chemotherapy was given. 59.6% of the neo-adjuvant group was down staged compared to 27.4% of the no neo-adjuvant chemotherapy group. Likewise, only 13.5% of the neo-adjuvant group was upstaged compared to 26.8% of the no neo-adjuvant chemotherapy group. (table 4.4)

The patients who were down staged that had received neo-adjuvant chemotherapy had similar overall survival to the neo-adjuvant chemotherapy naïve group with a 5-year overall survival of 76% vs 63%. This implies a durable down staging in the neo-adjuvant group that is not just a temporary effect after chemotherapy. (figure 4.5)

Survival	Neo-Adjuvant Group	No Neo-Adjuvant Group
5yr OS (%)	54	47
Median OS (months)	31	18
5yr RFS (%)	54	57
Median RFS	47	30

Table 4.3 Table to show the difference overall survival and recurrence free survival between patients who received neo-adjuvant chemotherapy and those that did not. There was no statistical difference between the two groups.

Change in Stage	Percentage of pts (%) in Neo-Adjuvant Group	Percentage of pts (%) in No Neo-adjuvant group	P-value
Downstaged	56.5	27.4	<0.000
Upstaged	11.3	26.8	<0.000
No Change	32.2	45.7	0.306

Table 4.4: Table to show the difference in the number of patients who were either upstaged, down staged or stayed the same at the point of cystectomy when either receiving or not receiving neo-adjuvant chemotherapy. Patients who received neo-adjuvant chemotherapy were more likely to be down-staged than those who did not. Likewise patients who did not receive neo-adjuvant chemotherapy were more likely to be up-staged a the time of surgery.

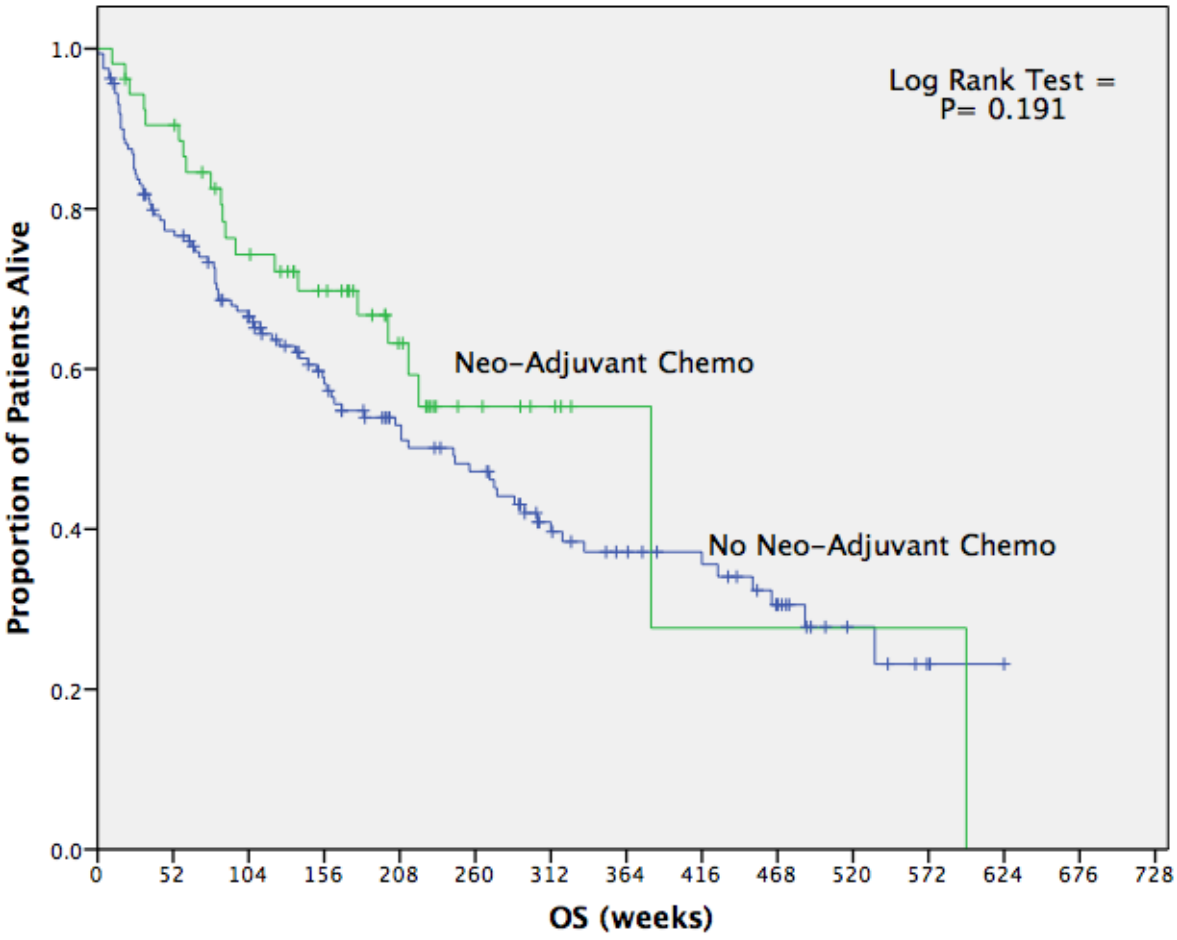


Figure 4.4 Graph to show the effect of Neo-adjuvant chemotherapy on overall survival in patients treated with radical cystectomy. There is a trend towards improved OS but there was no statistical difference between the two curves.

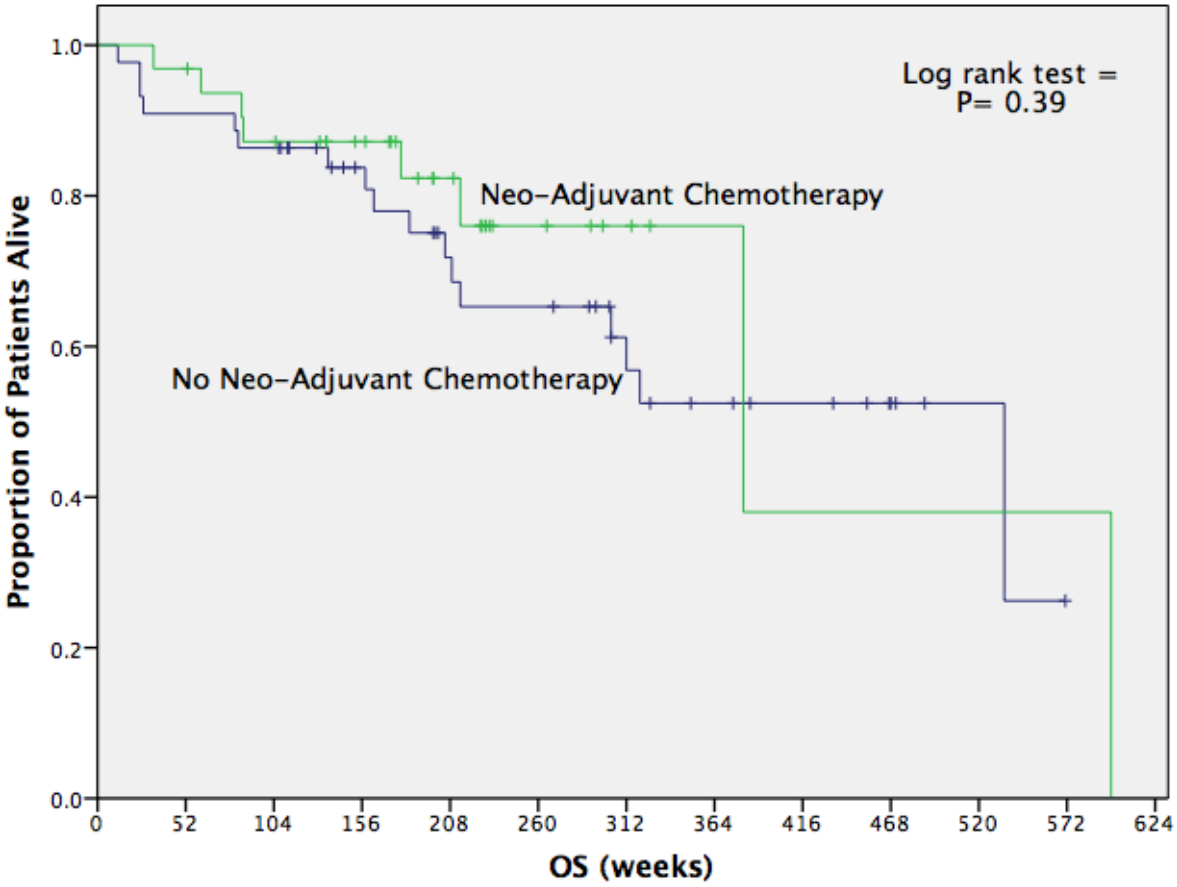


Figure 4.5: Graph to show the effect of neo-adjuvant chemotherapy on overall survival in the patients who were down staged at the time of cystectomy. There is no difference between the two curves implying that patients were robustly down-staged by neo-adjuvant chemotherapy and it was not a short, misleading effect.

4.2.4 Survival in patients over 80 years old

Radical cystectomy is a very morbid procedure with an associated mortality. As a result the age of the patient is taken into to consideration. Thirty-seven patients in this cohort were aged 80 or over (80-87 years). The 5-year overall survival was 24% in this group however; the 5-year recurrence free survival was 42%. (figures 4.6) When comparing survival for different stages of disease it can be seen that a good recurrence free survival can be achieved in patients with organ confined disease with 5-year recurrence free survivals of 44%-58%. (figure 4.6) It must be noted however, that there was likely a strong selection bias on these patients which may have biased the results.

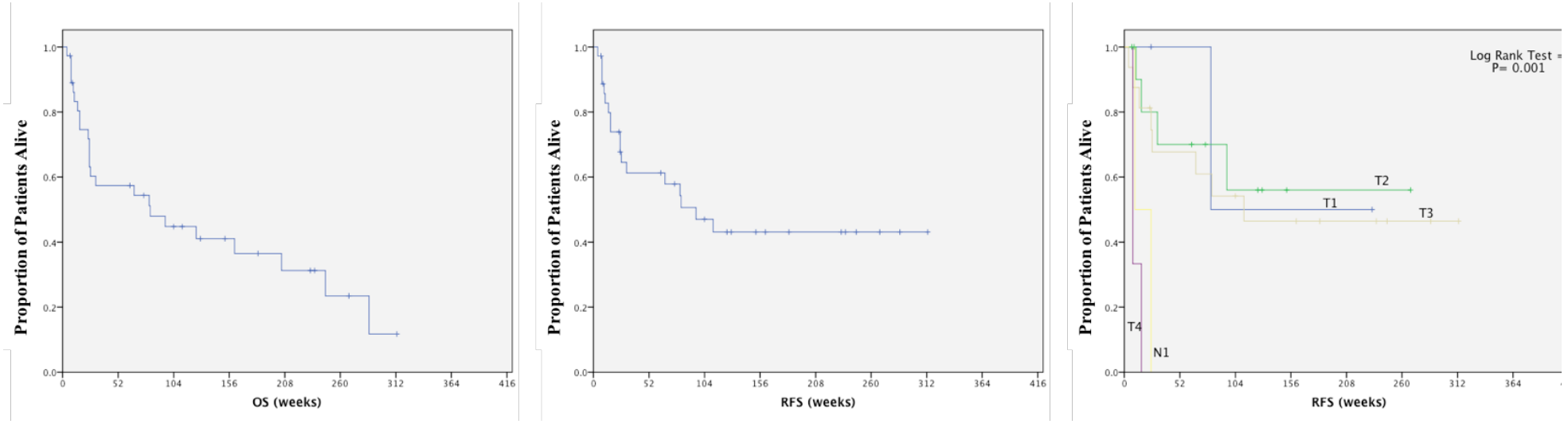


Figure 4.6: Survival curves for the patients undergoing radical cystectomy aged 80 years or older. The first graph represents overall survival, the second recurrence free survival and the third recurrence free survival when broken down for stage of disease. Patients aged over 80 had similar survival characteristics for T1, T2 and T3 disease as their younger counterparts but survival for locally advanced disease was very poor with all patients dying within 6 months of surgery.

4.3 Discussion

This retrospective database found that approximately 26 cystectomies were performed each year at University Hospital Southampton between 2001 and 2011. However, the hospital currently performs over 40 cystectomies a year implying that either more surgeries are being performed currently or there were potentially some cases that were missed. Every effort was made to avoid missed data but miscoding in the pathology department coupled with the surgery being performed in an unusual theatre are possible reasons for data not being recorded. Nonetheless this is a representative and close to comprehensive sample from the 10-year period. The mean length of follow up of 49 months is comparable to other large cystectomy series in the literature[180,181] and the survival figures are broadly in concordance.[180,181]

Radical cystectomy was performed for mainly muscle invasive, organ confined disease with 17.7% of cases for high-risk non-muscle invasive bladder cancer. The high-risk non-muscle invasive bladder cancer comprised of pure carcinoma in situ (CIS), G3pT1 or G3pT1 plus CIS and for the purpose of analysis all coded together <T2 disease. As expected both overall survival and recurrence free survival decreased with increasing stage of the disease. Although disease specific survival was looked at it was felt that the aim of the surgery was to either cure the patient or prevent them from dying with or from metastatic disease, as a result recurrence free survival was felt to be a more useful endpoint.

In the literature most series track survival based upon the pathology observed at the time of cystectomy. In this series there was a noticeable difference in pre-operative

staging based upon trans-urethral resection and CT imaging compared to the final pathological specimens (cystectomy stage). In this cohort only 42% of patients remained the same stage. This could be due to a number of reasons. Firstly, the CT imaging could be misleading or the disease process may have been modified in some way, perhaps by the quality of the initial trans-urethral resection (TUR) or the sensitivity of the tumour to neo-adjuvant chemotherapy. It has previously been shown that about 10% of cystectomy patients will be down staged to T0 purely due to the initial TUR and this will increase to 38% if neo-adjuvant chemotherapy is given.[182] In this cohort, 14.6% of the the cystectomy specimens were T0 if no neo-adjuvant chemotherapy was given compared to 35.5% if it had, implying a degree of chemo sensitivity. Overall when comparing the pre-operative stage to the cystectomy stage 35.4% were down staged and 22.5% were upstaged.

Using the pre-operative stage generally gave similar survival outcomes to the cystectomy stage for the muscle invasive samples with a 5-year overall survival for the T2s and T3s of 52% and 41% respectively in the pre-operative stage group and 60% and 38% in the cystectomy stage group. The biggest difference was in the <T2 group who had a 72% 5-year overall survival in the pre-operative stage group compared to 58% in the cystectomy stage group. This is difficult to explain. It is possible that patients who were truly T3 and T2 were down staged to <T2, or muscle invasive disease plus CIS down staged to pure CIS, by the quality of the initial resection or by chemotherapy after micro-metastatic disease had already occurred, thus accounting for the poorer than expected survival.

It is hard to distinguish which staging group is the most representative and both logically have their advantages. The pre-operative stage group is what we make our clinical decisions on and how we determine the treatment options, including neo-adjuvant chemotherapy. The cystectomy stage group is advantageous as it is definitive and also includes the T0 group that is a very good prognostic cohort.

The patients who were rendered T0 had by far the best outcomes with a 5-year overall survival of 86% (figure 4.2) and a recurrence free survival of 91% (figure not shown). It is interesting that 3 out of the 33 patients who were rendered T0N0 had disease recurrence. This is presumably due to micro-metastatic nodal disease that was either not seen in the histological specimens or were present in nodes that were not removed. Nonetheless it can be seen that T0 status is such a very good prognostic sign. This has also been noted in previous studies and as such is starting to be used in clinical trials as primary end point. For example, there is a national phase 2 trial called NEO-BLADE that is utilizing T0 as the primary end point to allow quicker results. If this is successful, then a phase 3 trial will follow using OS as the primary end point.

The effect of neo-adjuvant chemotherapy was investigated by comparing the overall survival between the two groups. The median overall survival was 31 months in the chemotherapy group compared to 18 months in the neo-adjuvant chemotherapy naïve group and likewise the median recurrence free survival was 47 months vs 30 months (table 4.3). The trend for a survival benefit was strong but did not quite meet statistical significance (figure 4.4). This is almost certainly due to insufficient numbers and was to be expected particularly as there have been many conflicting

papers looking at the benefit of neo-adjuvant chemotherapy and it wasn't until the 2003 meta-analysis that there was complete concordance on its overall benefit. [182]

As already stated some patients were down staged or upstaged and the effect of this on survival was looked at. The 5-year overall survival improved to 68% if patients were down staged and worsened to 22% if they were upstaged at the time of cystectomy. When comparing the three groups there were no significant differences in their pre-operative stages but not surprisingly when looking at the cystectomy stage the upstaged group had more higher stage disease.

As it was shown that there was a strong trend for improved survival in the neo-adjuvant chemotherapy group the association with down staging was investigated. 56.5% of patients who received neo-adjuvant chemotherapy were down staged and only 11.3% were upstaged. This is in comparison to 27.4% of the patients who did not receive neo-adjuvant chemotherapy being down staged and 26.8% being upstaged. (table 4.4) This shows that neo-adjuvant chemotherapy increases the chance of being down staged and we have shown that this increases the overall survival for the patients. This adds to the evidence for the benefit of neo-adjuvant chemotherapy and supports the previous conclusion that the survival figures did not reach statistical significance due to a lack of numbers.

The final subset of patients looked at was those aged 80 years or over. As cystectomy has a significant morbidity and mortality a patient's age is inevitably taken into consideration. Radical cystectomy has a reported mortality (within 30 days) of around 2-3%.[183] In this database there were 5 patients who died within 30 days (1.9%)

of which 1 died in the immediate peri-operative period. In the 80-years or older group 1 patient died within 30 days of surgery (2.7%). As one might expect the overall survival in this sub-group is disappointing with a 5-year overall survival of only 24%. However, the recurrence free survival is more encouraging with 42% of patients remaining disease free at 5-years with all recurrences occurring within the first 2 years. Indeed it appears that with stages 1-3 it is possible to achieve a durable disease free survival whereas those that are stage 4 or have nodal disease rapidly succumb. (figure 4.6) These results support radical cystectomy for a select cohort of patients aged 80-years or over and suggest that those with locally advanced disease will not do well with surgery. The findings are in keeping with the small number of papers regarding cystectomy in this age group. The papers include relatively small numbers of papers and mainly look at peri-operative complications and mortality. One cohort of 27 octogenarians, reported retrospectively, compared the outcomes of these patients treated with either radical cystectomy (11) or conservative surgery (16) (trans-urethral resection, partial cystectomy). They demonstrated similar 5-year OS with 27% in the radical cystectomy group compared to 19.4% in the conservative surgery arm, which was not statistically different. They divided their patients into organ confined disease and non-organ confined disease and like our results all patients with non-organ confined disease died rapidly.[184]

To summarise it can be seen that patients with increasing stage of disease have a worse overall and recurrence free survival. The addition of neo-adjuvant chemotherapy gives a strong trend for improved outcomes and in keeping with this it increases the probability of down staging the disease at the time of surgery. Being down staged particularly to T0 status is a very favorable prognostic sign. Overall this

data set appears to be reliable with survival data in keeping with previous published series. It is therefore reasonable to associate it with a tissue micro-array looking at prognostic information on novel biomarkers.

Chapter 5.

Development and Optimization of a novel 3-dimensional Organotypic tissue culture for the study of invasive bladder cancer

5.1 Introduction

To help fill the void between in-vitro 2-dimensional (2D) tissue culture and in-vivo experiments this thesis aimed to develop a 3-dimensional (3D) in-vitro cell culture technique for bladder cancer cell lines that accurately recreated the in-vivo environment to a greater degree, improving the ability to translate results from laboratory studies into clinically useful treatments.

Cells grown in monolayers on tissue culture dishes lack the 3D cell-cell and cell-matrix contact and communication present in intact tissue. The aim of organotypic cell culture is to recreate in-vivo conditions in an in-vitro model that represents a useful "halfway house" between 2D cell work and animal models. Experiments in 2D models can have different outcomes compared to those in animal or organotypic culture.[185] For example cells in organotypic culture are able to withstand drugs and toxins[176] better than in normal 2D culture and the morphology of cells can be completely different.[177] As an example; human embryonic stem cells in 3D culture, when stimulated by external factors to induce hepatic histogenesis, produced cells that more closely resembled true hepatocytes than those in 2D culture.[177]

In cancer the interactions between the cells and the cancer micro-environment is crucial in the development of invasion and metastasis. This appears to represent a change in a cells phenotype from epithelial like to mesenchymal like. The epithelial to mesenchymal transition (EMT) has been implicated as an essential part of cell invasiveness with cells developing a fibroblast like morphology with a lack of cell to cell adhesion and loss of apical polarity.

There is increasing evidence that the cancer cells' interactions with the other cells and growth factors in the surrounding matrix are crucial for the cell's invasive characteristics. The myo-fibroblast seems to be one of the most influential variables along with the integrins; αv6β and transforming growth factor β and have been shown to be able to induce EMT.[186] As part of this process the trans-membrane protein, E-cadherin, which is critical for the epithelial phenotype is lost. There is also increasing evidence of the role of EGFR and potentially HER2 in EMT with the non-invasive cell line, MCF-7, undergoing EMT after induction of EGFR signaling and this was further associated with resistance to Tamoxifen.[187]

In normal 2D culture many of these factors are disregarded and as a result it is difficult to translate results into the in-vivo environment. An organotypic model is able to allow for many of these important elements however, it still fails to fully mirror an in-vivo model due to the lack of other vital environmental factors, not least of which include an active immune system and blood supply.

Bladder organotypic models have not yet been fully utilized in bladder cancer research. A novel organotypic has been used to study the role of PTEN in bladder cancer cell lines. In this model the urothelium was removed from rat bladders and the remaining bladder tissue used as the base onto which the cells of study were cultured. They showed that PTEN inhibited invasion in the cell lines in both the organotypic model and also in vivo when the cells were introduced intra-vesically into nude-mice.[178]

The aim of this study therefore was to develop a useful organotypic model to study invasive bladder cancer in a more physiologically relevant fashion. Presented here is the first bladder organotypic to utilise primary human bladder cancer cells and its potential for use in the study of invasive bladder cancer is evident.

Due to the lack of models available the organotypic model was designed and optimized for this thesis based upon an original 3D culture designed by Professor Gareth Thomas for the study of another 'epithelial cancer', squamous cell carcinoma.[175] As described in the methods section this model was developed using a mixture of collagen, fibroblasts, Matrigel and cell media and processed over a period of 14 days. (figure 5.1) The resulting tissue was then fixed and processed, as would be the case with a normal human tissue sample.

To build an effective and relevant organotypic model, optimization needs to be performed to determine the correct proportion of reagents. Many experiments were performed to optimize the organotypic model including different ratios for Matrigel, collagen and numbers of fibroblasts. The final ratios used were as described in the methods section. The most important variables and their effect on the organotypic are presented in this chapter. The end point of such a process is to ensure that the natural in-vivo characteristics of a particular cell line are recapitulated. The initial intended focus for utilization of this model was in characterization of HER2 as a therapeutic target and as such the majority of experiments were performed using HER2 expressing cell lines.

Firstly, panels of cell lines were screened for their expression of HER2 using the western blotting technique and confirmed with immunofluorescence. The cells that overexpressed HER2 were then used in the optimization of the organotypic culture. The original stage and grade and hence the invasive properties of the cells were known and as a result the ability of the model to recreate the original in-vivo picture was observed. This was further confirmed on a primary cell line that was grown from fresh tissue obtained from a G3pT3 bladder cancer at the time of radical cystectomy. The essential role of myofibroblasts was established and finally the effect of knocking down HER2 expression was investigated in the organotypic setting.

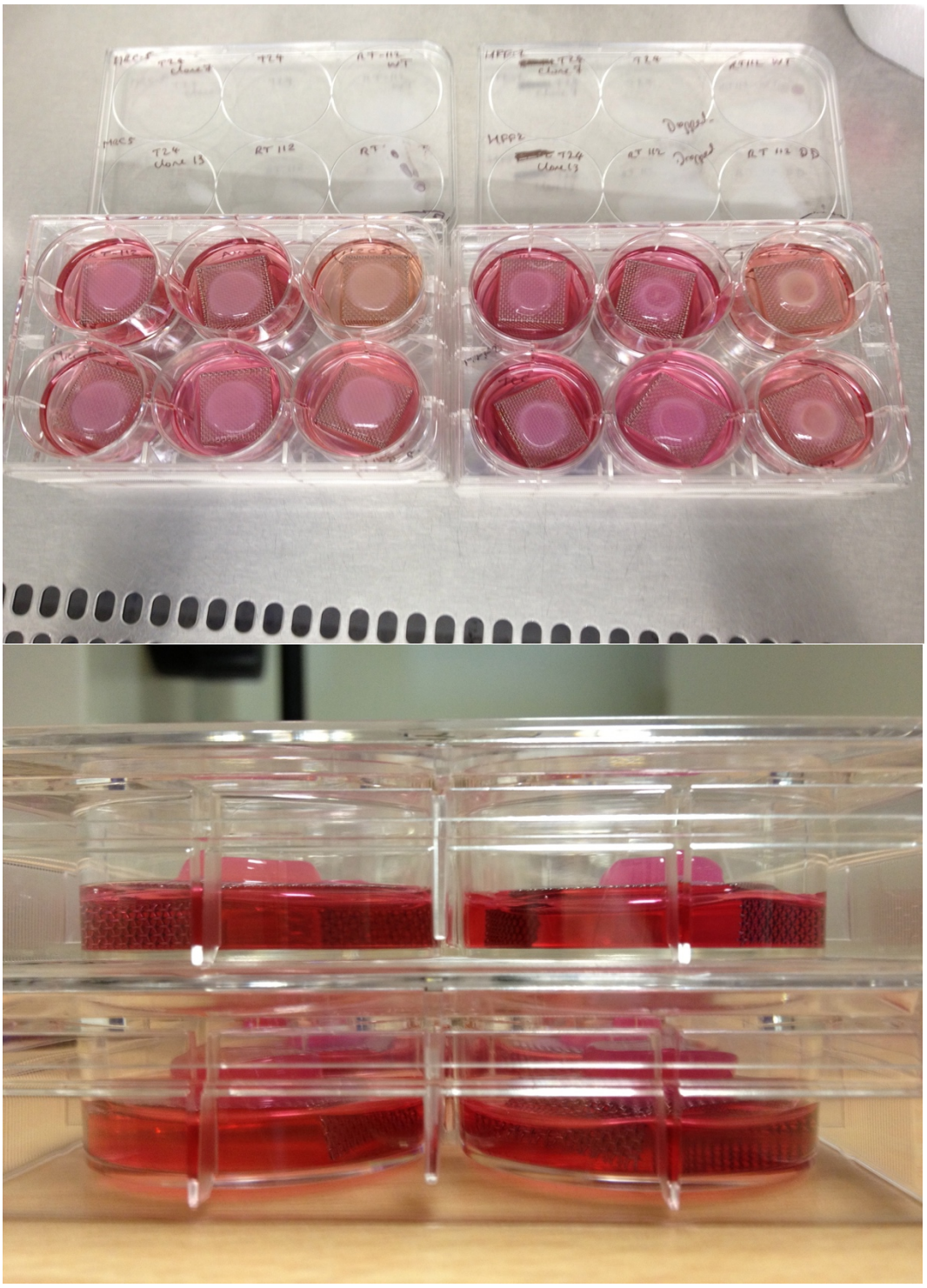


Figure 5.1 A set of Organotypics viewed from above and the side at 6 days from the start of culture. It can be seen that a white confluent layer of cells has started to grow on the surface when viewed from above. From the side view the air-liquid interface between the organotypic tissue substitute and cell media is demonstrated.

5.2 Identifying HER2 positive cell lines

It is felt that HER2 has a pivotal role in a subset of muscle invasive bladder cancers. As a result, a panel of available bladder cancer cell lines was screened for HER2 expression as well as for EGFR, HER3 and HER4 using western blot analysis and immunofluorescence.

The T24 cell line is derived from a G3pT2 transitional cell carcinoma and the RT112 cell line originates from a G3pT1 cell line. Additionally, the RT112CP cell line that has resistance to the chemotherapeutic agent Cisplatin was obtained.

Western blot analysis demonstrated that the T24 cell line expressed HER2 and HER4 with small amounts of EGFR. The RT112 cell line expressed each member of the EGF family. (figure 5.2) HER2 overexpressing cells were required and T24 and RT112 cell lines both overexpressed. There are widely divergent and complex differences in the biology of different bladder cancers and these two cell lines were chosen as cell lines likely to mirror the clinical picture of muscle invasive and non-muscle invasive cancer. As a result, these cell lines were chosen for the further experiments with regards to HER2 expression and optimisation of the organotypic model.

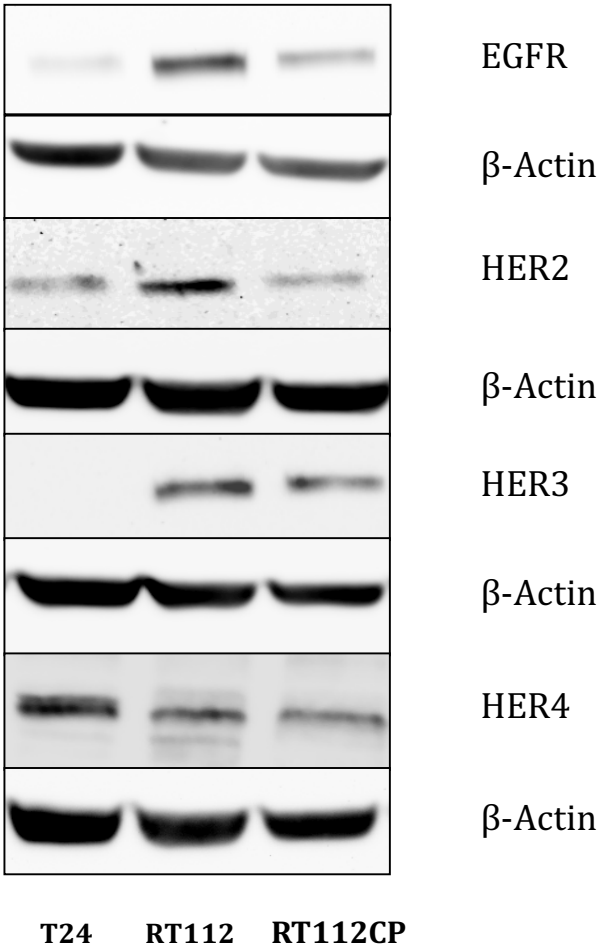


Figure 5.2: Western Blot demonstrating expression of HER2, HER4 and small amounts of EGFR in the T24 cell line. The RT112 and RT112CP cell lines expressed the whole EGF family. β-actin was used in the control wells.

5.3 Identifying muscle invasive cell lines and determining optimal length of time for invasion in an Organotypic bladder cancer model

The cell lines were cultured at the air-liquid interface upon the model to assess for invasion. T24 was selected as an example of HER2 overexpressing muscle invasive cell line and was used to determine the optimal time line for invasion and the RT112 cell line used as an example of non-muscle invasive HER2 overexpressing cell line. All the experiments were repeated at least 3 times. (figure 5.3)

To try and validate the organotypics ability to accurately mimic the cell lines' invasive characteristics, two further known muscle invasive cell lines were used along with T24 and RT-112. The J82 and UmUc3 cell lines were both known to originate from invasive tumours and were kindly donated by Dr. E Sayan.

5.3.1 Timeline for Invasion

With preliminary experiments it was quickly apparent that the T24 cell line was readily invasive in keeping with it's tumour origin, and as a result was used to determine the optimal length of time to culture the model. Organotypics growing T24 cells were grown in parallel and processed at different time points: 2 days, 4 days, 6 days, 9 days and 14 days. Progressively more invasion was observed the longer the organotypic was maintained and 14 days was determined to be optimal to demonstrate invasion. (Figure 5.3)

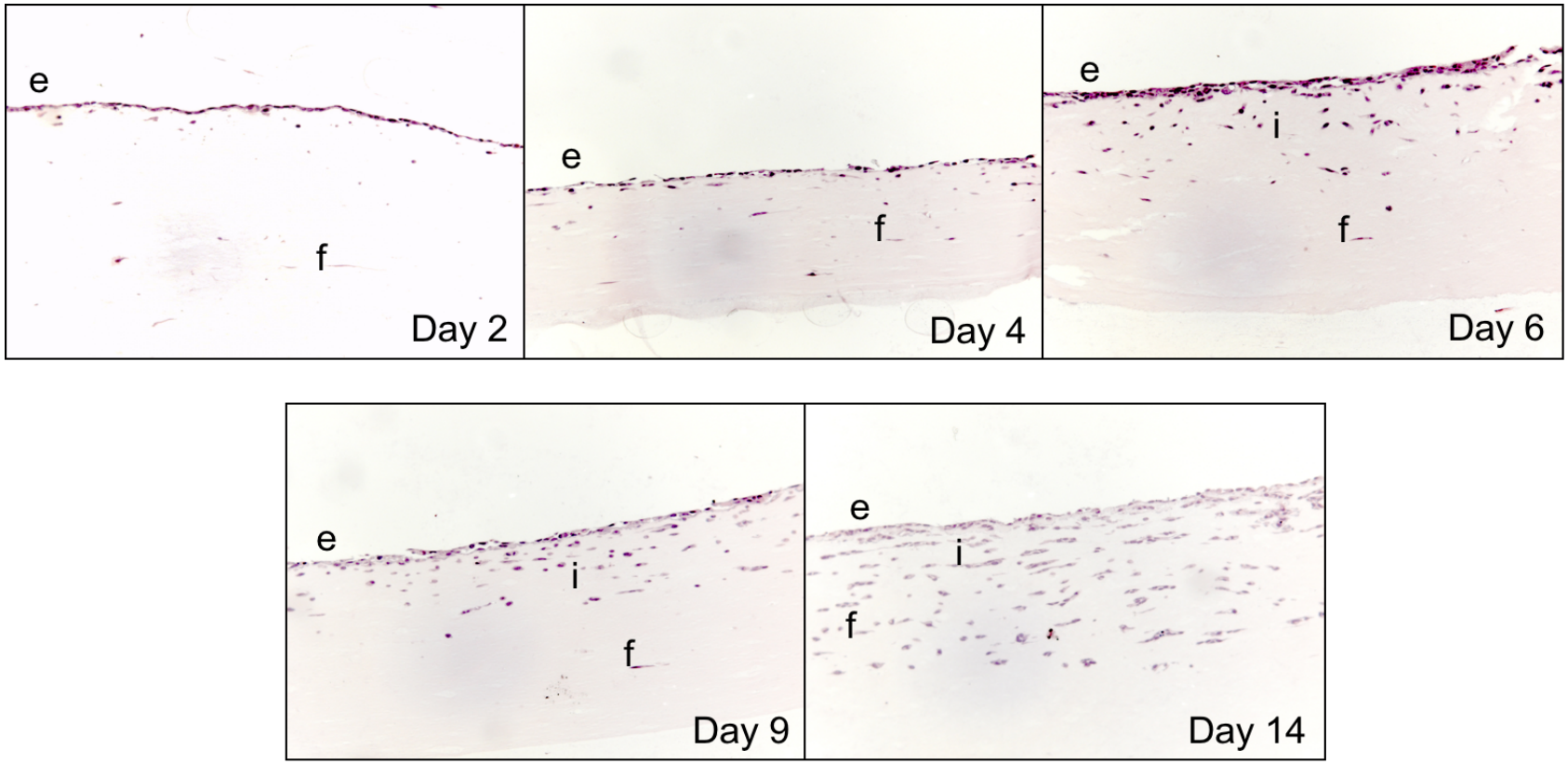


Figure 5.3: Timeline demonstrating H&E stained slides of cross sections from the organotypic model, culturing T24 cells, at different time points. On day 2 there is a thin epithelial layer (e) that has begun to grow. There is very little discernable invasion (i) and fibroblasts (f) are visible in the stroma. As the timeline progresses the epithelial layer (e) thickens and invasion (i) becomes more widespread with the most invasion (i) visible on day 14.

5.3.2 Non-invasive cell line

The RT-112 cell line originated form a high grade, non-muscle invasive bladder cancer and was used to confirm the organotypics ability to accurately recreate the original tumour characteristics. The RT-112 cell line formed a thick healthy epithelial layer and no sign of invasion into the organotypic. The cells are visibly more rounded and the cell to cell adhesion well established.

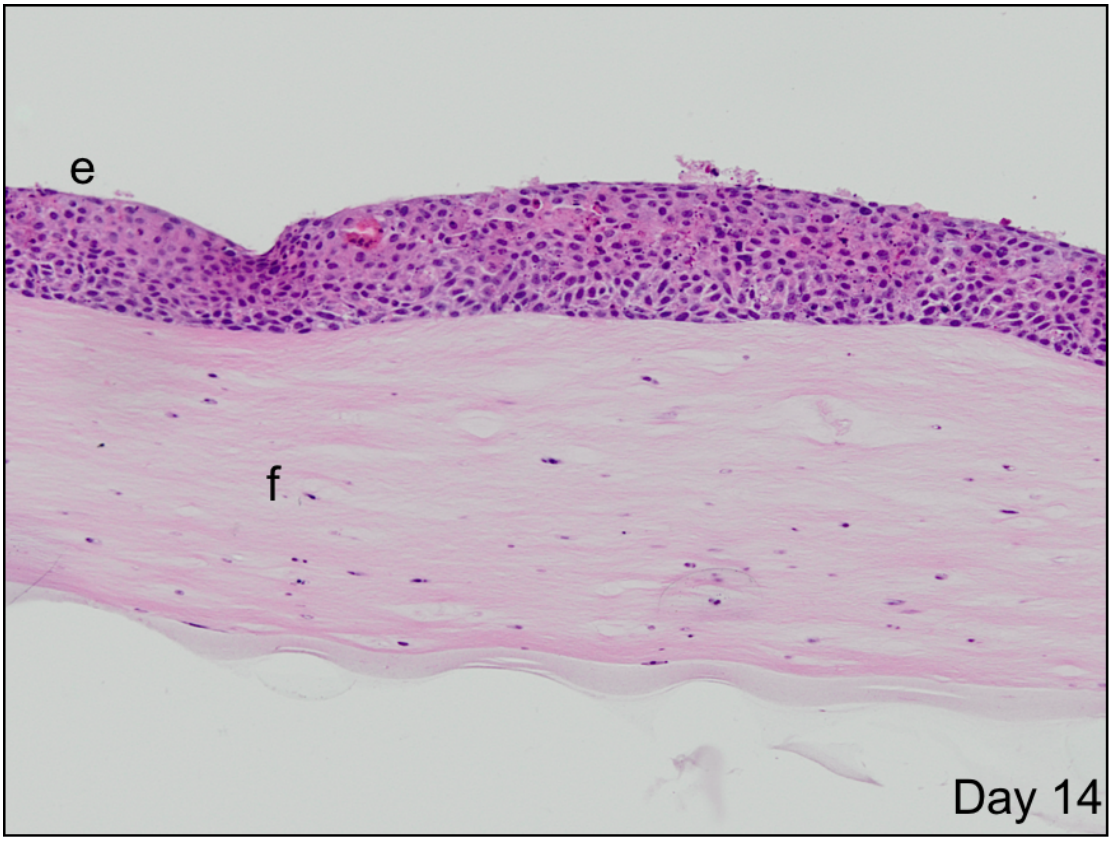


Figure 5.4: Cross section of an organotypic growing the RT-112 cell line at 14 days demonstrating a healthy epithelial layer (e) but no invasion. The fibroblasts (f) can be seen in the stroma of the organotypic.

5.3.3 Invasive cell lines

Additional to the HER2 expressing invasive cell line T24, two further cell lines, J82 and UmUc3 were also obtained. These cells were known to originate from muscle invasive tumours and to possess a mesenchymal phenotype. Therefore, a comparative experiment was performed to confirm the model's ability to re-create their muscle invasive characteristics.

The T24 cell line formed only a thin epithelial layer but quickly invades into the organotypic model. (figure 5.3) The J82 cell line forms a thick epithelial layer and invades as islands. (figure 5.5) UmUc-3 also forms a healthy layer and invades deep into the organotypic model. (figure 5.6) The morphology of the J82 and UmUc-3 cell lines are similar but it is clear to see that the cell to cell adhesions are being lost as the cells invade. Although not as extreme as the T24 cells these findings are very much in keeping with the mesenchymal phenotype associated with invasion compared to the epithelial phenotype of the RT112 cells.

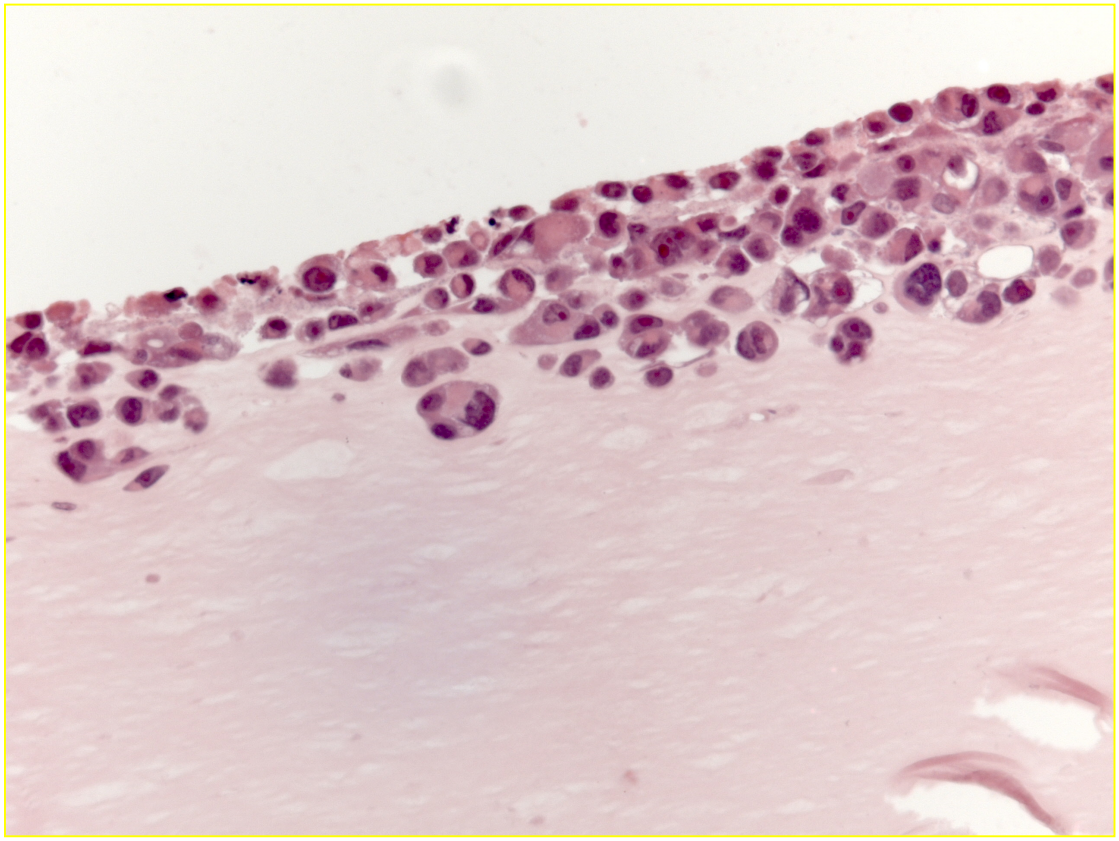


Figure 5.5: Cross section of an organotypic growing J82 cells. The tumour cells form a healthy epithelial layer and can be seen to invade as islands into the organotypic.

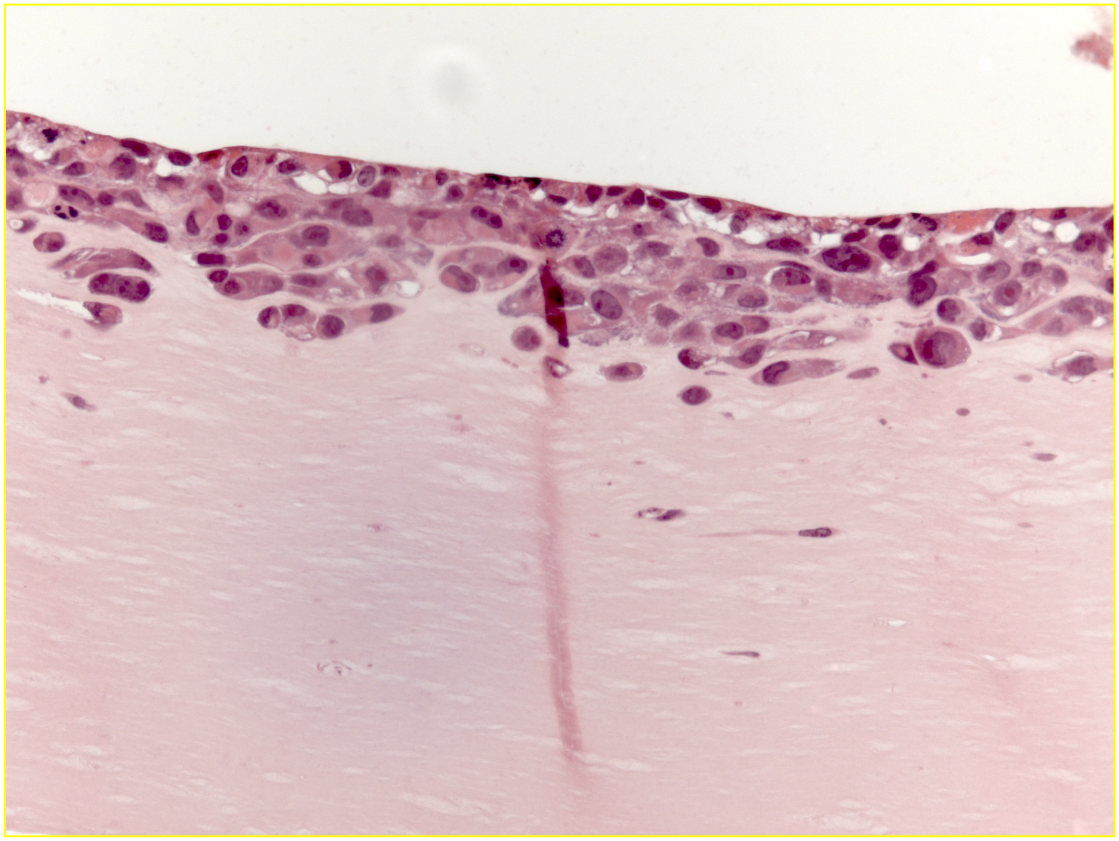


Figure 5.6: Cross section of an organotypic growing the UmUc-3 cell line. It can be seen that there is a healthy epithelial layer and invasion of tumour cells into the underlying organotypic.

5.3.3.1 Primary Muscle Invasive (G3pT3) cell line

Having established a working model for invasion with cell lines a novel model, using primary bladder cancer cells was established. G3pT3 cells were harvested from fresh bladder specimens at the time of cystectomy and cultured in the laboratory. These cells were grown on the organotypic for a 14-day period and the invasive characteristics were re-created. (figure 5.7) This provided further reassurance of the validity of this model.

5.4 Effect of Fibroblast type on invasion

As discussed earlier there is increasing evidence that cancer invasion is influenced by the ongoing interaction between the tumour cells and the stromal cells.[188] Fibroblasts and myofibroblasts seem to be some of the most important factors in the stromal matrix and their role in tumour invasion[189,190], angiogenesis[191,192], metastasis[189] and response to therapy is becoming increasingly recognized.

The exact role of fibroblasts is yet to be elucidated but it has been shown that fibroblasts can be transformed into myofibroblasts by paracrine signals generated by cancer cells including transforming growth factor-beta (TGF-β). Myofibroblasts, which express α-smooth muscle actin (α-SMA), can induce EMT and stimulate tumour invasion whereas non α-SMA producing fibroblasts do not.[189,193]

In order to optimize the organotypic model two fibroblast cell lines were used. The Human Foetal Foreskin fibroblast cell line, HFF-2, has been shown to be readily

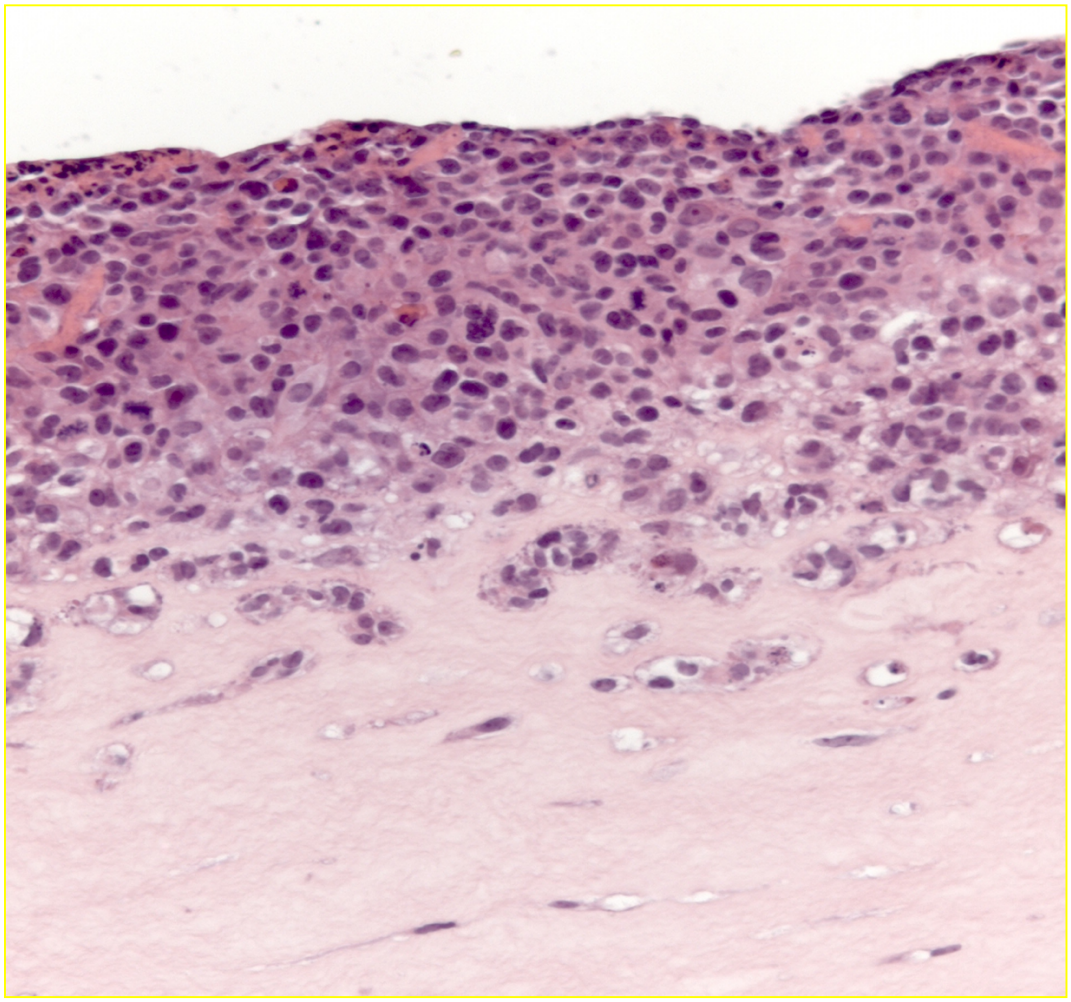


Figure 5.7: Cross section of an organotypic model growing the primary cells from a patient with G3pT3 bladder cancer. There is a healthy epithelial layer and invasion of tumour cells into the underlying organotypic.

transformed into myofibroblasts and to express α-SMA whereas the human foetal lung fibroblast cell line MRC-5 had been shown to be more resistant to transformation.

In order to confirm the role of fibroblasts in invasion in bladder cancer the invasive T24 cell line and the primary G3pT3 bladder cancer cells were cultured in the organotypic model for 14 days using both HFF-2 fibroblasts and MRC-5 fibroblasts. After harvesting the slides were stained with H&E and invasion studied. The HFF-2 fibroblasts recreated the known invasive properties of the cells, whereas the MRC-5 fibroblasts failed to facilitate invasion. (figure 5.8) This supports the essential role of fibroblasts in the invasive process.

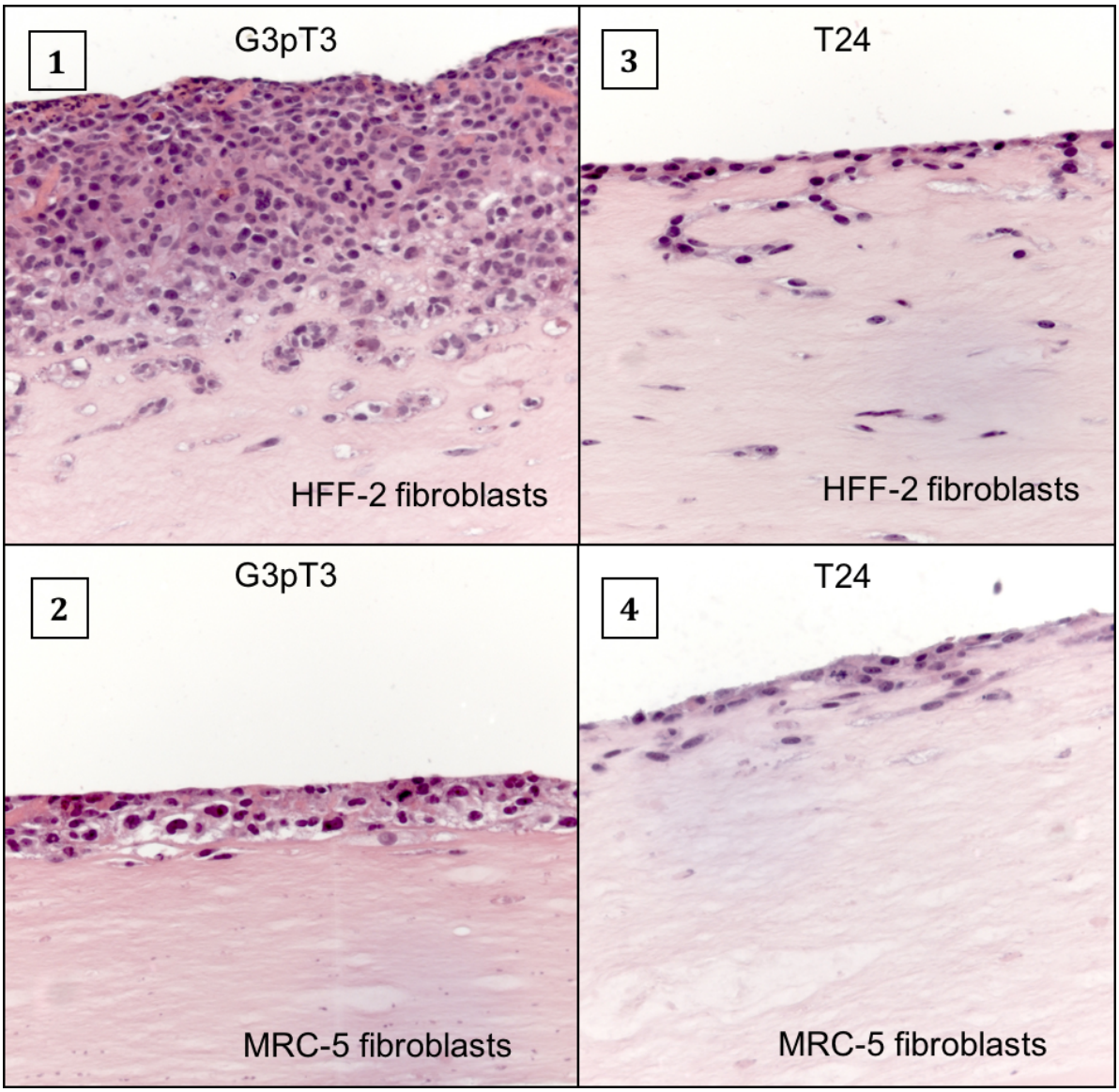


Figure 5.8: Organotypics demonstrating the role of fibroblast type on invasion. ***1*** *and* ***3****: Primary G3pT3 bladder cancer cells and T24 cell line readily invading with Human Foreskin Fibroblasts 2 (HFF-2) embedded in the organotypic.* ***2 and 4:*** *Primary G3pT3 cell line and T24 cell line not invading with MRC-5 fibroblasts.*

5.5 Effect of HER2 knockdown on invasiveness on T24 cells

To test whether overexpression of HER2 has a role in the invasive characteristics of a bladder cancer cell, HER2 levels were reduced using a lenti-viral knockdown model. The lenti-viral knockdown T24 cells (kindly provided by Prof. T Powles) were cultured on an organotypic model and compared to another normal T24 cell line (control for knockdown provided by Prof T Powles). Both cell lines were cultured for 14 days simultaneously on separate models using identical conditions.

There were three clones with differing levels of HER2 knockdown. When compared to the new, unaltered, T24 cell line, as a control, it could be seen that Clones 4 and 7 demonstrated a reduced quantity of HER2 protein on western blot with no obvious difference between clone 13 and control. (figure 5.9) When quantifying the reduction on protein expression using ImageJ software it can be seen that there is over 80% reduction in HER2 protein levels in clone 7 compared to the control. (figure 5.9)

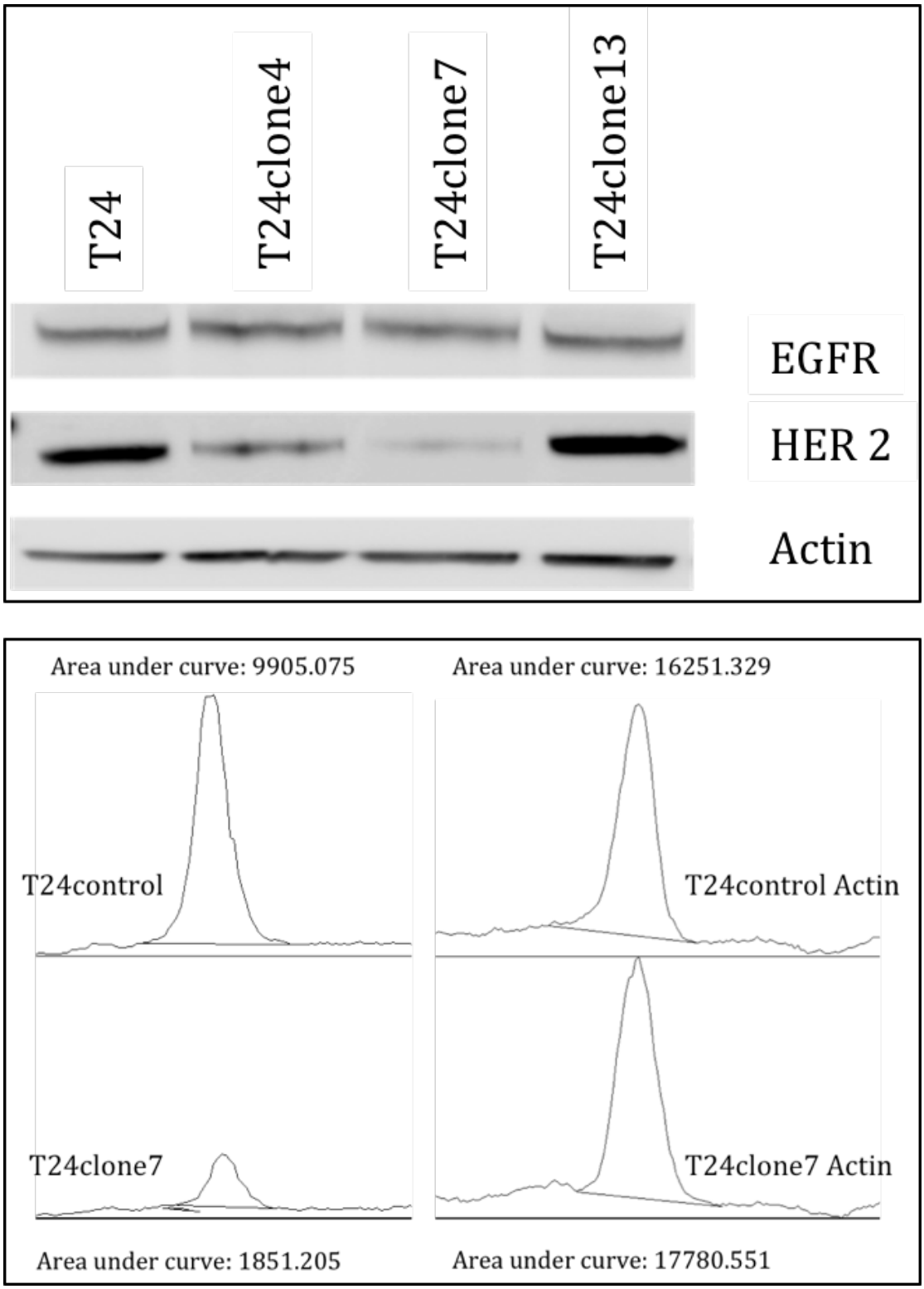


Figure 5.9: Western Blots demonstrating HER2 protein levels in the T24 cell line and T24 lenti-viral HER2 knockdown clones 4,7 and 13. Clone 7 visually had the most effective knockdown and this was quantified using ImageJ software. It can be seen that the intensity of the HER2 band is approximately 80% in clone 7 (area under the curve – 9905.075) of the T24 control band (area under the curve – 1851.205).

To confirm effective knockdown of HER2 expression after 14 days of culture on the organotypic model the slides with clone 7 cells and T24 cells were harvested and stained for HER2. There was expression of HER2 in the control T24 cells and any staining in clone 7 was hard to determine over background staining (figure 5.10). Although not quantifiable less HER2 staining was in keeping with the knockdown and subsequent western blot.

The same cell line organotypics were also stained for EGFR to see if there was any up regulation of EGFR to compensate for the lack of HER2. There did not appear to be any difference in the staining characteristics between the two cell lines. (figure 5.11)

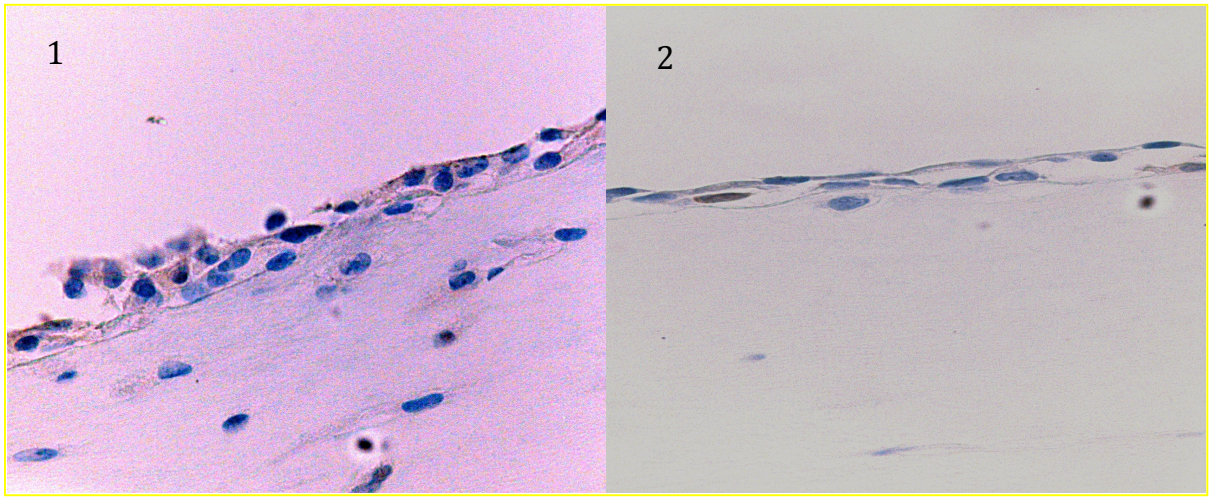


Figure 5.10: Immunohistochemical staining for HER2 in T24 control (image 1) and T24 clone 7 (image 2). There is reduced staining in clone 7 compared to the T24 control.

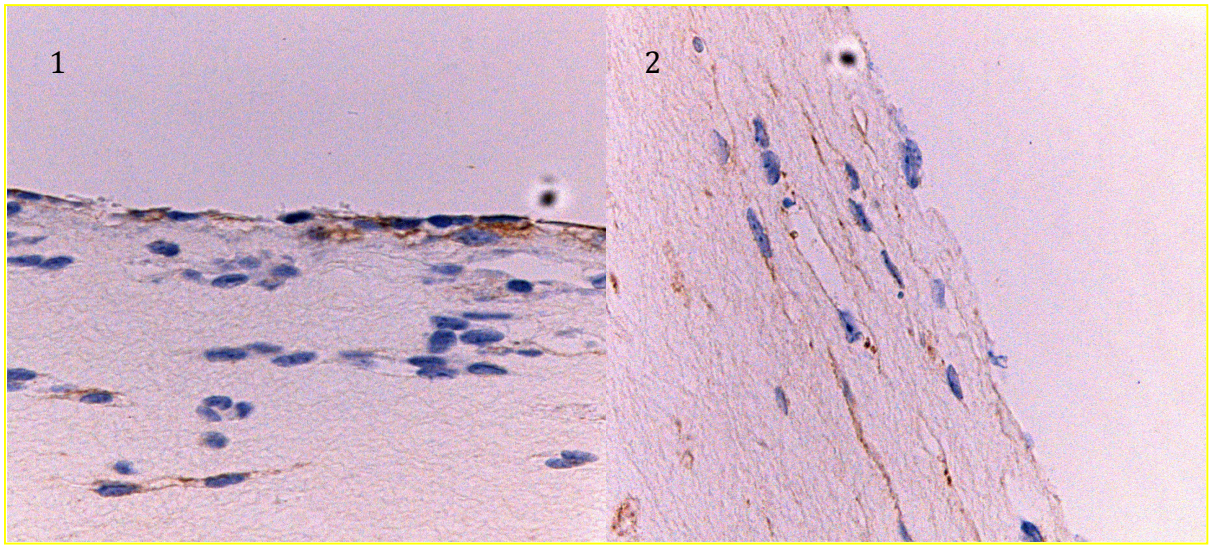


Figure 5.11: Immunohistochemical staining for EGFR in T24 control (image 1) and T24 clone 7 (image 2). There is back ground staining and no difference in membrane staining between the two cell lines.

The T24 control was grown along with clone 7 and clone 14 on the organotypic model for 14 days to see if this would demonstrate differing levels of invasion between the three groups in keeping with their expression levels of HER2. Clone 7 was used as this had the greatest reduction in HER2 protein load on western blot analysis and was postulated to show the least amount of invasion. The T24 control was used as was clone 13. Clone 13 was utilized in case the process of inducing a lenti-viral knockdown had any unknown effects on invasion.

Despite the knockdown of HER2 in clone 7, by 14 days there was good invasion in all three cell lines. No difference in invasion characteristics could be seen (figure 5.12).

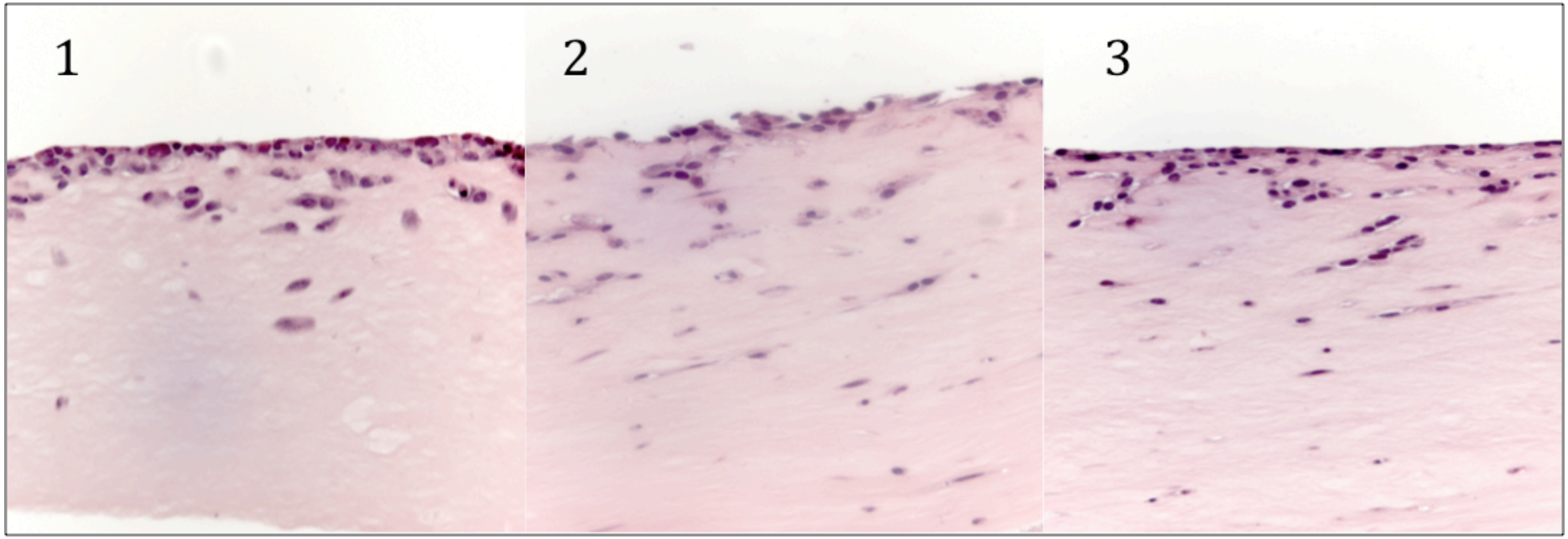


Figure 5.12: Pictures demonstrating the invasive characteristics of the T24 cell line (image1) and the T24 cell line after lenti-viral knockdown of HER2 in clone 13 (image 2) and clone 7 (image 3). All three demonstrate a thin epithelial layer and widespread invasion. There is no clear difference in invasive characteristics.

5.6 Discussion

The cell lines T24 and RT-112 were selected as they both expressed high levels of HER2 and represented a muscle invasive and non-muscle invasive cell type. The HER2 status was confirmed with immunoblotting and immunofluorescence. As with all bought cell lines it is almost impossible to know exactly how many passage life cycles the cells have been through with the result that small genetic changes would inevitably occur. It is therefore little more than assumption that these cell lines maintained the invasive properties that they originally possessed.

When growing the cells on the organotypic culture model, even after just 2-4 days, the T24 cells demonstrated some invasion and this was clearly obvious by 9 days. At 14 days there was substantial invasion and the cells still appeared healthy. (figure 5.3) It was decided that this was a sensible length of time to grow the cells in culture, allowing plenty of time for invasion in a functional and pragmatic system. Equally the non-invasive RT-112 cells grew into a confluent monolayer by 4 days and were still healthy at day 14. (figure 5.4)

To validate the model's ability to reproduce the invasive characteristics of the cells two further cell lines (J82 and UmUc3), known to be highly invasive, were also cultured on the organotypic. Reassuringly, it was observed that by day 14 the J82 cells had started to invade as small islands of cells and the UmUc-3 cells had invaded into the underlying stroma.

Finally, a primary bladder cancer cell line was grown from a fresh tissue specimen retrieved at the time of radical cystectomy for what turned out to be G3pT3 disease. This allowed for accurate knowledge of the true disease characteristics of the cell line. Growing the primary

cell line proved difficult. It was initially aimed to grow primary "cancer" fibroblasts and primary tumour cells from the specimen. Previous experience in our laboratory had revealed that it is normally much easier to select out the fibroblasts than the epithelial cancer cells. The converse was true for this experiment. Despite multiple sections of tissue being sampled and cultured as described previously it was not possible to grow sufficient fibroblasts for experiments as they all died after a couple of passages. Unusually the epithelial cancer cells were much more amenable and although they were not immortalized could be effectively cryo-preserved and quick-thawed successfully.

Culturing the primary cells on the 3D model resulted in a healthy epithelial layer and widespread invasion by 14 days. This was reproduced on three separate cultures and is felt to help validate the organotypic model.

The theory behind an organotypic culture is that the cell-cell contact and the interactions with the stroma are essential for cell invasion. Fibroblasts that exist in a cancer environment are commonly seen to be transformed into myofibroblasts by paracrine signals such as TGF-β and to express α-SMA. It was observed when optimizing the organotypic model that the myofibroblasts cell line, HFF-2, facilitated invasion whereas the fibroblast cell line MRC-5 which is known not to express α-SMA did not.

Therefore, the invasive T24 cell line and the invasive primary G3pT3 cells were cultured in identical conditions with either HFF-2 fibroblasts or MRC-5 fibroblasts. The HFF-2 fibroblasts showed widespread invasion whereas the MRC-5 fibroblasts did not facilitate any invasion, not even in the primary cell line. This shows for the first time with primary cells and also for the first time within a 3D bladder cancer model, that interaction with fibroblasts

may be key for invasion in some types of bladder cancer. It validates work done in other cancer types emphasizing the need of a cancer environment to allow cell invasion as well as the properties of the cancer cells themselves.[175,186]

The secondary aim of the experiment was to try and use primary cancer fibroblasts in the organotypic model and see if EMT and invasion could be induced in a previously non-invasive cell line. Results of such an experiment could provide a model system that could be utilized to gain insights into why some aggressive non-muscle invasive bladder (G3pTa) cancers stay superficial and some progress to invasive disease. Unfortunately, it was not possible to grow adequate levels of primary fibroblasts and the experiment could not be performed. Questions that remain unanswered include whether the paracrine signals originate in the tumour activating the surrounding stroma or whether it is primarily the surrounding stroma that transforms the cancer cells.

Finally, the effect of HER2 on cell invasion was investigated in the T24 cell line. A lenti-viral knock down cell line model of T24 cells was kindly donated by by Ms Christiana Kitromilidou and Prof Tom Powles of the Barts Cancer Center, London. There were three clones; 4,7 and 13. Upon Western Blotting it could be seen that clones 4 and 7 had substantial reduction in HER2 protein and clone 13 had not been effective. Clones 4 and 7 were therefore chosen to be cultured on the organotypic model. After measuring the blot intensity using ImageJ software it was decided that clone 7 had the most effective knockdown with over 80% reduction in HER2 expression. It was attempted to confirm this by performing immunohistochemistry for HER2 on the processed organotypics. The normal T24 cell line expressed HER2 on the cell surface and without the aid of a validated scoring system for organotypics there appeared to be a reduction in the HER2 expression in clone 7

(figure 5.10). EGFR is the most widely studied in the HER family and is associated with poor prognosis disease. It is also a known dimerising partner of HER2 and has the potential to be up regulated to counter the reduction of HER2. The HER2 knockdown organotypic was therefore stained for EGFR to see if expression increased as a mechanism for countering a reduction in HER2. This was not the case and there was little staining for EGFR in either the control T24 cell line or clone 7.

Culturing the two effectively knocked down clones onto the organotypic model resulted in a healthy confluence of T24 cells. There did not appear to be any difference in invasive characteristics between the clones and the control with all of them invading readily. This implies that in this cell line HER2 is not needed for cell invasion.

This experiment implies that HER2 is not essential for cell invasion in this cell line but certain limitations of the study need to be addressed in future experiments. It is possible that although the clones had low levels of HER2 at time point zero on the organotypic, after a period of incubation on the culture this may have changed. An attempt to confirm HER2 knock down was performed by staining the organotypics at 14 days in both the control and clone 7. The results from this are very difficult to interpret and an alternative may be to harvest the cells and perform western blot analysis on them to look at protein levels and perform a direct comparison to the original blots.

Finally, this is the first bladder cancer organotypic model to utilise primary bladder cancer cells and this is the first time that the role of fibroblasts has been shown to be essential for invasion for a subset of bladder cancers. Overall the organotypic created appeared to be a valid experimental model in bladder cancer with the ability to recreate known invasive

characteristics of various cell lines. In the T24 cell line it does not appear that HER2 is responsible for cell invasion.

There are many avenues for future work with this model. Firstly, we will aim to use the model to validate previous markers shown in studies of other cancer types with respect to EMT. Staining of the organotypic models for E-cadherin would be the first step towards showing the loss of epithelial phenotype of the invading cells and subsequently staining the sections for α-sma would confirm that the HFF2 fibroblasts are indeed active myofibroblasts.

Further to this repeat attempts at harvesting primary bladder cancer associated fibroblasts are likely to be successful. Integrating these into the organotypic model would bring greater physiological relevance to it. It should be possible to confirm the likely myofibroblasts phenotype of these types of fibroblasts and their role in EMT/ inducing invasion in different cell lines would be attempted.

The effect of various target agents could be introduced and their effect on cell growth and invasion measured. Once this becomes a reproducible model it may ultimately be possible to create a personalised organotypic culture whereby a patient's own bladder cancer cells can be grown in contact with a matrix containing their own fibroblasts. Although much validation would have to occur, not least within animal models, this has the potential to be used as a precision model allowing for testing of targeted therapies before translating them directly into the patient.

Chapter 6.

Creation of a Cystectomy TMA for biomarker discovery and to understand the role of HER2 and the EGFR Family Members as Biomarkers in Muscle Invasive Bladder Cancer

6.1 Introduction

One hypothesis of this thesis was that a tissue micro-array (TMA) would be a good model for biomarker discovery and for validating potential biomarkers that have been implicated from pre-clinical research. This was created using triplicate 1mm cores from archived cystectomy tissue. A clinical database of 226 patients was created for this study, however, 17 sets of tissue blocks were unable to be found as they were either lost or were unobtainable after being returned to other hospitals. This left a total of 209 patients with available tissue blocks for creation of the TMA.

A number of patients were down staged at the time of cystectomy to T0 disease with no remaining tumour in the bladder. These patients had no tumour to be able to stage and as a result immunohistochemistry was performed on the last pre-cystectomy bladder biopsy. If the previous biopsy was unavailable, the patient was excluded from the analysis. This is one of the problems with a retrospective TMA. To consider the pre-cystectomy biopsy as representative of the cancer's biomarker expression is reasonable however, it does introduce a clear bias. The pre-cystectomy biopsy is arguably the most important sample for tailoring a specific treatment strategy as the tumour is yet to be influenced by external disease modifying agents such as neo-adjuvant chemotherapy. However, it was not possible to identify the pre-cystectomy biopsies in the majority of cases for many reasons including referrals from other hospitals, lost slides and time constraints associated with interrogating the hospital records for the dates of previous biopsies.

Furthermore, there was concern that patients who were node positive on the pre-operative stage, with likely metastatic disease at the time of presentation, who were subsequently down staged by chemotherapy before being given the benefit of doubt and undergoing surgery may

cause a negative bias in the results. This small group, of 9 patients, was excluded from the final analysis as they fell out of the usual treatment pathway. For this reason, the inclusion criteria for the TMA analysis was a pre-operative staging of any tumour stage, no nodes and no metastasis (TanyN0M0).

HER2 and the other family members have potential roles as biomarkers in patients with bladder cancer being treated by radical cystectomy. This family of tyrosine kinase receptors is an attractive experimental therapeutic target as, on balance, the literature supports that HER2 is overexpressed in a subset of bladder cancers, as is EGFR[49,50,121,125]. Furthermore, there are now pharmacological agents in routine clinical use in other diseases that are targeted at HER2 or EGFR. Lapatinib is one of the newer tyrosine kinase inhibitors that act on both EGFR and HER2 that is already used in metastatic breast cancer patients. Its role in the management of patients with metastatic bladder cancer has recently been reported in a prospective randomized controlled trial.[158] This studied advanced patients who had failed 1st line palliative chemotherapy and identified patients who overexpressed EGFR and/or HER2 using the HercepTest^{R}. Following the same logic this TMA of radical cystectomy patients was interrogated to see how many patients would be suitable for therapy with Lapatinib, potentially as an adjuvant therapy and what the effect on survival was for patients overexpressing EGFR and/orHER2.

The TMA was cut and processed on the hospitals fully automated immunohistochemistry machines to reduce inter-slide variability. The validated HercepTest^{R} was used to score all the family members and the associations with survival are now presented.

6.2 Results

The TMA was successfully created and there was a variation in the staining patterns between different cores as expected. EGFR, HER2, HER3 and HER4 all demonstrated membrane staining when expressed (figure; 6.1). From the representative cores for 209 patients, there were lost cores secondary to the processing of the slides, with a lack of tumour tissue, for between 14-35 patients on the different TMAs.

EGFR demonstrated overexpression in 38.5% of patients' tumours. HER2 was overexpressed in 17%, HER3 in 24% and HER4 in 24.6% (Table: 6.1). When looking at the over-expression rates compared to tumour stage the numbers were not large enough to look at significant variations. However, the trend was for EGFR and HER4 to be overexpressed mainly in the higher stage disease, HER2 to be expressed relatively consistently across all stages and HER3 to be associated with the lower stages (table: 6.2).

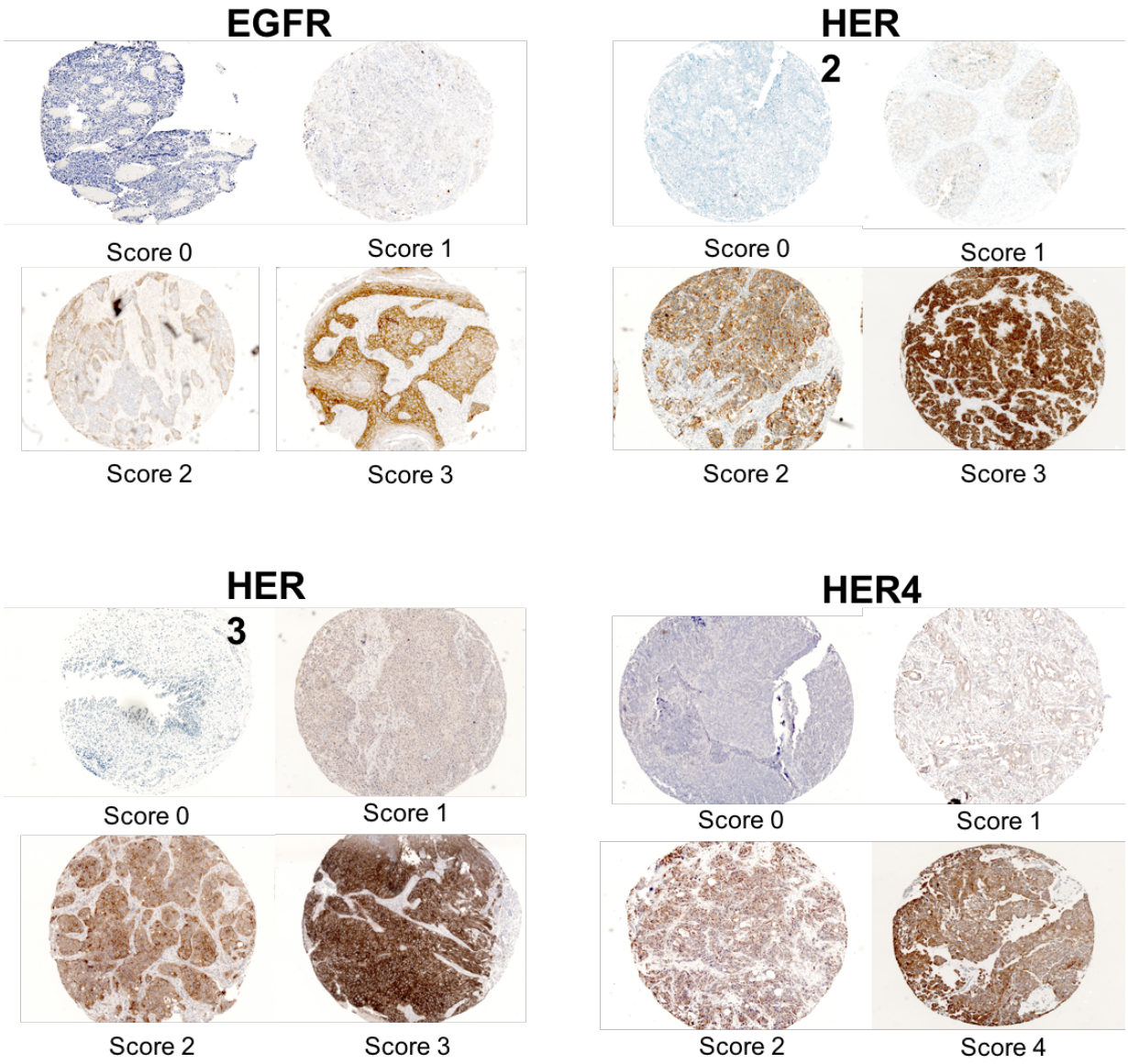


Figure 6.1: Representative cores taken from the tissue micro-array demonstrating different immunohistochemical staining for EGFR, HER2, HER3 and HER4. The HercepTest® was used throughout. Score 0 represents no staining or weak staining in less than 10% of the tissue, Score 1 represents weak membrane staining in more than 10%, Score 2 represents moderate staining in more than 10% of the tissue and Score 3 represents strong staining in more than 10% of the tissue. For analysis the cores were defined as positive if score 2 or 3 and negative if score 0 or 1.

Variable	Total number (%)
Available Tissue Blocks	209
Missing / No Tumour Cores	35
Representative Tissue Cores	174 (100)
EGFR +ve Cores	67 (38.5)
EGFR –ve Cores	107 (61.5)

Variable	Total number (%)
Available Tissue Blocks	209
Missing / No Tumour Cores	14
Representative Tissue Cores	194 (100)
HER2 +ve Cores	33 (17)
HER2 –ve Cores	161 (83)

Variable	Total number (%)
Available Tissue Blocks	209
Missing / No Tumour Cores	34
Representative Tissue Cores	175 (100)
HER3 +ve Cores	42 (24)
HER3 –ve Cores	133 (76)

Variable	Total number (%)
Available Tissue Blocks	209
Missing / No Tumour Cores	34
Representative Tissue Cores	175 (100)
HER4 +ve Cores	43 (24.6)
HER4 –ve Cores	132 (75.4)

Table 6.2: Four tables to demonstrate the number of patients with representative cores of tissue on the TMA, after processing and staining, and the percentage of patients with positive immunohistochemical scoring for EGFR, HER2, HER3 and HER4. The HercepTest^{R} was used and score 2 or 3 defined as a positive score. EGFR was overexpressed in 38.5% of patients, HER2 in 17%, HER3 in 24% and HER4 in 24.6%.

Stage	EGFR +ve (%)		EGFR –ve (%)	
T0	n/a	24	5	49
<T2	11		22	
T2	13		22	
T3	43	76	33	51
T4	4		7	
N+	28		11	

Stage	HER2 +ve (%)		HER2 –ve (%)	
T0	n/a	52	16	45
<T2	28		15	
T2	24		14	
T3	21	48	35	55
T4	15		4	
N+	12		16	

Stage	HER3 +ve (%)		HER3 –ve (%)	
T0	9	76	5	33
<T2	43		11	
T2	24		17	
T3	14	24	44	67
T4	10		6	
N+	0		17	

Stage	HER4 +ve (%)		HER4 –ve (%)	
T0	n/a	33	9	46
<T2	19		18	
T2	14		19	
T3	39	67	37	54
T4	5		6	
N+	23		11	

Table 6.3: Four tables demonstrating the different amount of over-expression of EGFR, HER2, HER3 and HER4 according to stage. The percentage scoring in the positive (+ve) groups relates to only the patients with overexpression. The EGFR +ve group comprises 67 patients compared to 107 patients in the EGFR –ve group. HER2 +ve represents 33 patients, HER3 +ve 42 patients and HER4 +ve 43 patients.

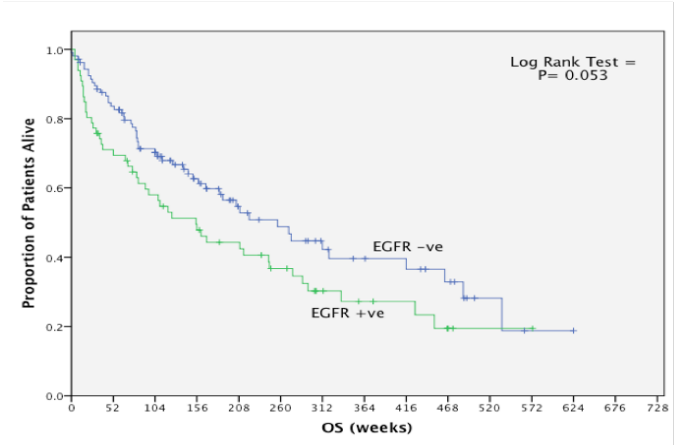
6.2.1 The effect of the HER family on overall survival

There were representative cores for 174 patients on the TMA slides used for the EGFR immunohistochemistry. There was over-expression of EGFR in 38.5% of patients and this was associated with a trend towards worse overall survival (OS). The median OS was only 155 weeks in the EGFR positive group compared to 256 weeks in the EGFR negative group. This equated to a 5-year OS of 37% vs 51% although this was not statistically significant. (figure: 6.2)

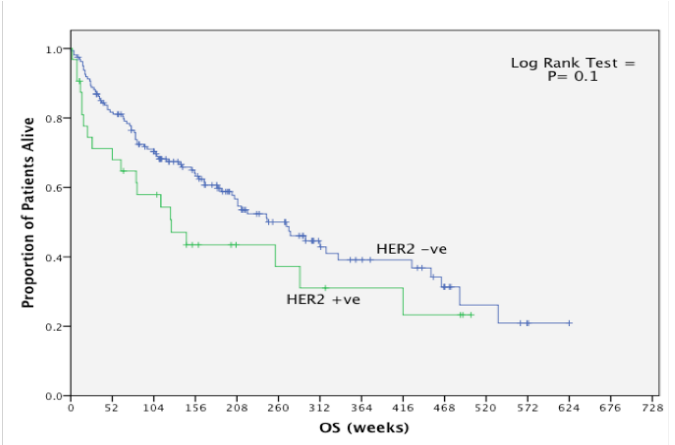
For the analysis of HER2 there were 194 patients with representative tissue on the TMA. HER2 was over-expressed in 17% of patients and again this was associated with a trend for a poorer survival with a median OS of 126 weeks in the HER2 positive group compared to 270 weeks in the HER2 negative group. This was associated with a 5-year OS of 37.5% vs 50.5% however; despite the trend this was not statistically significant with univariate analysis. (figure: 6.2) When multivariate analysis was performed, by accounting for known adverse factors such as patient age, sex and stage of disease it was found that HER2 was an independent variable for worse OS with a hazard ratio (HR) of 1.68 and a P-value of 0.048. (table: 6.3)

There were 175 patient cores for the HER3 staining. HER3 was over-expressed in 24% of patients with no difference in the OS between the HER3 positive and negative patients. The median OS was 205 weeks for the positive patients compared to 188 weeks in the negative patients. Overall the 5-year OS was 50% in the HER3 positive compared to 43% in the HER3 negative. (figure: 6.2)

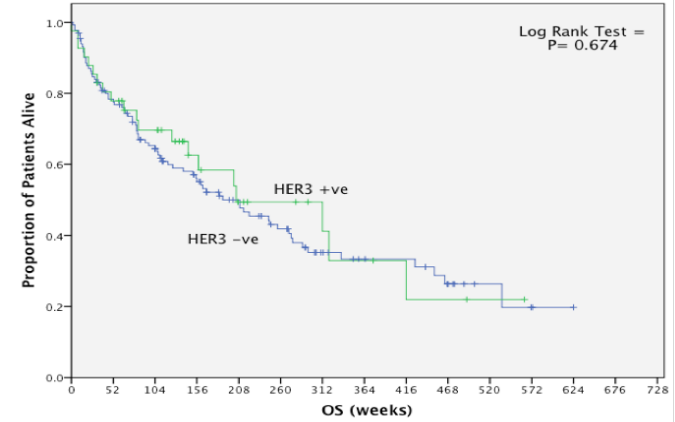
There were also representative tumour cores, for 175 patients, that were stained for HER4. Over-expression was apparent in 24.6% of cases. The trend for a worse OS was significantly worse in the HER4 positive patients with a median OS of only 155 weeks in the HER4 positive group compared to 214 weeks in the HER4 negative group. This equated to a 5-year OS of 28% in the HER4 positive patients compared to 48% in the HER4 negative patients. (figure: 6.2) This was statistically significant with a P-value of 0.025 and is the first time that HER4 has been shown to be a negative predictor for outcome in bladder cancer. This difference was re-enforced when multivariate analysis was performed by accounting for known adverse factors such as patient age, sex and stage of disease. It was found that HER4 was an independent variable for worse OS with a HR of 1.61 and a P-value of 0.031. (table: 6.4)



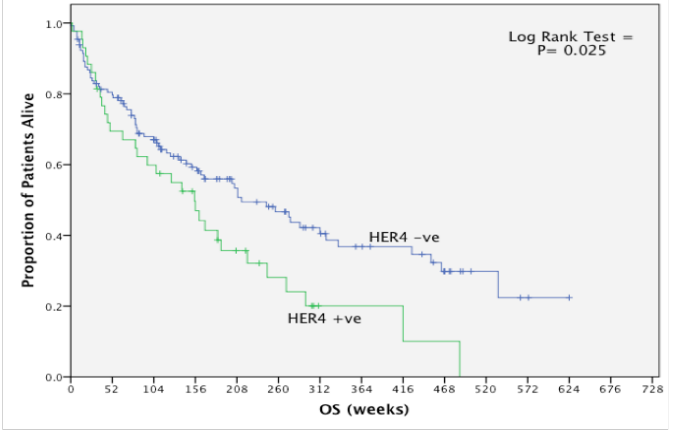
Variable	5yr Overall Survival (%)	Median Overall Survival (weeks)	95% Confidence Interval (weeks)
EGFR +ve	37	155	93-210
EGFR –ve	51	256	172-339



Variable	5yr Overall Survival (%)	Median Overall Survival (weeks)	95% Confidence Interval (weeks)
HER2 +ve	37.5	126	49-203
HER2 –ve	50.5	270	203-337



Variable	5yr Overall Survival (%)	Median Overall Survival (weeks)	95% Confidence Interval (weeks)
HER3 +ve	50	205	41-368
HER3 –ve	43	188	113-262



Variable	5yr Overall Survival (%)	Median Overall Survival (weeks)	95% Confidence Interval (weeks)
HER4 +ve	28	155	93-217
HER4 –ve	48	214	128-299

Figure 6.2: The Kaplan-Meier plots demonstrating the differences in overall survival in patients with marker (EGFR, HER2, HER3 or HER4) positive and marker negative disease. The tables demonstrate the5 year median overall survivals with confidence intervals. The log-rank test was applied to look for statistical difference between the survival curves and the p-value recorded.

	Hazard Ratio	95% C.I.	P-Value
EGFR +ve	1.23	0.8-1.9	0.343
Sex	1.5	0.9-2.55	0.12
Age	1.02	0.995-1.04	0.15
Pre-op stage <T2	1.99	0.95-4.16	0.07
T2	2.57	1.21-5.44	0.01
T3	7.14	2.32-21.98	<0.01
T4	NR	NR	NR

	Hazard Ratio	95% C.I.	P-Value
HER2 +ve	1.66	1.01-2.74	**0.048**
Sex	1.25	0.77-1.97	0.39
Age	1.03	1.00-1.05	0.02
Pre-op stage <T2	2.11	0.98-4.54	0.06
T2	3.14	1.47-6.74	0.03
T3	8.4	2.74-25.73	<0.01
T4	3.27	3.87-26.46	0.27

	Hazard Ratio	95% C.I.	P-Value
HER3 +ve	1.04	0.62-1.75	0.883
Sex	1.33	0.80-2.21	0.266
Age	1.01	0.99-1.04	0.126
Pre-op stage <T2	1.69	0.81-3.54	0.16
T2	2.31	1.11-4.8	0.025
T3	6.14	2.04-18.49	0.001
T4	NR	NR	NR

	Hazard Ratio	95% C.I.	P-Value
HER4 +ve	1.61	1.05-2.48	**0.031**
Sex	1.4	0.85-2.29	0.18
Age	1.02	0.99-1.04	0.06
Pre-op stage <T1	1.84	0.91-3.72	0.09
T2	2.25	1.11-4.53	0.02
T3	6.81	2.3-20.16	<0.01
T4	2.49	0.31-19.92	0.39

Table 6.4: Four tables to demonstrate the hazard ratio (HR) , after multi-variate analysis, associated with overexpression of one of the markers (EGFR, HER2, HER3, HER4). The variables to be adjusted for were patient sex, age and stage of disease. HER2 was predictive of poor outcome with a HR of 1.66 (P= 0.048) as was HER4 with a HR of 1.61 (P=0.031). NR = numbers not reached

6.2.2 The effect of the HER family on recurrence free survival

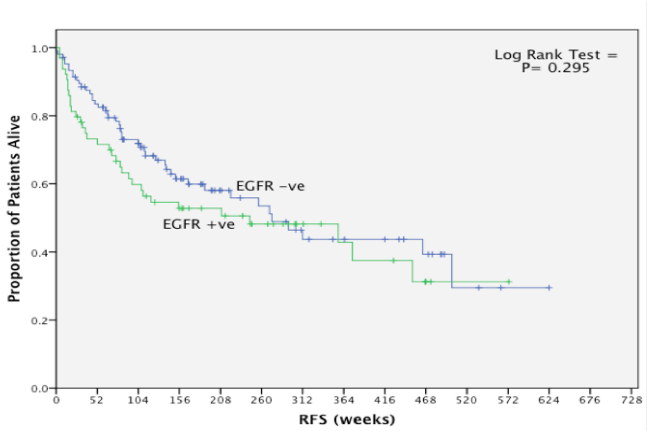
To ensure similarity between overall survival and cancer specific outcome the recurrence free survival (RFS) was calculated. The same trend was seen for RFS as for OS.

For the patients that overexpressed EGFR the median RFS was 245 weeks compared to 273 weeks for the EGFR negative patients. The 5-year RFS was 48% for EGFR positive patients' vs 53% for EGFR negative patients. The difference was not statistically significant. (figure: 6.3)

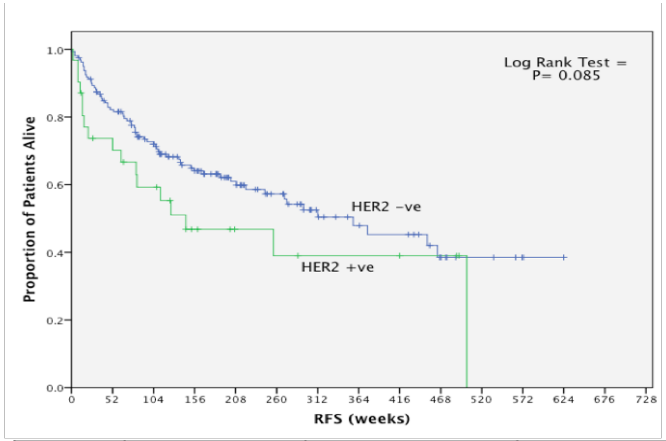
The trend again suggested a worse RFS for HER2 positive patients compared to HER2 negative patients with a median RFS of 145 weeks vs 357 weeks. However, despite a 5-year RFS of only 39% in HER2 positive patients vs 58% in HER2 negative patients, this difference was not statistically significant. (figure: 6.3)

The HER3 positive patients did not demonstrate any difference in RFS. Their median RFS was 312 weeks for HER3 positive patients vs 256 weeks for the HER3 negative patients. Likewise, the 5-year RFS was 59% vs 50%. (figure: 6.3)

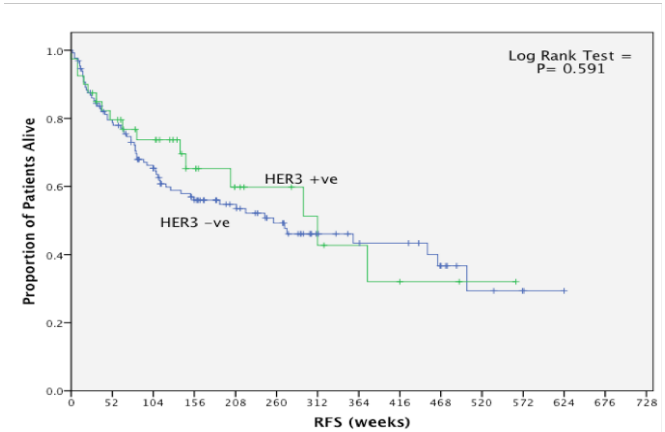
Overexpression of HER4 was associated with a median RFS of 139 weeks compared to 312 weeks for the HER4 negative patients. The 5-year RFS was 38% in the HER4 positive group compared to 56% in the HER4 negative group. The trend was seen in the survival curves but, unlike for OS, the difference did not make statistical significance with a P-value of 0.083. (figure: 6.3)



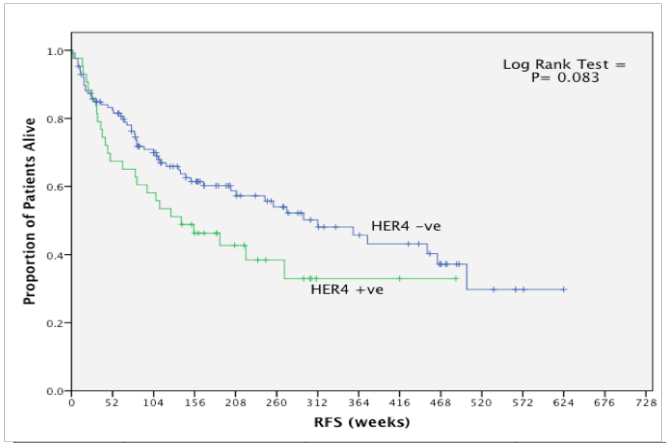
Variable	5yr Recurrence Free Survival (%)	Median Recurrence Free Survival (weeks)	95% Confidence Interval (weeks)
EGFR +ve	48	245	8-482
EGFR –ve	53	273	182-364



Variable	5yr Recurrence Free Survival (%)	Median Recurrence Free Survival (weeks)	95% Confidence Interval (weeks)
HER2 +ve	39	145	0-310
HER2 –ve	58	357	216-497



Variable	5yr Recurrence Free Survival (%)	Median Recurrence Survival (weeks)	95% Confidence Interval (weeks)
HER3 +ve	59	312	158-466
HER3 –ve	50	256	101-410



Variable	5yr Recurrence Free Survival (%)	Median Recurrence Free Survival (weeks)	95% Confidence Interval (weeks)
HER4 +ve	38	139	47-231
HER4 –ve	56	312	197-426

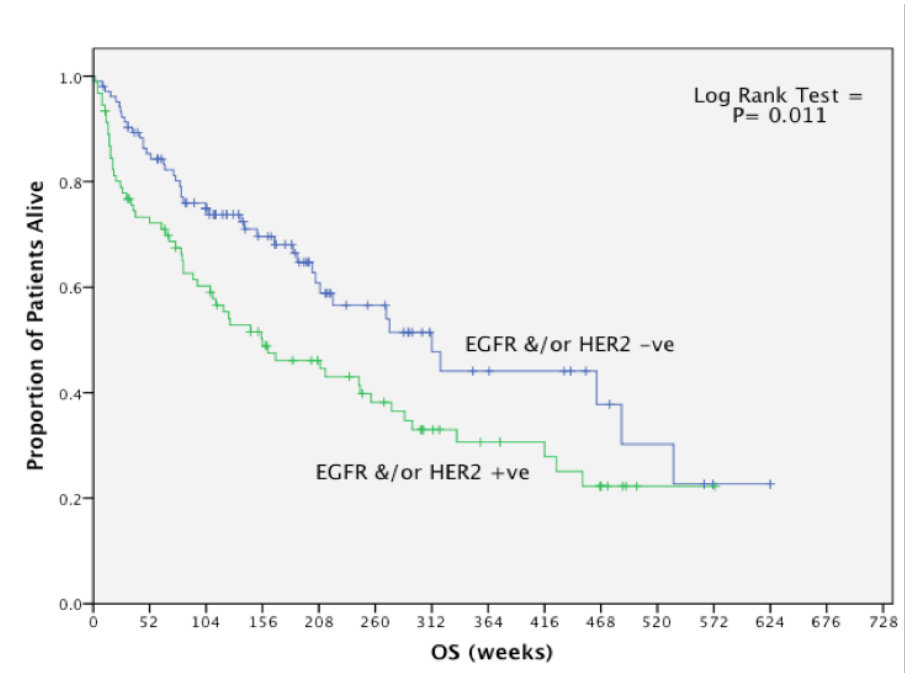
Figure 6.3: The Kaplan-Meier plots demonstrating the differences in recurrence free survival (RFS) in patients with marker (EGFR, HER2, HER3 or HER4) positive and marker negative disease. The tables demonstrate the 5 year median RFS with confidence intervals. The log-rank test was applied to look for statistical difference between the survival curves and the p-value recorded. There is a strong trend for worse survival in patients who over-expressed HER2 or HER4 but this was not statistically significant.

6.2.3 Overexpression of EGFR and/or HER2

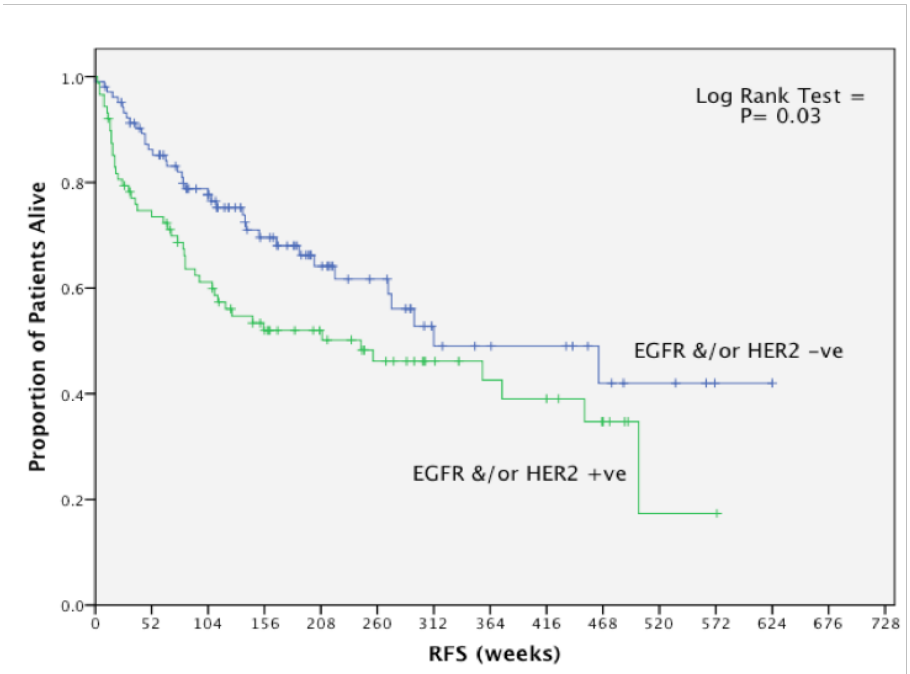
To assess the number of patients who would potentially be candidates for adjuvant therapy with the dual EGFR/HER2 tyrosine kinase inhibitor, Lapatinib, the TMA was interrogated. This pattern of expression was chosen due to the availability of target agents and to match the inclusion criteria for the LAMB study who used this expression profile to select patients. The effect of this expression pattern was also looked at with respect to survival outcomes.

It was found that 46.2% of patients overexpressed EGFR and/or HER2. The median OS was worse in the EGFR and/or HER2 group at 156 weeks compared to 312 weeks in the negative group. The 5-year overall survival was 38% in the positive group compared to 56% in the negative group. This was statistically significant with a p-value of 0.011. (figure: 6.4)

The RFS was worse if a patient overexpressed EGFR and/or HER2 with a median RFS of 245 weeks compared with 312 weeks if they did not. This equated to a 5-year RFS of 46% for the EGFR and/or HER2 positive group compared to 61% for the negative group. This was statistically significant with a P-value of 0.03. (figure: 6.4)



Variable	5yr Overall Survival (%)	Median Overall Survival (weeks)	95% Confidence Interval (weeks)
EGFR &/or HER2 +ve	38	156	67-244
EGFR &/or HER2 –ve	56	312	204-420



Variable	5yr Recurrence Free Survival (%)	Median Recurrence Free Survival (weeks)	95% Confidence Interval (weeks)
EGFR &/or HER2 +ve	46	245	47-443
EGFR &/or HER2 –ve	61	312	117-507

Figure 6.4: The Kaplan-Meier plots demonstrating the differences in overall survival and recurrence free survival (RFS) in patients who overexpressed EGFR and/or HER2. The tables demonstrate the 5-year median overall survival and 5 year RFS with confidence intervals. The log-rank test was applied to look for statistical difference between the survival curves and the p-value recorded.

6.3 Discussion

Tissue micro-arrays (TMAs) have inherent limitations that were discussed earlier (chapter 1.4.2). Cancers are very heterogeneous and taking a 1mm core may lead to biologically unrepresentative or under-representative sections of tumour being sampled. This has been illustrated in work in renal cell carcinomas where primary and metastatic tumours underwent exome sequencing, chromosome aberration analysis and ploidy profiling. [48] This study and others[194] have demonstrated significant heterogeneity both within the tumour itself and also compared to metastatic sites. This is visible immunohistochemically but also, as these studies demonstrate, at the genetic level. Different mutational phenotypes have been seen for multiple tumour suppressor genes within the spatially separated tissue samples. This lack of ability to see the whole picture is a continuing conundrum for which there is currently no satisfactory answer and for now we have to accept this limitation. Heterogeneity is a major concern for any precision therapy based upon a single biopsy approach with the distinct possibility that the targeted drug may be ineffective for a missed driver mutation in the primary or an evolved separate mutation in a metastasis. For this study the decision was made to simply perform triplicate cores to increase the chances of sampling representative tumour. This approach has been shown to have 91-98% concordance with matched whole tissue samples.[195] Another limitation of a TMA is that as serial slices are taken through a core it is possible to move out of tumour and into benign tissue or simply wax. Moreover, this dataset was complicated by the fact that some patients were down staged to T0 with no residual tumour in the cystectomy specimen. In these cases, the associated pre-cystectomy biopsy was used as representative tumor. If no pre-cystectomy biopsy was present or there was simply no tumour in the core, then scoring was not performed and the core rejected.

There were cases of differences in score between the triplicate cores for the same tumour. It was hypothesized that the biomarkers being studied were biologically active and therefore the highest score was taken as the definitive score for the individual tumour. Similarly, if one or two of the triplicate cores had been lost during processing the remaining core/cores were used as the representative sample. This must be an issue with all TMAs but is something that has not been highlighted in publications and as such the above tactic seemed reasonable.

During the processing of the slides some cores failed to adhere to the slide and were washed off in the staining process. This led to a further reduction in the total number of cores available for analysis. Unlike other markers in this thesis, the antigen retrieval and staining process for EGFR, HER2, HER3 and HER4 were performed in the hospital clinical histopathology department. This utilized the department's standardized procedures and fully automated machine processes. This was advantageous, as the optimized dose of antibody and type of antigen retrieval was already validated for these anti-bodies. The inter-slide variability that can occur due to experimental error such as the length of time the anti-body is left on the slide and the effectiveness of washes were vastly reduced by using the automated machines that processed all the slides simultaneously under identical conditions.

EGFR was the first member of this tyrosine kinase receptor family to be discovered and was the first to be stained for on the TMA. As described in the methods overexpression was defined as a HercepTest^{R} score of 2+ or 3+. The TMA had representative tissue cores for 174 patients. EGFR was overexpressed in 38.5% of tumours and there was a strong trend for worse overall and recurrence free survival in the EGFR positive group. Although this did not quite reach statistical significance it is reasonable to think that with a larger sample size it would. Previous papers have supported the view that EGFR is associated with higher stage

and grade of disease with associated poorer survival.[196] The data here supports this with EGFR overexpression being associated with a worsening stage of disease. (table: 6.2) When the patients were placed into two groups (stage 0,1,2 or stage 3,4 or N+) it could be seen that the patients who overexpressed EGFR were predominantly in the higher stage group.

HER2 has not been so well studied in bladder cancer in the world literature but is an established therapeutic target in breast and other cancers. As a result it is under clinical investigation in bladder cancer. The results here show that over-expression of HER2 is an independent risk factor for poor overall survival after performing COX regression analysis with a hazard ration of 1.66 (table: 6.3). This is despite the fact that the differences in overall and recurrence free survival were not statistically different in univariate analysis, although the trend was there. The 5-year overall survival was 37.5% in the HER2 positive group compared to 50.5% in the HER2 negative group with a median length of survival of 126 weeks versus 270 weeks. (figure: 6.2) This is most probably attributable to only 17% of patients overexpressing HER2 leaving only 33 patients in that arm thus limiting the statistical analysis. However, when studying the Kaplan Meir survival curves for both overall (figure: 6.2) and recurrence free survival (figure: 6.3), it is clear that the curves are technically divergent and again with a larger cohort significance would possibly be seen. Interestingly HER2 status did not seem to stratify for stage of disease and is predictive of survival independently to this factor. (table: 6.2)

HER3 is the member of the family without a tyrosine kinase and is the most frequent dimerising partner to HER2, which lacks a known ligand. In this study there was no significant difference in survival characteristics in HER3 positive or HER3 negative patients (figures: 6.2, 6.3). In contrast to the other HER family members the trend on the Kaplan

Meir curves is towards that of better outcomes. When looking at the spread of different stage disease between the HER3 positive and negative groups there is again a strong trend towards lower stage disease. 76% of the HER3 positive patients had stage 0,1 or 2 disease compared to 33% of the HER3 negative group. This is in keeping with previous literature suggesting a "protective role" role of HER3 as well as HER4 when co-expressed with HER2 or EGFR.[111,112] This appears logical as it may reflect normal physiological dimerization patterns between the HER family members with resultant normal downstream pathway activation, implying a less aggressive tumour. This is in comparison to HER2 overexpression without other family members, which would imply a more pathological scenario.

As mentioned there is a lack of published work on the role of HER4 in bladder cancer. The 3 papers in the world literature all point towards, what the authors describe as, a protective role for HER4 in bladder cancer.[112,197,198] In this thesis conflicting results to this have been shown for the first time. HER4 appears to be prognostic for poor overall and recurrence free survival (figures: 6.2, 6.3). The median overall survival was 155 weeks in the HER4 positive group with a 5-year overall survival of 28% compared to 48% in the HER4 negative group. After cox regression analysis HER4 is still an independent risk factor with a hazard ratio of 1.61 (p=0.031). (table 6.3)

The previous HER4 papers have looked at co-expression levels of HER3 and HER4 concluding that increased co-expression is beneficial. The papers by Memon et al. looked at mRNA expression levels and used an arbitrary cut off at the median concentration to define high and low levels. In this thesis a pre-defined immunohistochemical score, using the HercepTest^{R}, was used. This may explain some of the discrepancy between results. It was felt that it was best to use the HercepTest^{R} as this has been widely validated and is used on a

daily basis in the clinical setting. Memon et al. found that HER4 was overexpressed in 63% of samples (all stages and grades) whereas using the stricter HercepTest^{R} for over expression meant this thesis found that only 24.6% of patients overexpressed HER4.

Due to the reported possibility of co-expression revealing prognostic information this was looked at. Unfortunately, there were very few tumours that overexpressed more than one of the HER family members to make any meaningful statistical analysis. For example, only six of the patients overexpressed HER2 and HER4. When survival was plotted, the HER2:HER4 co-expressers did appear to do much worse with a median survival of only 21 weeks compared to 245 weeks but this did not quite reach significance with a p-value of 0.068. (data not shown)

Similarly, only 7 patients overexpressed EGFR and HER2. Again these patients did badly with a median overall survival of only 27 weeks compared to 256 weeks. This was statistically significant with a P-value of 0.000 (data not shown) however, with such very small numbers it is hard to draw meaningful conclusions.

It may be relevant however, to consider the patients who ever-express EGFR and or HER2 as there are clinically available drug inhibitors that target both EGFR and HER2 as discussed previously. 46.2% of tumours overexpressed EGFR and or HER2 and as a result would be potential candidates for targeted drug treatments. These patients may benefit from such personalized therapies especially as they comprise a worse prognostic group. When considered as a single group, patients who over express EGFR and or HER2 have a significantly worse 5-year overall (38% vs 56%) and 5-year recurrence free (46% vs 61%) survival (figures: 6.2, 6.3).

To summarise this thesis has added to the evidence that EGFR is a poor prognostic indicator for patients with bladder cancer requiring radical cystectomy. It has also confirmed that HER2 is a poor prognosticator and unlike previous published papers this dataset looks at just patients with disease requiring radical cystectomy. HER3 may have some positive prognostic implications but these were not conclusive. In comparison HER4 for the first time has been shown to predict poor survival and this is confirmed to be independent to stage of disease, age and sex of the patient. Further work into the biology of HER4 in bladder cancer needs be performed and the findings from this cohort need to be validated on an external dataset.

When considering the ultimate goal of personalizing treatments, this data would support the rationale for a prospective clinical trial for personalised adjuvant or neo-adjuvant therapies, for patients requiring radical cystectomy, utilizing the dual EGFR/HER2 inhibitors that we already have available. However, the recent phase II/III LAMB study has presented its results and it has failed to demonstrate any survival advantage in giving Lapatinib as a second line chemotherapy agent to advanced bladder cancer patients who overexpress EGFR and/orHER2. (ASCO 2105) This is disappointing and reasons for its failure are still being discussed. It may simply be that EGFR and HER2 have no significant targetable role. More likely, especially with such advanced metastatic disease, the tumour heterogeneity both intra-tumour and between primary and metastasis means that simply targeting one or other of these two tyrosine kinases is simply not enough. However, until this problem of tumour heterogeneity is overcome, 46% of patients undergoing radical cystectomy with curative intent overexpress EGFR and or HER2 and these remain a rational target for a personalised treatment approach.

Chapter 7.

Utilization of the TMA to understand the role of β-HCG, Nrf-2 and AIMP3 as biomarkers in a cystectomy cohort

7.1 Introduction

Having established the TMA, it was then utilized in three further projects. These were:

1: to investigate the role of the beta (β) sub-unit of the hormone, human chorionic gonadotrophin (hCG) both as a serum marker and as an immunohistochemical marker. (Internal project)

2: to investigate the role of the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf-2). (Internal project)

3: to look at the role of the novel tumour suppressor gene Aminoacyl-tRNA synthetase (ARS)-interacting multifunctional protein 3 (AIMP3). (External collaborative project)

7.1.1 β-HCG

The significance of serum β-HCG levels was noted simultaneously in this cystectomy database and also by another internal group creating a database of bladder cancer patients receiving chemotherapy. Like several other non-trophoblastic tumours bladder cancer has been associated with ectopic production of the free beta sub-unit of the hormone, human chorionic gonadotrophin (hCG).

HCG is a heterodimeric glycoprotein formed of two sub-units – alpha and beta. It is perhaps best known for its physiological role in pregnancy where it is initially secreted

by the developing embryo and later by syncitiotrophoblasts of the placenta. In pregnancy it has a number of described roles including promoting angiogenesis of the uterine vasculature and enhancing corticosteroid production and maternal immunosuppression to prevent rejection of the foetus and placenta.

HCG only has hormonal properties when it is in an intact α-β arrangement; in other words, the free subunits themselves are not sufficient to stimulate the hCG receptor and do not show gonadotrophic or thyrotrophic activity. However, several independent studies have shown the free beta subunit of hCG (β-hCG) to have growth effects, and pathological production of HCG is well recognized in trophoblastic tumours of placental and germ cell origin.

More recently, the ectopic production of free β-hCG in the absence of the alpha subunit has also been noted in many epithelial tumours. In this context, β-hCG production is usually a sign of aggressive disease and elevated levels of β-hCG have been linked with poor prognosis. Initially it was thought that it increased cell proliferation but more recent work by Butler et al in 2004[199] and Jankowska et al in 2008[200] supports the theory that β-hCG actually promotes cancer cell survival through inhibition of cancer cell apoptosis.

Most studies looking at the significance of β-hCG in bladder cancer were published in the late 80's and early 90's with generally small study sizes of 13-100 patients, looking predominantly at serum β-hCG levels and β-hCG expression on immunohistochemical staining of bladder cancer tissue. The β-hCG levels in serum have also been looked at with the reported prevalence of elevated serum β-hCG levels in bladder cancer of 10-

35%[201-204]. One study reported even higher figures, with findings of significantly elevated serum β-hCG in 76%[205] of patients with widespread metastatic bladder cancer.

The data is limited but demonstrates that elevated β-hCG is associated with more advanced disease and a worse prognosis. Tumours positive for β-hCG have also been found to be significantly less responsive to treatment with radiotherapy, irrespective of tumour stage and grade.[206] Overall, the studies are limited in numbers and are inconclusive and as a result we looked further at this marker.

7.1.2 Nrf-2

Cisplatin based regimes are the gold standard for chemotherapy in both the neo-adjuvant and advanced setting for bladder cancer patients. However, cisplatin resistance mechanisms that are either primary or acquired are a major clinical problem. Various mediators of anti-oxidant response and cellular detoxification such as glutathione have been associated with resistance to cisplatin chemotherapy. Also the different expression levels of various metallothioneins, which regulate heavy metal metabolism, have been associated with poor prognosis bladder cancer and cisplatin resistance.[207,208]

Nrf-2 is a transcription factor master regulator with 100-200 targeting genes. These include target genes controlling cellular responses to oxidative stress including glutathione mediators such as gamma-glutamylcysteine synthetase and anti-oxidants including glutathione peroxidase.[209] Therefore, Nrf-2 regulates many of the events involved in Cisplatin resistance but this has never been studied in bladder cancer. Further more it is known that Nrf-2 is negatively regulated by kelchlike ECH-associated protein 1 (Keap-1).[209]

As a result of the above one of the departments lead researchers, Dr A Hayden, postulated that Nrf-2 activation in bladder cancer might drive a mechanism for cisplatin resistance. Using the wild type RT-112 cell line and a cisplatin resistant RT-112 cell line (RT-112 CP) it was demonstrated that there was significantly more Nrf-2 expression in the resistant cell line, which had approximately a 10-fold less growth inhibition to cisplatin. After depleting the RT-112 CP cell line of Nrf-2 by siRNA partial restoration in cisplatin sensitivity was achieved.

Due to the promising results collaboration occurred and the TMA was used to look for the prevalence of Nrf-2 in a cystectomy population and the clinical database to see if Nrf-2 status was predictive of survival or response to chemotherapy.

7.1.3 AIMP3

A project investigating resistance of bladder cancer to radiotherapy identified a novel tumour suppressor gene that was predictive of response. The lead researcher at UCL, Dr P Gurung (in Prof. J Kelly's laboratory) mined through a global gene-expression dataset using a modified algorithm selecting for low-expressing genes. They identified the loss of expression of a novel tumor suppressor gene, AIMP3 as a common feature in MIBC. AIMP3 is an auxiliary component of the macromolecular multisynthetase complex that mediates protein translation but it has also been implicated as an upstream activator of p53 in response to DNA damage or oncogenic stimuli[210,211]. AIMP3-homozygous mice are embryonic lethal, while AIMP3-heterozygous mice spontaneously develop tumors[210]. Normal urothelial cells

all express moderate to high levels of AIMP3 but reduced AIMP3 expression had previously been reported in some cancers but not in bladder cancer[210,212].

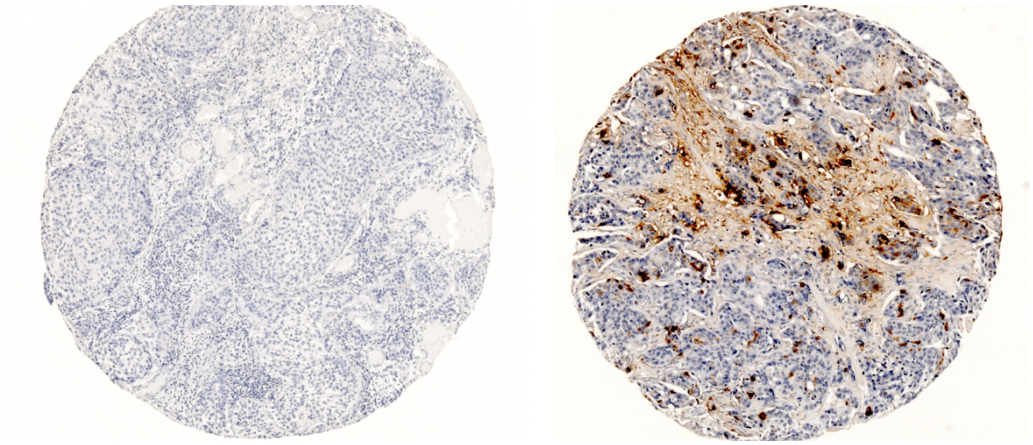
They looked at AIMP3 expression in the TMA from BCON (bladder carbogen and nicotinamide) trial and found a reduction in expression to predict resistance to radiotherapy. As a result, the expression levels of AIMP3 were determined on our TMA to see if its effects were unique to radiotherapy patients or present on all muscle invasive bladder cancers.

7.2 Results

7.2.1 HCG staining on TMA

The TMA was stained by immunohistochemistry for β-hCG as described previously (see chapter 3.9). A previously validated scoring system for β-hCG was used.[213] This scoring system had two main components; the intensity of scoring and the percentage of the tumour stained. No staining = 0, weak = 1, moderate =2 and strong = 3. The extent of the staining was split into quartiles with 0-25% = +, 26-50% = ++, 51-75% = +++ and 76-100% = ++++. This gave a score range of 0+ to 3++++. The results were then dichotomized into positive (≥2++) or negative (<2++). (figure 7.1)

There were 190 cores available for analysis with 20% of cases overexpressing β-hCG. (figure 7.1, table 7.1) The male to female ratio was 3.4:1 and the median age was 72 years. There was no difference in overall or recurrence free survival between the two groups. (figure 7.2)

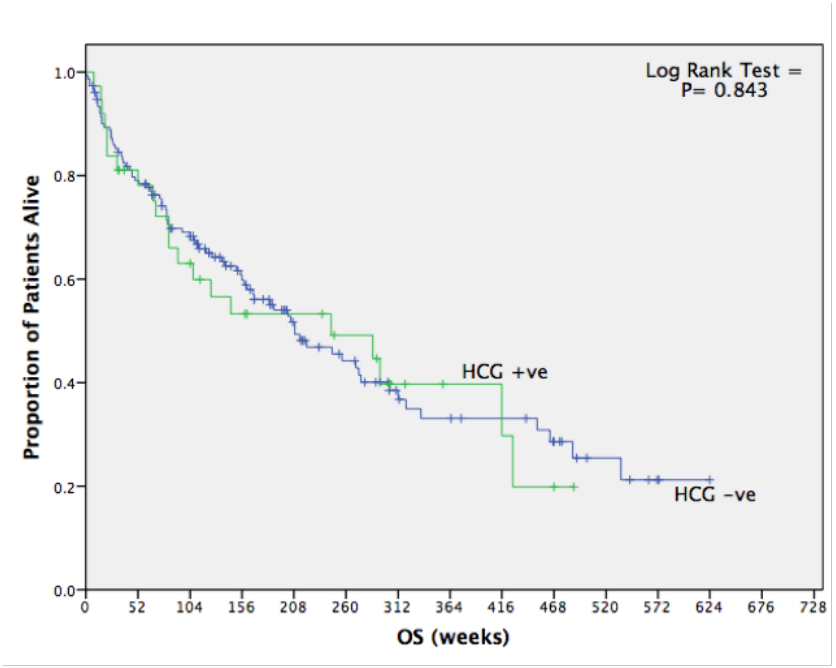


Negative Positive

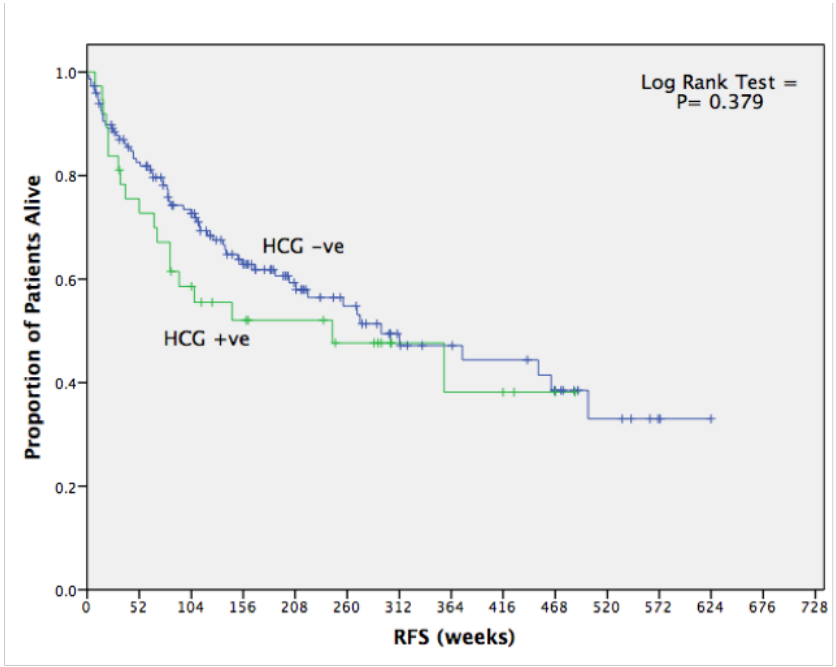
Figure 7.1: Representative images of two TMA cores. The first representing a negative stain for β-hCG and the second core is positively stained for β-hCG.

Variable	Total number (%)
Available Tissue Blocks	209
Missing / No Tumour Cores	19
Representative Tissue Cores	190 (100)
β-hCG +ve Cores	38 (20)
β-hCG –ve Cores	152 (80)

Table 7.1: Number of patients with representative tissue on the TMA and the associated levels of β-hCG expression.



Variable	5yr Overall Survival (%)	Median Overall Survival (weeks)	95% Confidence Interval (weeks)
β-hCG +ve	49	245	7-483
β-hCG –ve	44	209	148-270



Variable	5yr recurrence free survival (%)	Median recurrence free survival(weeks)	95% Confidence Interval (weeks)
β-hCG +ve	48	245	0-495
β-hCG –ve	55	294	161-427

Figure 7.2: The Kaplan-Meier plots demonstrating the differences in overall survival and recurrence free survival in patients with immunohistochemically HCG positive and HCG negative disease. The tables demonstrate the 5 year median overall survivals with confidence intervals. The log-rank test was applied to look for statistical difference between the survival curves and the p-value recorded.

7.2.2 The Effect of a Positive Serum β-HCG on Survival and Recurrence

It was postulated that a raised serum β-hCG might be an indicator for disease recurrence. The results were accessed from the electronic laboratory records. A serum β-HCG was measured in a subset of patients as part of their clinical follow-up. It is not a routine blood test in cystectomy patients; however, it is now commonly done in a non-systematic fashion. A total of 99 patients had at least one serum β-HCG measured after their radical cystectomy in this cohort. (figure 7.3)

It is possible for men and non-pregnant women to produce low levels of HCG from the pituitary.[214] Therefore it was necessary to define a raised or abnormal β-HCG. The hospital uses a Beckman-Coulter™ Access® Total β-hCG radioimmunoassay to measure serum β-hCG levels. The hospital had a reference normal population of 250 people where by 95% of the population had a serum β-hCG of <0.5 – 2 IU/L with a median value of 0.5, irrespective of gender. For the majority of the period studied the laboratory machines had a lower reference of 2IU/L. This based the rationale to dichotomize the results into levels greater than or equal to 2 IU/L considered as positive and less than 2 IU/L considered negative.

The median age of the patients at the time of cystectomy was 67 years and the male to female ratio was 3.3:1. Forty-seven patients had a serum level of 0 IU/L. Fifty-two patients had a reading with the range of values from 1.6 – 557 IU/L with a median value of 6 IU/L. Three patients were excluded from the data as they had either concomitant upper tract disease or died of metastatic disease within 30 days of their surgery. (figure 7.3)

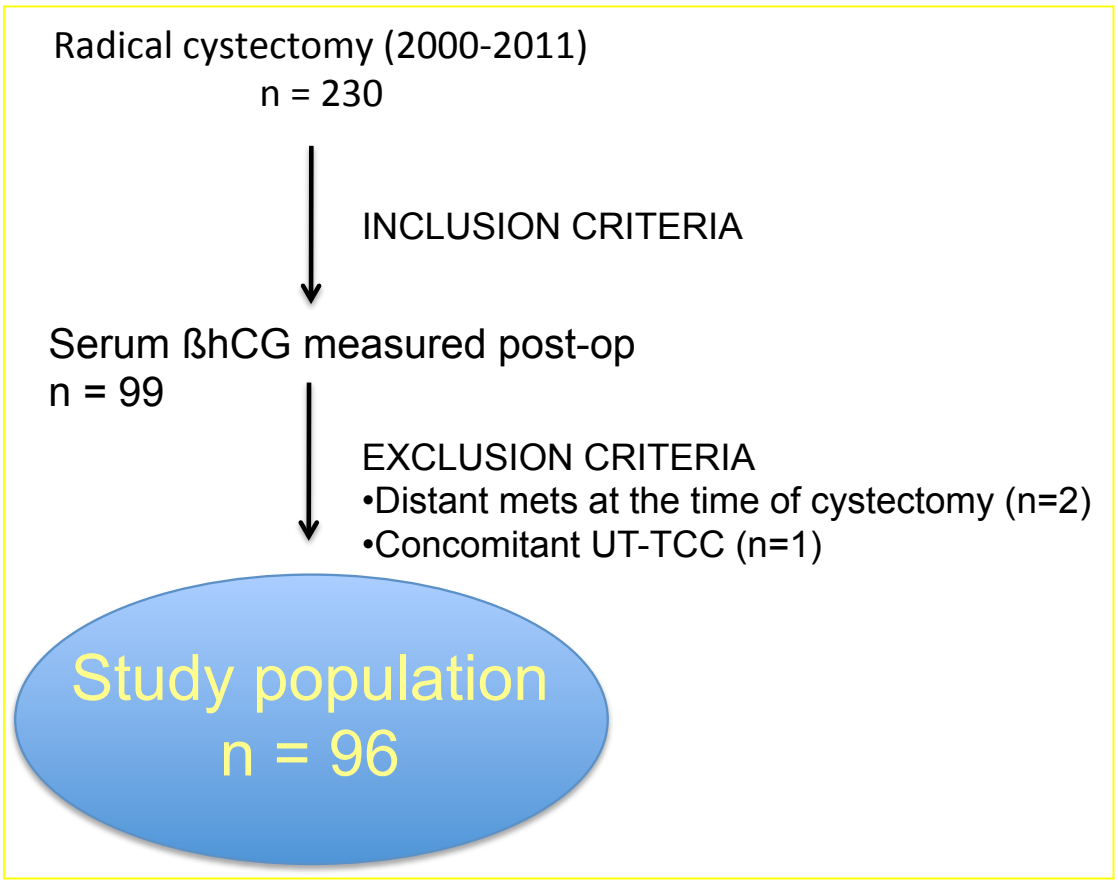


Figure 7.3: Schematic demonstrating the numbers of patients who had a serum β-hCG measured in the post-operative follow up period and who were included and excluded. Of the initial cohort 99 patients had a post operative β-hCG and 96 were included.

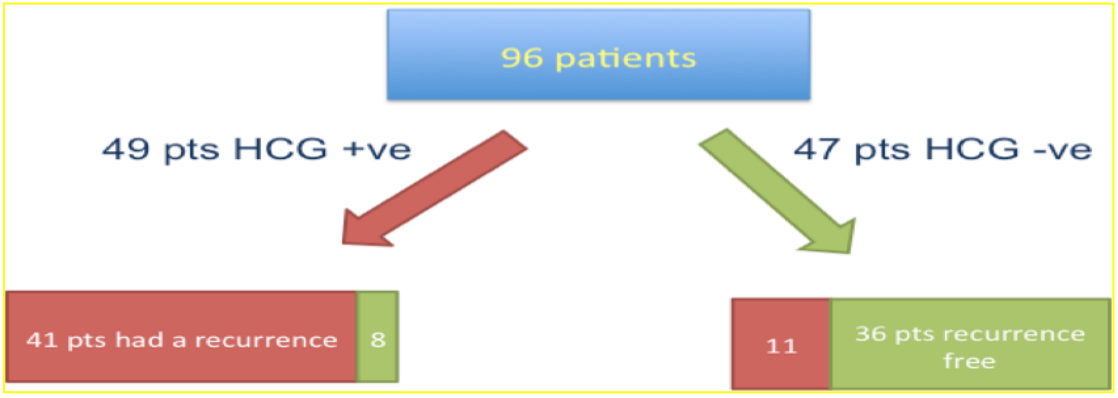


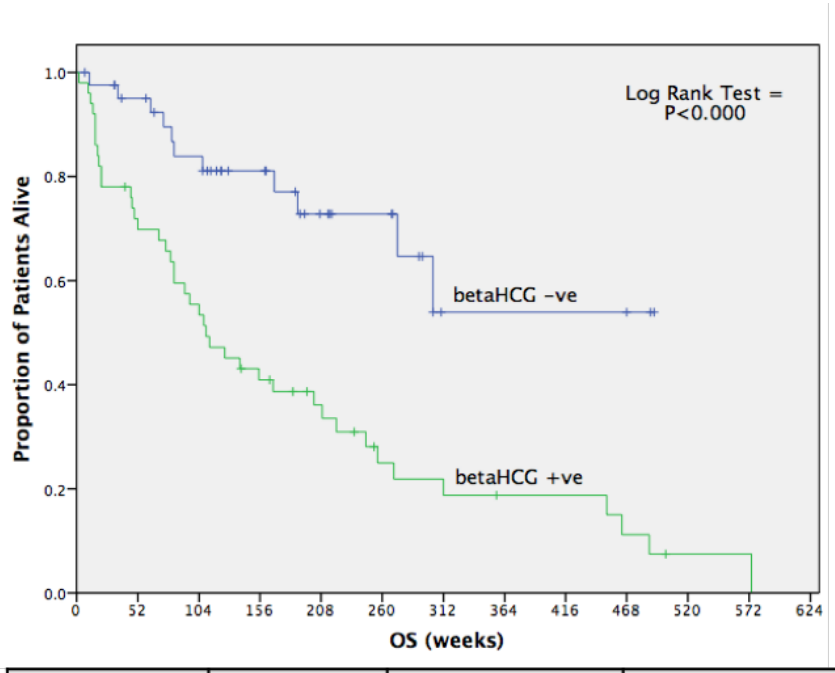
Figure 7.4 Schematic demonstrating the proportion of patients who subsequently develop a recurrence after positive and negative serum β-hCG tests. 41 of 49 patients with a raised β-hCG also had a recurrence compared to only 11 of the 47 patients who did not have a raised β-hCG.

It can be seen that the spread of different stages in the serum β-hCG group is reasonably similar to the remainder of the database implying that there is no major stage effect on HCG levels. (table 7.2)

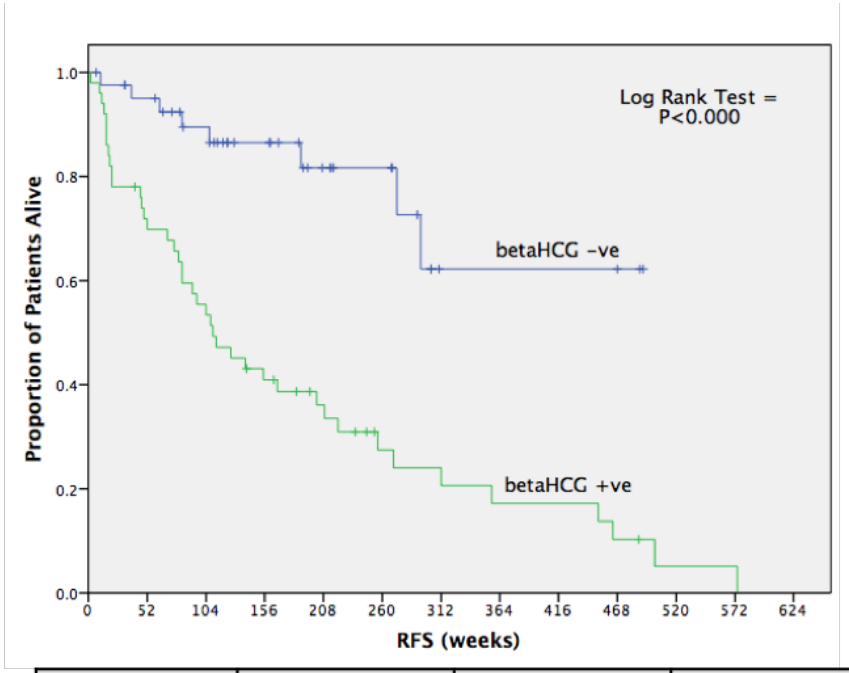
Stage	Number of Pts (%) Cystectomy group	Number of Pts (%) in HCG group
<T2	78 (35)	25 (18.2)
T2	36 (15.9)	18 (18.2)
T3	69 (30.5)	27 (27.3)
T4	11 (4.9)	9 (9.1)
N+	32 (14.2)	20 (20.2)

Table 7.2: Table to show the different stages of disease seen in the β-hCG positive patients compared to the overall relative spread of stages in the cystectomy group as a whole. Patients with a raised post operative β-hCG (taken at an arbitrary time point) appeared to be represented across all stages of disease with no indication that it was associated with patients with higher stages of disease at the time of cystectomy.

The overall survival was significantly better in the serum β-hCG negative group with a 5-year overall survival of 68% compared to only 25% in the serum β-hCG positive group. The same difference is observed in the recurrence free survival with 76% of the serum negative group recurrence free at 5-years compared to 27% in the positive group. (figure 7.5)



Variable	5yr overall survival (%)	Mean overall survival (weeks)	95% Confidence Interval (weeks)
Serum β-hCG +ve	25	183	131-234
Serum β-hCG –ve	68	325	256-394



Variable	5yr recurrence free survival (%)	Median recurrence free survival (weeks)	95% Confidence Interval (weeks)
Serum β-hCG +ve	27	110	70-150
Serum β-hCG –ve	76	501	NR

Figure 7.5: The Kaplan-Meier plots demonstrating the differences in overall survival and recurrence free survival in patients with serumβ-HCG positive and serum β-HCG negative disease. The tables demonstrate the 5 year median overall survivals with confidence intervals. The log-rank test was applied to look for statistical difference between the survival curves and the p-value recorded. Patients with a raised serum β-hCG had significantly worse OS and RFS.

A further subgroup of 86 patients from the database had representative cores that underwent staining for β-HCG and had subsequently had a serum raise recorded in follow up. 48% of the positively stained cores had a serum rise and 57% of the negatively stained cores had a rise. (table 7.3) Therefore, there was no relationship between serum levels of β-HCG and the original immunohistochemical expression on the TMA implying that β-HCG expression in the primary tumour does not predict whether a recurrence will be β-HCG secreting.

	IHC β-HCG +ve (%)	IHC β-HCG –ve (%)
Serum rise – no	52	43
Serum rise - yes	48	57

Table 7.3: table demonstrating the relationship between the percentage of patients who had a serum rise of β-HCG and their β-HCG staining characteristics on the tissue microarray.

7.2.3 Nrf-2 staining on TMA

It was postulated that Nrf-2 expression might predict survival in cystectomy patients or predict response to chemotherapy. The TMA was stained for Nrf-2 as described previously (see chapter 3.9).

A previously used scoring system for Nrf-2 was used.[215] This scoring system differentiated between nuclear and cytoplasmic staining and had two main components; the intensity of scoring and the percentage of the tumour stained. No staining = 0, weak = 1, moderate =2 and strong = 3. The extent of the staining expressed as a percentage and the two scores multiplied together to give a potential score of 0-300. For the purpose of this thesis it was decided that only moderate to strong staining should be considered as positive and this should be in at least 10% of the tumour sample. Therefore, the results were then dichotomized into positive (≥20) or negative (<20). (figure 7.6)

There were representative tissue cores for 177 patients, 57 (32%) of which stained positive for Nrf-2. (table 7.3) Of these patients only 3 had positive nuclear scoring and the remaining 54 had cytoplasmic scoring. There was no difference between these two groups and were considered together as one group. There was no difference in overall survival between positively and negatively stained groups. The median overall survival was 270 weeks in the Nrf-2 positive group and 221 weeks in the Nrf-2 negative group (P= 0.786). (7.7)

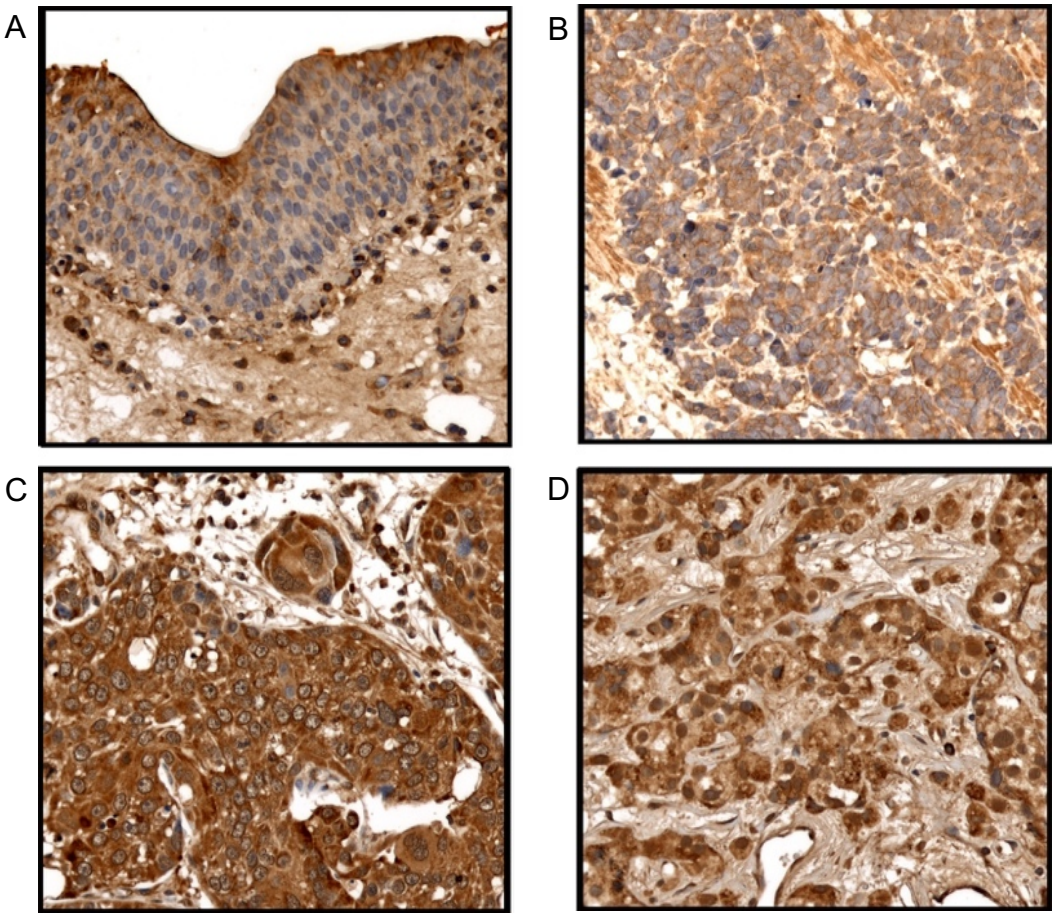


Figure 7.6: Representative immunohistochemical stains for Nrf-2 taken from cores on the TMA.

Variable	Total number (%)
Available Tissue Blocks	209
Missing / No Tumour Cores	32
Representative Tissue Cores	177 (100)
Nrf-2 +ve Cores	57 (32)
Nrf-2 –ve Cores	120 (68)

Table 7.3 Table to show the total number of available tissue blocks associated with the database and how many cores were left after staining that had either representative tumour or had not been washed off during Nrf2 staining.

Likewise, recurrence free survival was similar with a median recurrence free survival of 145 weeks in the positive group compared to 205 weeks in the negative group (P= 0.894). (figure 7.7, table 7.4)

However, Nrf-2 is implicated in cisplatin resistance and when looking at the 71 patients in the subset of this cohort who had neo-adjuvant (cisplatin based) chemotherapy, it can be seen that the patients who overexpressed Nrf-2 did significantly worse. The median overall and recurrence free survivals were 188 weeks and 117 weeks respectively in the Nrf-2 negative group compared to only 21 weeks for both in the Nrf-2 positive group. (figure 7.7, table 7.5))

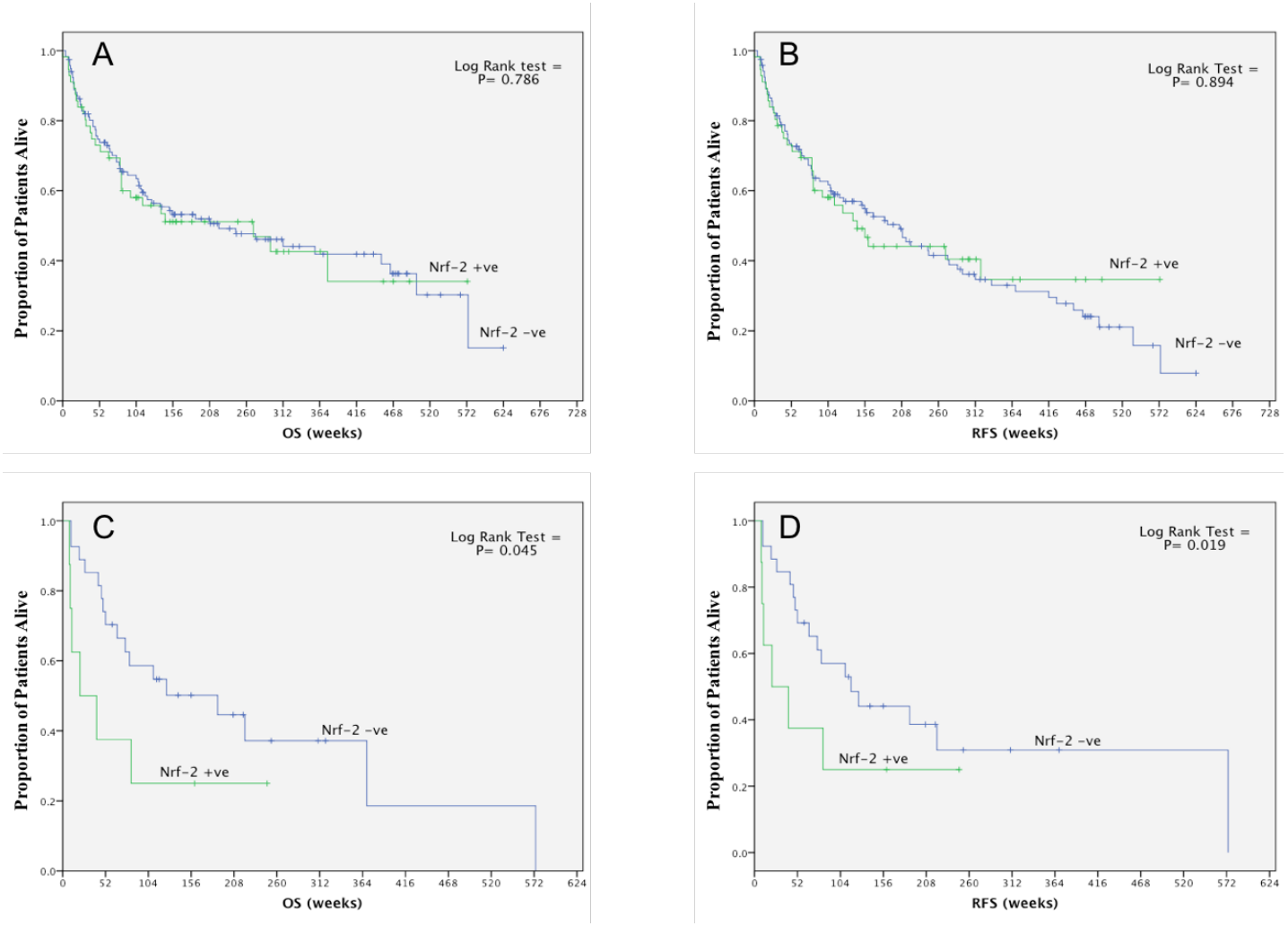


Figure 7.7: The Kaplan-Meier plots demonstrating the differences in overall survival and recurrence free survival in patients with Nrf-2 positive and Nrf-2 negative disease. Graphs A and B represent cystectomy patients who did not receive neo-adjuvant chemotherapy. Graphs C and D represent those who received neo-adjuvant chemotherapy. Patients who had neo-adjuvant chemotherapy had worse overall and recurrence free survival if they overexpressed Nrf-2. The log-rank test was applied to look for statistical difference between the survival curves and the p-value recorded

Variable	5yr overall survival (%)	Median overall survival (weeks)	95% Confidence interval (weeks)
Nrf-2 +ve	49	270	89-201
Nrf-2 -ve	49	221	148-262

Variable	5yr recurrence free survival (%)	Median recurrence free survival (weeks)	95% Confidence Interval (weeks)
Nrf-2 +ve	43	145	66-474
Nrf-2 –ve	41	205	69-373

*Table 7.4: Tables to demonstrate the 5 year overall survival and recurrence free survival as well as median overall and recurrence free survival between Nrf2 +ve and Nrf2 –ve tumours in patients who **did not** receive chemotherapy.*

Variable	5yr Overall Survival (%)	Median Overall Survival (weeks)	95% Confidence Interval (weeks)
Nrf-2 +ve	25	21	0-62
Nrf-2 –ve	38	188	35-341

Variable	5yr Recurrence Free Survival (%)	Median Recurrence Free Survival (weeks)	95% Confidence Interval (weeks)
Nrf-2 +ve	38	21	0-63
Nrf-2 –ve	56	117	48-186

*Table 7.5: Tables to demonstrate the 5 year overall survival and recurrence free survival as well as median overall and recurrence free survival between Nrf2 +ve and Nrf2 –ve tumours in patients who **did** receive chemotherapy.*

7.2.4 AIMP3 staining on TMA

Reduced expression of AIMP3 was shown to be predictive of resistance of bladder cancer to radiotherapy by Gurung et al. working at UCL. To attempt to validate this work the expression levels of AIMP3 were measured on the cystectomy TMA to look for an association with survival. The immunohistochemistry was performed at UCLs laboratory to minimize variation between their previous work. The TMA was then scored using their scoring system by myself and Dr P Gurung with any discrepancy discussed with pathologist Dr M Sommerlad. I uploaded the data onto my database and analysed the results.

AIMP3 demonstrated moderate to high expression in 51% of cases. This did not confer any benefit in terms of overall or recurrence free survival in patients treated with radical cystectomy. (figure 7.8) This is in contrast to their findings of a reduced survival in patients with absent AIMP3 expression in patients treated with radical radiotherapy.

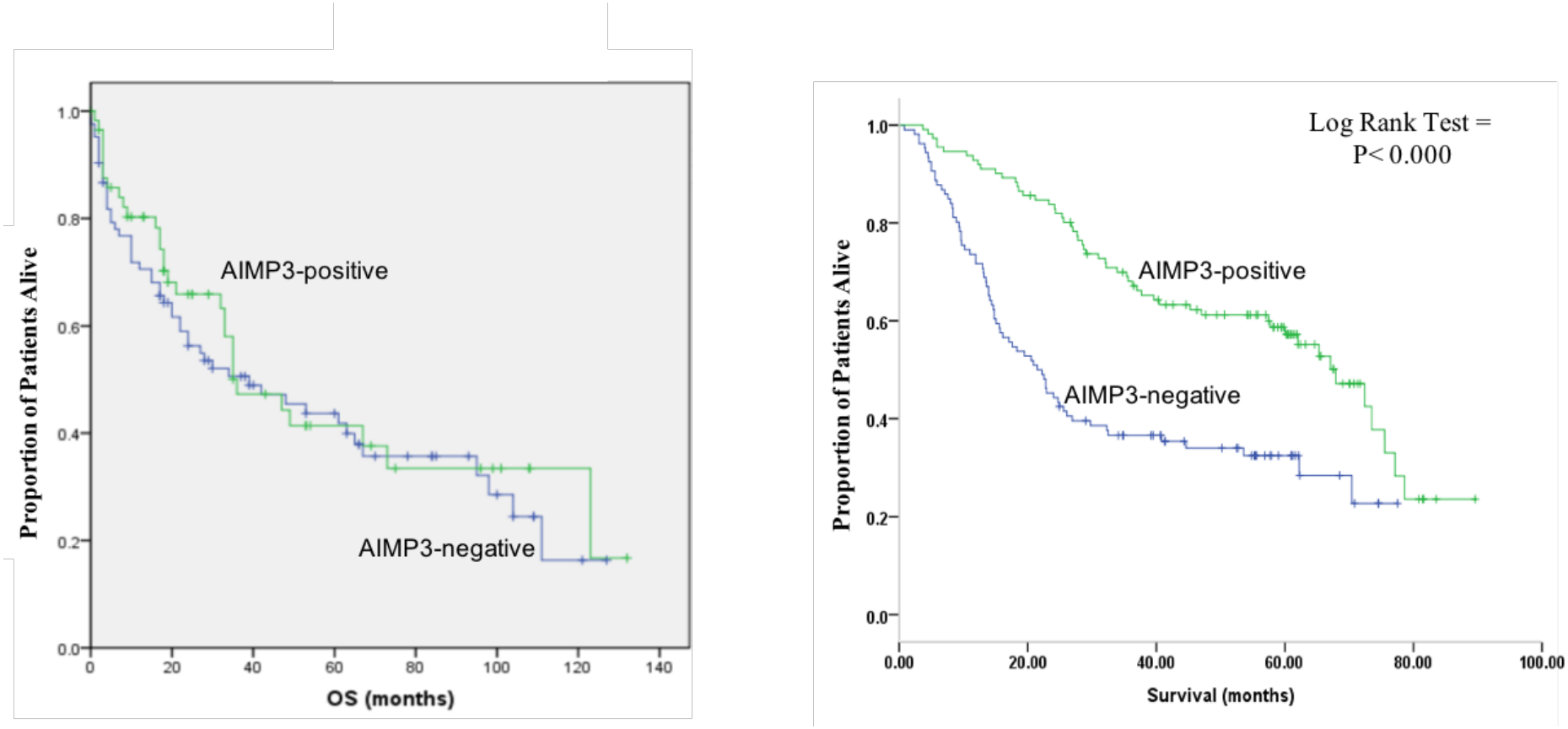


*Figure 7.8: The graph on the **left** shows the Kaplan-Meier plots demonstrating the differences in overall survival between patients with AIMP3 positive and negative disease when treated with radical **cystectomy**. The graph on the **right** shows the Kaplan-Meier plots demonstrating the differences in overall survival between patients with AIMP3 positive and negative disease when treated with radical **radiotherapy.** The log-rank test was applied to look for statistical difference between the survival curves and the p-value recorded. (modified with permission from published work by Gurung et al.)*

7.3 Discussion

The TMA and associated database proved themselves as a valuable resource for screening for potential biomarkers and demonstrated how it assists in translating laboratory findings into more clinically relevant results. β-hCG, Nrf-2 and AIMP3 all appear to be promising biomarkers and the results will now be discussed in more detail.

7.3.1 The role of immunohistochemical overexpression and serum levels of β-hCG in bladder cancer

Serum, urine and immunohistochemical β-hCG levels have been previously shown elevated in 30-76% of patients with bladder cancer.[148,216,217] There have been associations with increasing stage and grade as well as poor response to radiotherapy. [206] However, with a combination of the results from this thesis and a separate database of 235 transitional cell carcinoma patients who received chemotherapy from the same institution, we have demonstrated serum β-hCG to be an independent prognostic indicator for worse overall survival in patients receiving first line chemotherapy with a hazard ratio of 3.47. [147]

To look at the prognostic implications of immunohistochemical β-hCG expression the TMA was stained. Overall 20% of 190 patients had β-hCG overexpressing tumours but this did not have any impact on overall or recurrence free survival. The role for β-hCG in the host tumour is therefore not clear. It is possible that although biologically active the majority of the β-hCG

overexpressing tumours were fully excised and as a result failed to exert an influence, alternatively the sample size may be too small, tumour heterogeneity may have masked the driver mutation or there is genuine disconnect between expression levels and survival.

This is in stark contrast to the prognostic value of a raised serum β-hCG post operatively. In the post operative follow up period 97 patients who fitted the inclusion criteria had a serum β-hCG measured. Of the 49 patients who had a positive serum level, 41 had a confirmed recurrence of their disease. (figure 7.4) This would imply that a raised serum β-hCG has a sensitivity and specificity of 83.3% and 76.1% respectively, with a positive predictive value of 78.4% and a negative predictive value of 81.4% of disease recurrence. This implied that in the metastatic setting β-hCG was being secreted by the recurrent tumour and this was a remote process to the original immunohistochemical expression on the cystectomy specimen.

It is logical to assume that the cystectomy specimens that over-expressed β-hCG would belong to the same patients that subsequently secreted a serum β-hCG upon disease recurrence. However, this did not appear to be the case with only 48% of the patients' β-hCG overexpressing cystectomy specimens subsequently sustaining a serum rise. Equally 57% of the cystectomy specimens that did not overexpress β-hCG subsequently produced a serum β-hCG producing recurrence.

There are clearly a few obvious limitations to a retrospective cohort of patients that were not in any way randomized. Equally there was no control over which

patients received a post-operative serum β-hCG measurement or when it should occur. The clinician responsible for care of the patient made these decisions at the time. There has been an increase in the number of serum β-hCG measurements performed in this department on an ad hoc basis due to the cross-fertilization of ideas between the oncology and urology departments primarily at the weekly multi-disciplinary meeting.

For this study it must be made clear that the time point at which the serum β-hCG was measured varied significantly. This variability may reflect a clinician's suspicion of recurrence and this would significantly bias the results. Conversely, some patients had an ad hoc β-hCG performed early in their surveillance, which was negative, and subsequently relapsed without a further measurement. This will have limited the interpretation of results in the opposite direction if the there was a significant time lapse between the two events.

Despite the limitations the findings suggest an evolutional role for β-hCG in the survival mechanisms of bladder cancers. As previously mentioned some β-hCG negative tumours subsequently developed a secreting recurrence. It could be postulated that β-hCG's known roles in immunosuppression and angiogenesis may have been recruited by the recurrent tumour as one of the mechanisms for metastasis survival.

This data needs to be validated in a prospective randomized study, particularly as measuring it would be attractive due to the wide availability of serum β-hCG measurement in hospitals and its relative cheapness. All the patients had serum

levels measured after their radical surgery. In order to try and determine the prognostic value pre-operatively and establish the serum kinetics future studies should measure serum β-hCG upon first diagnosis, at the time of cystectomy and at each subsequent clinical follow up. As an individual case example there was one patient in this cohort who presented 3 years post surgery with a para-stomal hernia. He had a contrast CT scan for pre-operative planning that showed no sign of disease recurrence and also had an ad-hoc serum β-hCG measured at 6IU/L. At the next surveillance CT scan 6-months later he had developed a left pelvic sidewall nodal recurrence and his HCG had risen further to 11. It is possible therefore that serum β-hCG could conceivably have a role in follow up for bladder cancer similar to that in testis cancer although the effective treatment for these patients is not yet clear.

The relationship between immunohistochemical expression and serum levels needs to be established and future studies should aim to take serum levels at the time of surgery to allow for comparisons between immunohistochemical staining and serum levels. Similarly, they should aim to take tissue from metastatic lesions in patients with a raised serum β-hCG to be able to fully understand the relationship. Studies such as these need to be prospective, ideally randomized, correctly powered and to have tissue from primary and metastatic sites. Unfortunately, this is probably not possible and perhaps in the future it is where genomic and exomic profiling will help answer these type of questions.

This study has shown that β-hCG is overexpressed immunohistochemically in 20% of cystectomy patients and furthermore it has been shown that serum β-hCG is produced in a significant subset of patients requiring chemotherapy both with organ confined and metastatic disease.[147] With promising results of a preliminary phase 1 human trial, of a β-hCG vaccine for advanced epithelial malignancies[218], that demonstrated minimal side effects, it is possible that there may be personalized therapeutic treatments that could be developed for tumours that recruit β-hCG in the future.

7.3.2 The role of Nrf-2 as a biomarker in bladder cancer

As discussed previously cisplatin-based chemotherapy is the mainstay of treatment for patients with advanced bladder cancer. Resistance to cisplatin is a major clinical problem leading to rapid disease progression and death. Understanding how resistance to cisplatin occurs is vital to attempts to improve patients' survival. Nrf-2 has been identified as a transcription factor master regulator and controls many of the genes involved in oxidative stress and the expression of metallothioneins that regulate heavy metal metabolism (which would include cisplatin). Nrf-2 activation and mutations have been implicated in lung, pancreatic[215] and breast cancers.

As a result, it was hypothesized that Nrf-2 may have a role in cisplatin resistance in bladder cancer. Work performed in Dr Crabb's laboratory had shown that Nrf-2 was overexpressed in a bladder cancer cell line that were resistant to cisplatin

(RT112CP) whereas before resistance to cisplatin (RT112) it did not. Furthermore, when Nrf-2 levels were depleted with siRNA the sensitivity to cisplatin was partially restored.[151] Therefore, this interesting cell line data was taken forward by looking at the expression and prognostic significance in this TMA.

Nrf-2 staining in the TMA revealed that the majority of staining occurred in the cytoplasm and not at the nuclear level. The numbers were too small to be able to interpret this difference further but future studies with larger numbers may be able to see a difference. 32% of the available tissue cores stained positive and when comparing the positive and negative groups there was not an appreciable difference in either overall or recurrence free survival (figure 7.7). However, 71 patients had received cisplatin based neo-adjuvant chemotherapy. When interrogating this relatively small group it became apparent that patients who overexpressed Nrf-2 had a significantly worse overall and recurrence free survival (figure 7.7). This is particularly relevant as it was shown in chapter 4 (table 4.8) that patients who received neo-adjuvant chemotherapy were more likely to be down staged at the time of cystectomy implying a degree of chemo sensitivity for some bladder cancers. However, the finding that the Nrf-2 overexpressing tumours, in this subgroup of 71 neo-adjuvant chemotherapy patients, did significantly worse is compatible with a resistance to the cisplatin-based chemotherapy that they received. This is logical and fits with the original assumption and findings, from the cell line work performed by Dr Crabb's group, that Nrf-2 is associated with resistance to cisplatin chemotherapy.

It is important again to accept that these findings are based upon a retrospective clinical dataset and as such there are potential sources of bias and as a consequence these results need to be validated externally. The small subset of patients who received neo-adjuvant chemotherapy was not randomized and as such these results only add to the hypothesis that Nrf-2 contributes to cisplatin resistance and it is not possible to tell if it has a prognostic or predictive role as a biomarker. This data needs external validation, the difference in the findings between the chemotherapy and non-chemotherapy patients implies a possible predictive role and future wok will hopefully confirm this.

7.3.3 The role of AIMP3

Finally, the utility of a TMA to be used collaboratively was demonstrated with the work on AIMP3. This novel tumour suppressor, which works up stream of and has a role in regulation of P53, appears to be an important marker for response to radiotherapy. Mechanistically this is logical because low AIMP3 expressing cells have been demonstrated to exhibit reduced p53 activity and resistance to cell death[210,212]. Upon exposure to genotoxic stress (such as may occur with radiotherapy), AIMP3 directly interacts and activates the ataxia-telangectasia mutated (ATM) and ataxia-telangectasia and Rad3-related (ATR) tumour suppressing kinases in the nucleus, subsequently resulting in p53 up regulation. Hence, suppression of AIMP3 accompanies the down regulation of ATM although a feedback mechanism seems to link ATM and AIMP3[210-212,219]. Gurung et al. were also able to induce partial resistance to radiotherapy by knocking down AIMP3 in bladder cancer cell lines.

Although it appears that AIMP3 may be predictive of response to radiotherapy it is important to know if down regulation of AIMP3 has a prognostic role in muscle invasive bladder cancer patients who were not treated with radiotherapy. We were able to demonstrate that despite a high number of patients (49%) exhibiting down regulation of AIMP3 this did not appear to give any prognostic information of outcome. This re-enforces the specificity of AIMP3 as a predictive marker for response to radiotherapy.

To summarise, the TMA and associated database were created with the aim of finding novel biomarkers that may help improve the clinical outcomes of patients with bladder cancer. This utility has now been demonstrated and although the above work needs validating all three of the above biomarkers have the potential to be used directly in clinical practice. We have published the results from the β-hCG, Nrf-2 and AIMP3 work (see appendices). With further well-designed prospective studies serum β-hCG could have a potential use as a marker of recurrence post cystectomy. Nrf-2 may reveal further insights into cisplatin resistance mechanisms as well as perhaps being part of a signature predictive of response to chemotherapy and finally AIMP3 may be used to help screen which tumours may potentially be treated with radiotherapy.

8. Summary and Conclusions

There has been little improvement in the overall survival of patients with muscle invasive bladder cancer over the last 50 years. The TMA sample set in this thesis provides an accurate representation of survival rates for the disease when treated by radical cystectomy, with a 5-year overall survival of only 48.8% and a 5-year disease specific survival of 62.1%. This reflects the findings of most real world clinical series. Despite a much greater understanding of the tumour biology we have been unable to translate this into useful clinical treatment strategies with better, targeted drugs. One way to try and improve these disappointing survival figures is to find methods of personalising therapies, targeted at biological pathways specific to the individual cancer and to do this we need better model systems to probe the biology. Here I have developed two useful tools to aid the transition; a TMA and the first bladder cancer organotypic model utilizing primary bladder cancer cells.

Tissue micro-array is an effective tool to rapidly process large amounts of data on a specific marker to see if it is prognostic or predictive within a particular cohort of patients. The majority of large histological datasets in bladder cancer have looked at patients of all stages and grades. It is known that invasive and non-invasive bladder cancers not only have dramatically different outcomes but also have different genetic alterations.[27] It was therefore logical to look at just the patients with invasive disease so as to avoid bias from potentially very different tumours. For this thesis a TMA was constructed for patients who underwent radical cystectomy linked to the clinical outcomes from the associated database. This TMA was primarily created for biomarker discovery both for this project and for future studies. HER2 and its family members are an attractive target in bladder cancer and as such were screened on the TMA to look for their role as predictors of survival.

A TMA of 209 cystectomy patients is considered to be of reasonable size and compares favorably with other bladder TMAs available. There is always an attrition rate in the number of representative cores due to the processing of the slides but this TMA held up well with tissue consistently available for over 175 patients. When looking at the expression rates of the HER family it was reassuring that the rates were very similar to previous published work. For example an overexpression rate of 38.5% for EGFR sits in the middle of most published papers.[196,215] This goes someway to validate the quality of the TMA.

The hypothesis of this thesis was that HER2 overexpression is predictive of survival and recurrence in patients with bladder cancer requiring radical cystectomy. Previous studies have shown conflicting data and it was postulated that in a pure cystectomy dataset the effect might be more apparent. In keeping with previous literature (table 1.2) 17% of samples overexpressed HER2 and although there was a strong trend for worse overall and recurrence free survival there were insufficient numbers to show significance (figures 6.2, 6.3). However, upon multi-variate analysis overexpression of HER2 stood up as an independent risk factor with a hazard ratio of 1.66 (P=0.048) (table 6.3).

Differences in immunohistochemical scoring systems applied in biomarker expression experiments are one reason for a lack of agreement in results between different published papers. With regards the HER family there is still not a universally agreed scoring system. However, the HercepTest^{R} is the agreed validated score used in every day clinical practice with regards breast cancer and as such is now becoming the most

commonly used test in bladder cancer research also. For this reason, the HercepTest^{R} score was used on this TMA for HER2 but also, due to the lack of any good validated alternative, for the other family members. This was a reasonable decision however, highlights the need to validate these results in external data sets.

The other members of the HER family have a vital role in co-dimerization and also have potential roles as biomarkers. It was therefore also important to stain for them on the TMA. As expected EGFR was overexpressed with increasing stage of disease but despite a strong trend it was not possible to show significance for worsening survival (figure 6.2). It was however, surprising to see that HER4 was predictive of worse overall and recurrence free survival (figures 6.2, 6.3). The small amount of previous literature has suggested a protective role of HER4 when co-expressed with EGFR or HER2.[112,197,198] The findings in this TMA are contradictory to this and after multivariate analysis HER4 overexpression was an independent risk factor with a hazard ratio of 1.61 (P=0.031) (table 6.3). Little is known about the biology of HER4 and we know little about its dimerising characteristics and how it may have a role in cell transformation. This finding needs to be validated in an external cohort and then should be subject to further research for its role in bladder cancer.

It is known that the HER family members co-dimerise or homo-dimerise to affect their intra-cellular response but unfortunately the numbers of patients that overexpressed more than one was too small to allow any reliable interpretation. It is not possible to draw any conclusions from this as the HercepTest ^{R} scoring system used was chosen as a validated score for protein overexpression, however, the intensity that a co-dimerizing HER partner would produce is not known and may have

been dismissed on this analysis as a negative score. Furthermore, the score used had no way of quantitating homo-dimerising patterns that are known to occur particularly with HER2. To look at co-dimerising patterns, an alternative technique such as confocal microscopy may well be better suited but this may be difficult to achieve with a TMA.

The number of patients overexpressing EGFR and or HER2 was looked at as this would be a specific cohort of patients that would be suitable for targeted treatment with a dual EGFR/HER2 tyrosine kinase inhibitor such as that used currently in some breast cancer patients with advanced disease. A significant number of patients would be suitable for such a therapy with over 46% of patients fitting the criteria. Moreover, as a group of patients they do significantly worse (figure 6.4) and are therefore positioned to benefit from such an adjuvant or neo-adjuvant treatment strategy. This has already being investigated in the metastatic setting by the recently reported LAMB study where patients with advanced disease who had failed first line chemotherapy were stratified according to their EGFR/HER2 expression and given the targeted therapy Lapatinib.[158] The unexpected finding was that administration of Lapatinib did not improve overall survival although it was well tolerated. Although disappointing there are several possible reasons why this could be the case and anti-EGFR/HER2 therapies should not be dismissed. The patient cohort in the LAMB study had very advanced, end stage disease with a high tumour burden. The patients were stratified primarily from EGFR/HER2 expression from a single biopsy and this may simply be inadequate due to tumour heterogeneity (both locally and remotely). The problem here is a concern for all potential personalised therapies. As cancers evolve they become invasive and develop a mesenchymal phenotype that can,

subsequently, be seen to reverse back to an epithelial phenotype in metastatic sites. Different genetic alterations can be seen from separate areas within a primary and likewise between a primary and a metastatic tumour[194]. This level of tumour heterogeneity indicates that although a tumour may overexpress EGFR/HER2 in particular biopsy sample, when considered as part of the whole tumour burden, it may be nothing more than a small mutation that is in no way driving the tumour in its current state of evolution. For targeted bladder cancer treatments however, it still remains possible that at a much earlier stage of disease, targeting EGFR/HER2 may be biologically much more effective in a subset of patients and still needs further study. In the future the ability to see the 'whole picture' of a tumour and get over some of the problems associated with tumour heterogeneity may be overcome with the advent of genomics, however, genome and exome sequencing will not always identify all the proteins that have been translated, due to post translational modifications and methylations, and as such a tissue microarray will always be valuable to confirm expression levels of biomarkers in a real clinical data-set.

It is agreed that overexpression of HER2 in patients with bladder cancer, correlates with increased stage and grade of disease. In contrast to most studies this TMA has looked at just cystectomy patients and has now found it to be an independent risk factor for worse overall survival (table 6.3). It is not clear how or if HER2 creates this outcome. Working on the hypothesis that HER2 may be critical for invasion an experiment was designed to look at the effect of HER2 on invasion in a cell line.

Traditional in-vitro cell line work lacks the 3D cell to cell and cell to stroma interaction that is becoming increasingly recognised as an essential factor in cancer cell behavior. This may be one of the reasons that it can be hard to translate laboratory-based findings into clinically relevant results. By far the best intermediary model between cell line and human studies is the genetically engineered mouse model but this has specific hurdles, both ethical and financial, that limit its availability. To try and bridge the gap between in-vitro and in-vivo studies I have optimised a novel bladder organotypic adapted from a squamous cell carcinoma model.

In this thesis the novel 3D organotypic model was used for the first time in bladder cancer to recreate the invasive characteristics of the cell line used. A primary muscle invasive cell line was successfully cultured from a patient at the time of radical cystectomy and it was possible to recreate invasion within the organotypic. As part of the optimisation of the model it was confirmed that the type of fibroblast used was essential to recreating accurate invasion.

It has been previously demonstrated in other cancers such as in oesophageal, skin and breast, that cancer-associated fibroblasts or myofibroblasts are associated with tumourigenesis and invasion and can induce what is known as epithelial to mesenchymal transition (EMT). The transformation of fibroblasts into tumour-associated myofibroblasts is partially controlled by autocrine signaling via transforming growth factor β (TGF-β).[186] In order to investigate the integral relationship between the stroma and the invasive cell lines two different fibroblast cell lines were used. Hff-2 fibroblasts are known to readily undergo differentiation into myofibroblasts whereas MRC-5 fibroblasts do not. When using the MRC-5 fibroblast

line it was not possible to induce invasion whereas invasion was rapidly apparent with the HFF-2 fibroblast line. This crucial role for myofibroblasts has not been previously investigated or shown before in a bladder organotypic. Future work in this area should aim to confirm these findings using other cell lines and fibroblast types, ideally using primary fibroblasts from normal urothelium as well as from bladder cancer stroma. An understanding of how the stroma can induce invasion may have great clinical relevance, as it would potentially allow differentiation between the aggressive non-muscle invasive cell types (G3pTa/1/Tis). If there is a cancer environment that would progress these tumours, ultimately, into muscle invasive disease then earlier aggressive therapies, such as radical cystectomy could be offered in this cohort of patients.

The organotypic was used as the experimental model to look at the role of HER2 in invasion. The T24 cell line that was shown to overexpress HER2 was grown on the organotypic, exhibiting widespread invasion (figure 5.3). A second T24 cell line that had been lenti-virally transfected to reduce HER2 levels by approximately 80% was also grown on the organotypic (figure 5.12). This second HER2-deficient cell line had virtually the same invasive characteristics as the normal cell line. A reduced HER2 expression was confirmed by staining the TMA (figure 5.10). These results imply that HER2 is not essential for invasion in this cell line.

In retrospect, it was too simplistic and optimistic to expect that simply knocking down HER2 would prevent invasion. The process of invasion is not understood and involves many complex interactions causing different types of cellular movement including amoeboid, collective and mesenchymal motility. There are interactions

between the cancer cells, which may undergo EMT, and the stromal environment. Pathways may be created by metalloproteases for invasive cells to follow and chemokines have been associated with various tumour types. However, various genes have been associated with invasiveness and within this the HER family have been firmly implicated.[220]

HER2 activation leads to a complex series of downstream pathways ultimately leading to transformation of the cell and plays a critical role in the regulation of tissue development and growth. The pathways are influenced by the pattern of HER family dimerization and interconnecting inhibitory or excitatory pathways such as mTOR and PTEN. Exactly how HER2 may cause cell invasion is not known. It is likely that there will be many causative factors in invasion and HER2 is unlikely to be more than just one of the involved mechanisms. The T24 cell line may be invasive for reasons completely unrelated to HER2 but this does not mean that other invasive cell lines will not utilise HER2 in the invasive process.

The TMA has proved to be a useful tool for studying the HER family and its utility was further demonstrated by studying other putative markers that had been discovered in this database or with local collaborations. The data-set identified a group of patients who had had a post-operative serum β-HCG measured. If a patient had a positive serum β-HCG they had a 78.4% chance of disease recurrence and 81.4% chance of remaining disease free if the serum level was negative (figure 7.4). In keeping with this, patients had a significantly worse overall and recurrence free survival if they had a positive serum level. These results are subject to significant potential bias as it was a retrospective cohort with no control over

who did and did not get a blood test. The value of serum β-HCG was further re-enforced with a simultaneous study in Dr Crabb's group that demonstrated the prognostic role of serum β-HCG in bladder cancer patients undergoing chemotherapy. Taking these results together are convincing and require future validation in a prospective study.

To look at the relationship between β-HCG serum levels and immunohistochemical staining the TMA was interrogated. Over expression of β-HCG occurred in some 20% of patients but this had no association with stage of disease or survival. There were patients who did not overexpress β-HCG in their cystectomy specimens but subsequently developed a β-HCG producing tumour recurrence and equally the reverse was true. This implies that β-HCG may have a role in the survival of metastases but not necessarily in their initiation. As part of the evolutionary development of a metastatic deposit, in order for it to survive outside the host tumour, it needs to recruit mechanisms to feed and protect itself against the body's natural defences. The ability of β-HCG to encourage angiogenesis and induce immunosuppression may attract many solid tumours to recruit it to enable metastasis survival. Future work in this area could be exciting as there are promising β-HCG vaccines on the horizon that have proved to be well tolerated in early human trials. It will be important to try and harvest representative tissue from a metastatic lesion to try and elucidate the relationship between serum and immunohistochemical levels.

When discussing personalisation of therapies in bladder cancer it is mainly with the aim of determining the correct chemotherapeutic agent for the individual

cancer. Until we develop new, targeted agents the mainstay of chemotherapy for urothelial cancers is centred on cisplatin with resistance to cisplatin being a major problem. It was promising therefore, when cell line work in this laboratory demonstrated high Nrf-2 levels (a transcription factor master regulator) in a cisplatin resistant cell line that were not present in the same cell line prior to resistance. The sensitivity to cisplatin could be partially restored by depleting Nrf-2 levels in the resistant cell line. To see if this was relevant in clinical samples Nrf-2 staining was therefore performed on the TMA. A significant number of patients (38%) had a Nrf-2 expressing tumour but overall this did not predict outcome. However, when looking at the subset of patients who received cisplatin based neo-adjuvant chemotherapy it predicted a worse survival outcome in the patients who overexpressed Nrf-2. (figure 7.7) This fits with the in-vitro work that Nrf-2 confers resistance to cisplatin. There is much to learn in understanding the biological role of Nrf-2, how it connects with other pathways and the importance of its regulation by KEAP-1. Furthermore, this data has the limitation that it has been performed in a retrospective cohort and needs future validation in a prospective study but it provides promising new avenues of research into understanding the resistance mechanisms to cisplatin.

Further personalisation of therapies would include determining the correct radical therapy whether that is surgical excision or a bladder preservation approach as could be offered with external beam radiation. The concern with this approach is that a significant number of invasive bladder cancers are not cured and local recurrence from radiotherapy resistant tumours is one common event. The novel tumour suppressor AIMP3 has been identified by the research

group at University College London and loss of expression appears to be predictive of resistance to ionising radiation. My TMA has proved valuable, in aiding their work through collaboration, to help show the potential for this biomarker. Further validation work needs to be performed and further insights into the biology are needed but we have progressed towards translating the application of AIMP3 as a predictive biomarker.

To conclude it can be stated that this TMA has confirmed the hypothesis that HER2 is an independent prognostic marker in patients with bladder cancer treated with radical cystectomy and as such should be targeted in future prospective studies. I was unable to show that HER2 was critical for cell invasion. The unexpected finding of HER4 as a prognostic factor may prove it to be the "Cinderella" of the HER family.

The organotypic model has proved its utility for studying invasion in bladder cancer. It is the first bladder cancer model of this type and will provide a useful intermediate step between 2D cell culture and animal models. The TMA is a highly valuable tool and will be used repeatedly in future biomarkers studies as demonstrated with its application for Nrf-2 and AIMP3.

Appendices

Appendix 1: Variables recorded in database

ID

hospitalnumber

pathnumber

NRF2CorN

Nrf2Cyst

NrF2cystYn

Nrf2Bx

NrF2LN

NrF2Hi

P4

P3

P2

HER4ln

HER4CYST

HER4bx

HER4ynhighest

HER4cysyn

HER2HER4yn

HER3ln

HER3Cys

HER3bx

HER3ynhighest

HER3cysyn

bag1NucBx

nbb

nbc

BAG1nuclearCys

nCysB

nCysC

BAG1cytoplasmicCys

ccysb

ccysc

BAG1cytoBX

cbxb

cbxc

EGFRcyst

EGFRBx

EGFRLn

AIMP3

EGFRhigh

AIMP3EGFR

HCGCYST

HCGbx

HCGln

HCGIHC

blank2

Sex

hcgCODE

dead

RecurrenceYN

Lastseen

Alivenodiseasedate

date_death

DeathBCYN

Presentation

Date_diag

Date_muscleinvasion

Date_cystectomy

Age_cys

BCG

Chemo

Date_chemo_diag

Date_chemo

date_chemofinished

Cyclesofchem

Treatment_cat

Prior_RT

G_1st

T_1st

CIS_1st

G_treatmentdecission

T_treatmentdecission

N_treatmentdecission

CIS_treatmentdecission

G_cyst

T_cyst

N_cyst

CIS_cyst

TCYSThigh

preopstage

Downstaged

Thigh

Tcis

LNdissection

LengthofopMIN

Intraopcompl

Bloodlossmls

BloodTf

Ileus

Hospstay

postop_infec

definepostopcomplafterdischarge

ASA

Precreat

postcreat

Lastcreat

preopeGFR

posteGFR

lasteGFR

Hist_cat

Squam_diff

PreopImaging

Dateofrecurrence

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Hydroneph

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HCGserumriseyn

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HER2CYST

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HER2lnYN

SerumHCGyn

Appendix 2: Associated Publications

FULL PAPER

BJC

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Keywords: hCGβ; chemotherapy; cystectomy; prognosis; transitional cell carcinoma; urothelial carcinoma

Serum total hCGβ level is an independent prognostic factor in transitional cell carcinoma of the urothelial tract

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Background: Serum total human chorionic gonadotrophin β subunit (hCGβ) level might have prognostic value in urothelial transitional cell carcinoma (TCC) but has not been investigated for independence from other prognostic variables.

Methods: We utilised a clinical database of patients receiving chemotherapy between 2005 and 2011 for urothelial TCC and an independent cohort of radical cystectomy patients for validation purposes. Prognostic variables were tested by univariate Kaplan–Meier analyses and log-rank tests. Statistically significant variables were then assessed by multivariate Cox regression. Total hCGβ level was dichotomised at $<$ *vs* $\geqslant 2\,\mathrm{IU\,l^{-1}}$.

Results: A total of 235 chemotherapy patients were eligible. For neoadjuvant chemotherapy, established prognostic factors including low ECOG performance status, normal haemoglobin, lower T stage and suitability for cisplatin-based chemotherapy were associated with favourable survival in univariate analyses. In addition, low hCGβ level was favourable when assessed either before (median survival not reached *vs* 1.86 years, $P=0.001$) or on completion of chemotherapy (4.27 *vs* 0.42 years, $P=0.000002$). This was confirmed in multivariate analyses and in patients receiving first- and second-line palliative chemotherapy, and in a radical cystectomy validation set.

Conclusions: Serum total hCGβ level is an independent prognostic factor in patients receiving chemotherapy for urothelial TCC in both curative and palliative settings.

Approximately 10 000 new bladder cancers are diagnosed annually in the United Kingdom and over 90% are transitional cell carcinomas (TCC) (Crabb and Wheater, 2010). Perioperative cisplatin-based chemotherapy provides a 5–6% absolute survival advantage for operable muscle invasive bladder TCC and a modest survival gain in metastatic disease (which may include TCC occurring in other parts of the urothelial tract) (Logothetis *et al*, 1990; von der Maase *et al*, 2000; Sternberg *et al*, 2001; Grossman *et al*, 2003; von der Maase *et al*, 2005; Advanced Bladder Cancer (ABC) Meta-analysis Collaboration, 2005a, b; Crabb and Wheater, 2010; International Collaboration of Trialists *et al*, 2011). Improved prognostic characterisation to facilitate stratification for treatment would be valuable.

Various prognostic factors are established for urothelial tract TCC on treatment with chemotherapy. In the neoadjuvant setting (bladder TCC), favourable prognostic factors are pathological complete response in those undergoing cystectomy and lower T stage (Grossman *et al*, 2003). In advanced disease, performance

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status and disease extent are key prognostic factors (Mead *et al*, 1998; Bajorin *et al*, 1999). Bajorin *et al* (1999) described a retrospective cohort where Karnofsky performance status $<80\%$ and visceral metastases were independent poor prognostic factors. In a phase III trial comparing cisplatin-based regimens, good prognostic factors included Karnofsky performance status $>70\%$, no M1 disease, low/normal alkaline phosphatase, $\leqslant 3$ disease sites and no visceral metastases (von der Maase *et al*, 2005). Predictive biomarker development for TCC has been unsuccessful to date (Stadler *et al*, 2011). Various prognostic or predictive molecular characterisation models in retrospective cohorts have been proposed warranting prospective validation of their potential for therapy selection (Dyrskjot *et al*, 2003; Takata *et al*, 2005; Sanchez-Carbayo *et al*, 2006; Mengual *et al*, 2009; Mitra *et al*, 2009; Smith *et al*, 2011).

Human chorionic gonadotrophin (hCG) is a heterodimeric glycoprotein secreted by trophoblastic cells during gestation with placental, uterine and fetal regulatory roles. The α subunit is common to hCG, LH, FSH and TSH, whereas the β subunit (hCGβ) is distinct to the variant hCG forms. Serum hCGβ levels are a key tumour marker for trophoblastic and germ cell cancers. In addition, hCGβ is elevated in various solid epithelial malignancies, including TCC, with links in some to poor prognosis (Iles, 2007).

We hypothesised that total hCGβ level would function as an independent prognostic marker in patients undergoing chemotherapy for urothelial TCC and report data to demonstrate this.

MATERIALS AND METHODS

Patients and data collection. We undertook retrospective analysis of consecutive patients treated with systemic chemotherapy for invasive urothelial tract cancer at University Hospital Southampton NHS Foundation Trust, UK, between 2005 and 2011. Eligibility criteria were age $\geqslant 18$, confirmed pure or mixed histology TCC, and muscle invasive disease and/or nodal or metastatic spread (staged T_{2-4} and/or N_{1-3} and/or M_1) at first use of chemotherapy. Data collection was through retrospective case note review with data lock on 5 January, 2013. An independent validation set of patients undergoing radical cystectomy for bladder TCC but not perioperative chemotherapy was also created with data lock of 2 August, 2013.

Patients receiving chemotherapy or surgery were managed by oncologists and urologists with specialist interests in urothelial cancer and consistent with regionally approved treatment guidelines. The treating institution undertook specialist multidisciplinary review of all diagnostic and staging investigations for all patients.

Chemotherapy analyses were undertaken in three prospectively defined patient cohorts. The 'neoadjuvant cohort' received chemotherapy before either radical surgery or radiotherapy with curative intent for disease staged T_{2-4} N_0 M_0. The 'first line cohort' either received chemotherapy for newly diagnosed disease staged T_{any} N_{1-3} M_0 or T_{any} N_{any} M_1 or previously received perioperative (adjuvant or neoadjuvant) chemotherapy and were then subsequently treated again at disease relapse. The 'second line cohort' comprised all patients from the first-line cohort treated with subsequent chemotherapy at disease progression.

This research had UK National Research Ethics Service committee approval (10/H0405/99).

Statistical analyses. Overall survival was from the first day of the relevant course of chemotherapy, or the date of cystectomy in the validation set, to death. Progression free survival (first- and second-line cohorts) was from the first day of the relevant course of chemotherapy to disease progression or death from any cause. Relapse free survival (RFS) was from the first day of chemotherapy (neoadjuvant cohort), or cystectomy (validation set), to the first local, regional or distant recurrence or death from any cause. Statistical analysis was performed with SPSS, version 20.0 (IBM, Portsmouth, UK). Univariate analyses of survival outcomes were by the Kaplan–Meier method and log-rank tests. Statistically significant prognostic factors in univariate overall survival analyses were included in multivariate Cox regression analyses to determine hazard ratios as previously described (Crabb *et al*, 2008a, b). *P* values <0.05 were considered statistically significant.

hCGβ measurement. Total hCGβ levels in serum samples were determined by an accredited UK National Health Service chemical pathology department using a quantitative chemiluminescent immunoassay on a Beckman Coulter DxI immunoassay system (product number 33500, Beckman Coulter, High Wycombe, UK). Blood samples used were those taken as part of routine clinical practice before and immediately following a course of chemotherapy. We prospectively dichotomised hCGβ levels at $<$ *vs* $\geqslant 2\,\mathrm{IU\,l^{-1}}$. hCG$\beta$ levels were the most recent before initiation, or the first following completion, of a course of chemotherapy within a 28-day window. Levels outside these time constraints were excluded.

RESULTS

Chemotherapy cohorts and hCGβ levels. A total of 244 patients received chemotherapy for urothelial TCC between 2005 and 2011, of whom 235 met the inclusion criteria (Figure 1). A total of 92 and 149 received chemotherapy within the neoadjuvant and first-line cohorts, respectively. A total of 16 patients received adjuvant chemotherapy following radical cystectomy. A total of 14 and 8 patients receiving neoadjuvant or adjuvant chemotherapy, respectively, were also treated within the first-line cohort at disease relapse. A total of 63 patients had second-line chemotherapy.

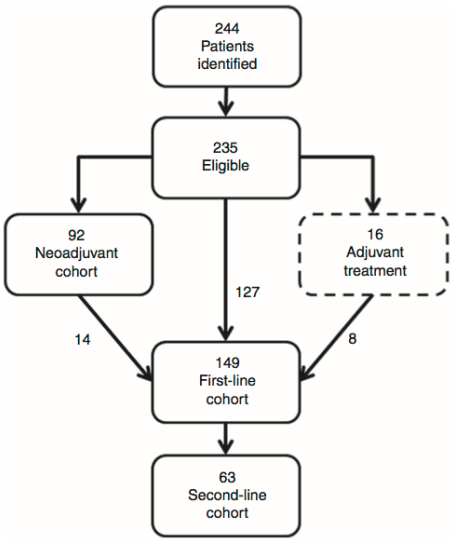

Figure 1. Flow diagram for chemotherapy cohorts used in this study.

Table 1 shows clinico-pathological characteristics and hCGβ levels before, and on completion of, chemotherapy. In the neoadjuvant cohort, 90.2% received gemcitabine/cisplatin (GC) and the rest gemcitabine/carboplatin (GCarbo). For the first-line cohort corresponding figures were 51% and 33.6%, respectively, with the remainder mostly receiving gemcitabine alone due to poor performance status. hCGβ level, where available, was $<2\,IU\,l^{-1}$ before neoadjuvant, first-line and second-line chemotherapy in 68%, 44% and 35%, respectively. The percentage with low ($<2\,IU\,l^{-1}$) hCGβ levels fell with respect to line of treatment with a higher proportion with more advanced disease presenting with intermediate (2 to $<10\,IU\,l^{-1}$) or higher ($\geqslant 10\,IU\,l^{-1}$) levels (Figure 2).

Neoadjuvant chemotherapy. We assessed potential prognostic factors with regard to overall survival following neoadjuvant chemotherapy (Table 2). Favourable factors in univariate analyses for survival were ECOG performance status (0–1 *vs* ⩾2), haemoglobin (⩾ lower limit of normal, LLN), lower T stage (⩽2 *vs* 3 *vs* 4) and suitability for GC (*vs* GCarbo). In addition, hCGβ level $<2\,IU\,l^{-1}$ before neoadjuvant chemotherapy was associated with improved survival (median survival not reached *vs* 1.86 years, $P=0.001$, Figure 3A). Likewise, hCGβ level $<2\,IU\,l^{-1}$ following neoadjuvant chemotherapy was also associated with favourable median survival (4.27 *vs* 0.42 years, $P=0.000002$, Figure 3B).

We undertook multivariate analyses for overall survival incorporating factors reaching statistical significance in univariate analyses including hCGβ level either before, or on completion of, neoadjuvant chemotherapy. hCGβ level in each model remained a statistically significant factor (hazard ratios (HR) 3.41, 95% confidence interval (CI) 1.49–7.83, $P=0.004$ and 15.36, 95% CI 2.13–110.65, $P=0.007$, respectively), along with haemoglobin level in the first model (Table 3).

In addition, low hCGβ level in the neoadjuvant cohort, assessed before chemotherapy was associated with RFS of 7.38 *vs* 1.45 years, but this was not statistically significant ($P=0.07$). However, low hCGβ level on completion of neoadjuvant chemotherapy was associated with RFS of 7.37 *vs* 0.51 years, $P=0.0003$.

First-line chemotherapy. For first-line chemotherapy, favourable ECOG performance status, normal serum alkaline phosphatase (⩽ULN), absence of visceral metastases, receipt of GC (*vs* other regimens), absence of Bajorin risk factors (Bajorin *et al*, 1999) and no prior perioperative chemotherapy were each associated with longer survival in univariate analyses (Table 2).

In addition, hCGβ level $<2\,IU\,l^{-1}$ before, or on completion of, chemotherapy was associated with improved survival (median 1.53 *vs* 0.86 years, $P=0.04$ and 1.68 *vs* 0.84 years, $P=0.00005$, respectively, Table 2, Figure 3C and D).

We undertook multivariate models for overall survival including ECOG performance status and presence of visceral metastases as separate factors (and so omitting the Bajorin index). hCGβ level on completion of first-line chemotherapy remained a statistically significant prognostic factor (HR 3.47, 95% CI 1.97–6.10, $P=0.00002$, Table 4) along with performance status and the presence of visceral metastases. It did not retain statistical significance for hCGβ levels taken before chemotherapy ($P=0.25$, Table 4). We also assessed the impact of hCGβ level on progression free survival and found low levels to be associated with improved outcomes in univariate analysis both before, and on completion of, chemotherapy (0.86 *vs* 0.64 years, $P=0.03$ and 0.86 *vs* 0.50 years, $P=0.0004$, respectively).

Second-line chemotherapy. In patients receiving second-line chemotherapy, low hCGβ level on completion of chemotherapy was associated with improved survival (median 1.78 *vs* 0.29 years, $P=0.003$), but not with levels before chemotherapy ($P=0.3$).

Validation cystectomy cohort. Finally we assessed hCGβ level in an independent sample set following radical cystectomy, but without chemotherapy, (Supplementary Table 1) and found that low levels were associated with improved overall survival (median not reached *vs* 2.18 years, $P=0.002$, Figure 4) and RFS (median not reached *vs* 0.87 years, $P=0.00002$).

DISCUSSION

Chemotherapy for muscle invasive TCC improves cure rates in combination with radical treatment options (Grossman *et al*, 2003; Advanced Bladder Cancer (ABC) Meta-analysis Collaboration, 2005a, b; International Collaboration of Trialists *et al*, 2011) and extends survival in metastatic disease (von der Maase *et al*, 2000, 2005). However, outcomes are poor following disease relapse or progression. We sought to extend the prognostic information available on initiation, or completion, of chemotherapy. In bladder cancer/TCC, previous studies indicate elevated hCGβ levels of 30–76% in serum, 35–73% in urine and 35% by immunohistochemistry, and possible associations to grade, stage and survival (Moutzouris *et al*, 1993; Iles, 2007). hCGβ-expressing TCC appears to act in a biologically aggressive manner with poor survival outcomes, increased risk of disease relapse and poor radiotherapy response (Martin *et al*, 1989; Marcillac *et al*, 1993; Moutzouris *et al*, 1993; Dobrowolski *et al*, 1994; Iles, 2007). Cook *et al* (2000) investigated hCGβ level within a tumour marker panel including carcino-embryonic antigen, CA125 and CA19.9, and response to chemotherapy in advanced bladder cancer. Neither clinical response nor survival differed between marker-negative and marker-positive patients, however clinical response was strongly related to marker response. Only 19 patients (24%) were evaluable for hCGβ response and so its relevance in this cohort remains uncertain (Cook *et al*, 2000). Urinary total hCG levels were found to be elevated in a subgroup of patients referred for cystoscopy who were found to have bladder cancer but none of those with benign conditions. It was a poor prognostic factor in those with muscle invasive disease (Iles *et al*, 1996).

Our work establishes raised total hCGβ level as a poor prognostic factor in chemotherapy-treated urothelial TCC and confirms independence from other established prognostic factors. To our knowledge this is the first time this has been demonstrated for a malignancy other than testis/germ cell cancer. We also demonstrated its association with a poor prognosis in patients who undergo cystectomy as a first step towards validation of hCGβ as a prognostic factor. We would now propose prospective validation in a chemotherapy-treated group of patients before clinical utilisation. Such development would be attractive as hCGβ level is routinely available to clinicians as a relatively cheap, commercially available, validated clinical test with known performance characteristics. Thus the path to establishing this biomarker for use in TCC may be less tortuous than other options. It is important to note that our assessment of hCGβ level utilised an automated, routine, clinically available immunoassay to detect total hCGβ. This is, in essence, a surrogate measurement of the free hCGβ presumed to be expressed by the tumour but will have included all forms of hCG present including intact, free, nicked and glycosylated forms. Future work should look to dissect the relevance of these using assays available with the sensitivity and specificity to do so (Cole and Butler, 2012).

Strengths of our study include that it represents a complete set of sequentially treated patients according to common management criteria, with all patients treated where clinically appropriate with cisplatin-based chemotherapy. These are 'real world' data however (which we view as a strength) and so also include those unfit for cisplatin-based regimens who are frequently omitted from research in this disease despite representing 40–50% of the population.

Table 1. Patient characteristics in the chemotherapy cohorts

	Neoadjuvant chemotherapy cohort, $n=92$	First-line chemotherapy cohort, $n=149$
Age		
Median	69	69
Range	48–84	34–92
⩽70	55 (59.8%)	77 (51.7%)
>70	37 (40.2%)	72 (48.3%)
Sex		
Male	65 (70.7%)	109 (73.2%)
Female	27 (29.3%)	40 (26.8%)
ECOG PS		
0 or 1	84 (91.3%)	106 (71.1%)
⩾2	5 (5.4%)	28 (18.8%)
Unknown	3 (3.3%)	15 (10.1%)
Hb		
Median ($g\,l^{-1}$)	135	125
Range	94–177	80–170
⩾LLN	61 (66.3%)	69 (46.3%
<LLN	29 (31.5%)	74 (49.7%)
Unknown	2 (2.2%)	6 (4.0%)
ALP		
Median (Ul^{-1})	88	104
Range	36–342	38–1181
⩽ULN	79 (85.9%)	96 (64.4%)
>ULN	9 (9.8%)	43 (28.9%)
Unknown	4 (4.3%)	10 (6.7%)
LDH		
Median ($IU\,l^{-1}$)	429	439
Range	321–703	272–2115
⩽ULN	21 (22.8%)	42 (28.2%)
>ULN	8 (8.7%)	23 (15.4%)
Unknown	63 (68.5%)	84 (56.4%)
Grade		
2	10 (10.9%)	14 (9.4%)
3	81 (88.0%)	122 (81.9%)
Unknown	1 (1.1%)	13 (8.7%)
Primary tumour site		
Bladder	92 (100%)	111 (74.5%)
Renal pelvis	—	22 (14.8%)
Ureteric	—	13 (8.7%)
Urethral	—	3 (2.0%)
T stage		
⩽2	44 (47.8%)	40 (26.8%
3	35 (38.0%)	48 (32.2%)
4	13 (14.1%)	16 (10.7%)
X	0	43 (28.9%)
N stage		
0	92 (100%)	70 (47.0%)
1	—	24 (16.1%)
2	—	54 (36.2%)
3	—	1 (0.7%)
M stage		
0	92 (100%)	62 (41.6%)
1	—	87 (58.4%)
Visceral metastases		
No	—	95 (63.8%)
Yes	—	54 (36.2%)
Bajorin risk factors (Bajorin *et al*, 1999)		
0	—	70 (47.0%)
1	—	47 (31.5%)
2	—	17 (11.4%)
Unknown	—	15 (10%)
Chemotherapy regimen		
GC	83 (90.2%)	76 (51.0%)
GCarbo	9 (9.8%)	50 (33.6%)
Other	0	23 (15.4%)
Prior chemotherapy		
No	—	127 (85.2%)
Yes	—	22 (14.8%)
Radical local therapy		
Surgery	48 (52.2%)	10 (3.4%)
Radiotherapy	28 (30.4%)	5 (6.7)
None	14 (15.2%)	134 (89.9%)
Unknown	2 (2.2%)	0
hCGβ level before chemotherapy		
$<2\,IU\,l^{-1}$	58 (63.0%)	61 (40.9%)
$\geqslant 2\,IU\,l^{-1}$	27 (29.3%)	77 (51.7%)
Unknown	7 (7.6%)	11 (7.4%)
hCGβ level on completion of chemotherapy		
$<2\,IU\,l^{-1}$	28 (30.4%)	56 (37.6%)
$\geqslant 2\,IU\,l^{-1}$	5 (5.4%)	40 (26.8%)
Unknown	59 (64.1%)	53 (35.6%)

Abbreviations: ALP = alkaline phosphatase; ECOG PS = Eastern Cooperative Oncology Group performance status; GC = gemcitabine/cisplatin; GCarbo = gemcitabine/carboplatin; Hb = haemoglobin; LDH = lactate dehydrogenase; LLN = lower limit of normal for the treating institution's reference range; ULN = upper limit of normal for the treating institution's reference range.

Thus our results are relevant to current standard-of-care management for this disease rather than the rarefied world of a clinical trial.

A number of questions arise from our data. We demonstrated a prognostic impact for hCGβ levels at completion of, as well as before, chemotherapy. This is despite higher numbers where hCGβ level was not recorded (64.1% neoadjuvant, 35.6% metastatic) which we acknowledge holds potential for bias. Thus there might be a role for hCGβ level as a predictive biomarker for chemotherapy benefit, either as an absolute level before, or following, chemotherapy, or in a dynamic sense as hCGβ 'normalisation' during treatment. One could also consider what hCGβ dynamics might imply for required duration or type of chemotherapy or if subsequent hCGβ rise might be utilised to detect disease recurrence/relapse. Anecdotally we have experience

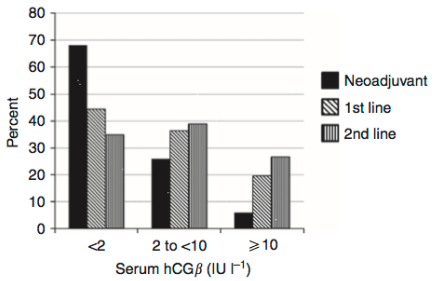

Figure 2. Total hCGβ levels before chemotherapy for patients undergoing neoadjuvant, first-line or second-line chemotherapy.

of the latter representing early indication of disease activity. These are hypotheses however and should be tested prospectively. It would also be of interest to investigate the relevance of hCGβ level in other settings, for example in non-muscle invasive disease to test risk for relapse and progression. In this, and the neoadjuvant/adjuvant chemotherapy settings, the question of whether a raised hCGβ level reflects those with micro-metastases arises and warrants further prospective work, possibly with comparison with experimental imaging methodologies. A further question is the relevance of hCGβ expression in the primary tumour which we are investigating in our radical cystectomy cohort.

Our work raises the question of the biological role of hCGβ in TCC. hCGβ may have a functional role in cancer progression as a transforming growth factor, an immunosuppressive agent, an inducer of metastasis or as an angiogenic factor (Iles, 2007; Cole, 2010). hCGβ, but not intact hCG, hCGα or hCGβ core fragment, stimulated TCC cell line growth which could be inhibited by hCGβ

Table 2. Univariate analyses to assess individual potential prognostic factors with respect to overall survival following chemotherapy

		Neoadjuvant chemotherapy		First-line chemotherapy	
Factor	**Division**	**Median OS, years (95% CI)**	***P*-value**	**Median OS, years (95% CI)**	***P*-value**
Age	⩽70 >70	NR 3.36 (0.91–5.81)	0.08	1.13 (0.66–1.61) 0.98 (0.68–1.30)	0.21
Sex	Male Female	5.26 (4.37–6.15) 4.05 (0.88–7.23)	0.49	1.16 (0.79–1.53) 1.02 (0.71–1.33)	0.84
ECOG PS	0 or 1 ⩾2	5.26 (4.52–6.01) 0.42 (0.33–0.50)	0.000008	1.49 (1.08–1.90) 0.63 (0.47–0.79)	0.0000005
Hb	⩾LLN <LLN	NR 1.22 (0.94–1.51)	0.00005	1.39 (0.97–1.82) 0.96 (0.69–1.24)	0.15
ALP	⩽ULN >ULN	NR 1.14 (0.87–1.41)	0.08	1.35 (1.05–1.65) 0.66 (0.52–0.80)	0.003
LDH	⩽ULN >ULN	4.27 (0.10–8.44) NR	0.34	1.23 (0.80–1.66) 1.04 (0.53–1.55)	0.58
Grade	2 3	4.05 (2.71–5.40) 5.26 (4.49–6.03)	0.90	1.02 (0.44–1.90) 1.14 (0.87–1.41)	0.87
Primary tumour site	Bladder Other	— —		1.07 (0.78–1.35) 1.07 (0.66–1.49)	0.57
T stage	⩽2 3 4	NR 5.27 (4.15–6.39) 1.09 (0.61–1.57)	0.006	— — —	
Visceral metastases	No Yes	— —		1.49 (1.03–1.95) 0.78 (0.67–0.88)	0.004
Chemotherapy regimen	GC GCarbo Other	NR 0.89 (0.48–1.31) —	0.001	1.49 (1.05–1.93) 0.80 (0.41–1.20) 0.57 (0.40–0.74)	0.00005
Bajorin risk factors (Bajorin *et al*, 1999)	0 1 2	— — —		1.74 (1.41–2.08) 0.98 (0.78–1.17) 0.47 (0.31–0.65)	0.0000001
Prior perioperative chemotherapy	No Yes	— —		1.15 (0.90–1.41) 0.60 (0.50–0.70)	0.001
hCGβ level before chemotherapy	<2 IU l^{-1} ⩾2 IU l^{-1}	NR 1.86 (0.51–3.21)	0.001	1.53 (1.17–1.89) 0.86 (0.67–1.05)	0.04
hCGβ level on completion of chemotherapy	<2 IU l^{-1} ⩾2 IU l^{-1}	4.27 (1.65–6.89) 0.42 (0.14–0.70)	0.000002	1.68 (1.25–2.11) 0.84 (0.68–1.00)	0.00005

Abbreviations: ALP = alkaline phosphatase; CI = confidence interval; ECOG PS = Eastern Cooperative Oncology Group performance status; GC = gemcitabine/cisplatin; GCarbo = gemcitabine/carboplatin; Hb = haemoglobin; LDH = lactate dehydrogenase; LLN = lower limit of normal for the treating institution's reference range; NR = not reached; ULN = upper limit of normal for the treating institution's reference range.

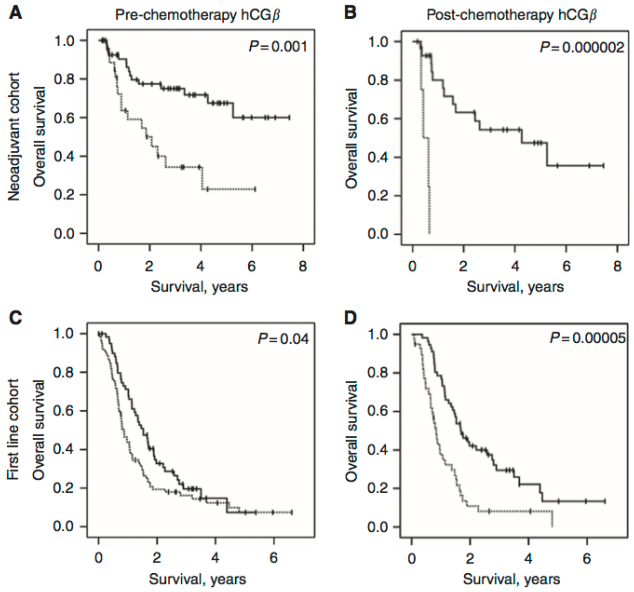

Figure 3. Kaplan–Meier plots to show overall survival according to total hCGβ level in the neoadjuvant (**A**, **B**) or first-line (**C**, **D**) chemotherapy cohorts either before (**A**, **C**), or on completion of (**B**, **D**) chemotherapy. Broken line – hCGβ level ⩾2; continuous line – hCGβ level <2.

Table 3. Multivariate analyses of potential prognostic factors for overall survival in patients undergoing neoadjuvant chemotherapy incorporating hCGβ level either before, or on completion of, chemotherapy

Factor	HR	95% CI	*P*-value	HR	95% CI	*P*-value
ECOG PS						
⩾2 *vs* 0 or 1	2.10	0.41–10.89	0.38	2.14	0.31–14.95	0.44
Hb						
<LLN *vs* ⩾LLN	3.56	1.69–7.46	0.001	1.17	0.25–5.45	0.84
Chemotherapy regimen						
GCarbo *vs* GC	1.48	0.40–5.42	0.56	1.79	0.42–7.57	0.43
T stage						
T3 *vs* T2 T4 *vs* T2	1.29 1.76	0.55–3.00 0.47–6.61	0.56 0.40	0.98 0.66	0.23–4.15 0.07–6.04	0.98 0.71
hCGβ level before chemotherapy						
⩾2 *vs* <2	3.41	1.49–7.83	0.004	—	—	—
hCGβ level on completion of chemotherapy						
⩾2 *vs* <2	—	—	—	15.36	2.13–110.7	0.007

Abbreviations: CI = confidence interval; ECOG PS = Eastern Cooperative Oncology Group performance status; GC = gemcitabine/cisplatin; GCarbo = gemcitabine/carboplatin; Hb = haemoglobin; HR = hazard ratio; LLN = lower limit of normal for the treating institution's reference range.

antibodies (Gillott *et al*, 1996). There is some evidence to suggest that, in part, hCGβ might act in TCC, and potentially other malignancies, by inhibition of TGFβ-induced apoptosis by virtue of their structural homology (reviewed by Iles (Iles, 2007)). Whether these putative biological mechanisms are relevant, or if hCGβ simply acts as a surrogate for poorly differentiated, biologically aggressive disease remains uncertain and further investigation of the biological role in TCC is warranted. hCGβ

Table 4. Multivariate analyses of potential prognostic factors for overall survival in patients undergoing first-line chemotherapy incorporating hCGβ level either before, or on completion of, chemotherapy

Factor	HR	95% CI	*P*-value	HR	95% CI	*P*-value
ECOG PS						
⩾2 *vs* 0 or 1	2.39	1.38–4.13	0.002	2.09	1.01–4.33	0.047
ALP						
<LLN *vs* ⩾LLN	0.98	0.59–1.60	0.92	1.44	0.78–2.68	0.24
Visceral metastases						
Yes *vs* no	2.01	1.29–3.13	0.002	2.65	1.49–4.70	0.001
Chemotherapy regimen						
GCarbo *vs* GC	0.83	0.83–0.66	0.11	1.86	0.52–6.66	0.34
Prior perioperative chemotherapy						
Yes *vs* no	2.16	0.81–5.79	0.82	1.07	0.60–6.66	0.34
hCGβ level before chemotherapy						
⩾2 *vs* <2 IU l^{-1}	1.28	0.84–1.96	0.25	—	—	—
hCGβ level on completion of chemotherapy						
⩾2 *vs* <2 IU l^{-1}	—	—	—	3.47	1.97–6.10	0.00002

Abbreviations: ALP = alkaline phosphatase; CI = confidence interval; ECOG PS = Eastern Cooperative Oncology Group performance status; GC = gemcitabine/cisplatin; GCarbo = gemcitabine/carboplatin; HR = hazard ratio; LLN = lower limit of normal for the treating institution's reference range.

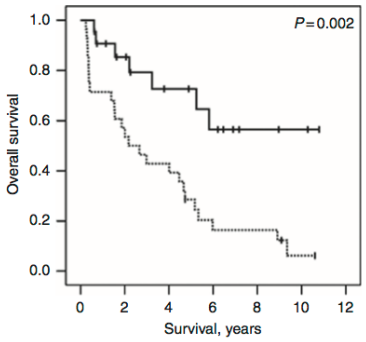

Figure 4. Kaplan–Meier plot to show overall survival according to total hCGβ level in a radical cystectomy cohort. Broken line – hCGβ level ⩾2; continuous line – hCGβ level <2.

might also represent a therapeutic target with vaccination strategies currently under development (Delves *et al*, 2007).

We utilised a prospectively defined hCGβ cut point of < *vs* ⩾2 IU l^{-1}. This was in part pragmatic as, during the period in question, this was the lower level of quantification at our institution. It would be of interest to undertake future analysis either of other cut points to optimise a dichotomous variable or to analyse as a continuous variable.

Certain limitations of our data exist. First, these were retrospective analyses. Second, patients were included on the basis of receipt of chemotherapy for urothelial TCC. The study therefore holds bias for those suitable to commence chemotherapy and our patient cohorts are somewhat heterogeneous, which future prospective validation should seek to address and control for. Our validation cohort was a cystectomy-treated group. We chose this primarily for pragmatic reasons as, to our knowledge and after some effort to find an alternative, there is no current sample set available of chemotherapy-treated patients with hCGβ data available. Prospective validation in both treatment settings is therefore now required and warranted. Finally our cohort was not randomised to treatment and so we cannot establish whether a predictive factor role for hCGβ levels exists from this sample set.

In conclusion, serum hCGβ level is an independent prognostic factor for outcome in patients undergoing chemotherapy for TCC of the urothelial tract. Prospective validation is warranted to determine its value for patient stratification in this disease.

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Original article

The Nrf2 transcription factor contributes to resistance to cisplatin in bladder cancer

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Abstract

Objectives: Cisplatin is the key systemic chemotherapeutic agent used for bladder cancer, but chemoresistance is a major clinical problem. The transcription factor nuclear factor erythroid 2–related factor 2 (Nrf2) regulates various critical cellular processes, including cellular antioxidant response, cellular detoxification, and drug uptake/efflux. These processes, and the expression of multiple Nrf2 target genes, have been found to be associated with bladder cancer prognosis and chemotherapy resistance. We, therefore, investigated whether Nrf2 might regulate cisplatin resistance in bladder cancer.

Materials and methods: We first used bladder cancer cell lines, including a cisplatin-resistant RT112 subline (RT112-CP), to investigate Nrf2 expression and activation and its association with cisplatin response. We then undertook immunohistochemical analysis of a tissue microarray of archival bladder cancer radical cystectomy specimens to test the relevance of clinical Nrf2 expression to outcomes following either neoadjuvant chemotherapy and cystectomy or cystectomy alone.

Results: Bladder cancer cell lines showed variable Nrf2 expression. Nrf2 expression was greater in RT112-CP cisplatin-resistant cells compared with that in parental RT112 cells. Nrf2 overexpression was functional in this model as it was associated with increased antioxidant response element reporter construct activity, Nrf2 target gene expression (metallothionein and glutathione reductase), and basal glutathione levels. Cisplatin resistance was associated with Nrf2 expression, and in RT112-CP cells, its depletion partially restored cisplatin sensitivity. We demonstrated increased cytoplasmic or nuclear Nrf2 expression or both in 32% of clinical bladder cancer samples compared with that in normal tissue samples. Expression of Nrf2 in bladder cancer following radical cystectomy was associated with unfavorable overall (median = 0.65 vs. 2.11 y, $P = 0.045$), bladder cancer–specific, and recurrence-free survival in those patients who also received neoadjuvant cisplatin-based chemotherapy but not in those treated with cystectomy alone.

Conclusions: Nrf2 overexpression in bladder cancer is associated with clinically relevant cisplatin resistance that is reversible in experimental models and should now be tested in prospective studies.

Keywords: Keap1; Nrf2; Cisplatin; Bladder cancer; Chemotherapy; Resistance

1. Introduction

Transitional cell carcinoma of the bladder causes approximately 5,000 deaths annually in the United Kingdom. Cisplatin-based chemotherapy provides a survival advantage in both the perioperative curative setting and advanced disease [1–4]. However, cisplatin resistance is a major clinical problem.

In bladder cancer, various mediators for antioxidant response, cellular detoxification, and drug uptake/efflux are linked either directly to cisplatin resistance or to clinical parameters. For example, pretreatment glutathione (GSH) level correlated negatively with response to cisplatin-based chemotherapy [5]. Various GSH (e.g., GSH reductase [GR] and γ-glutamylcysteine synthetase) and antioxidant

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mediators (e.g., GSH peroxidase and superoxide dismutase) are overexpressed in bladder cancer samples than in normal urothelium, correlating with grade and invasiveness [6]. Expression of metallothioneins, which regulate physiological and xenobiotic heavy metal metabolism, correlates with poor prognosis in bladder cancer and cisplatin resistance in cell lines [7,8]. Expression of heme oxygenase 1, an inducible heme-degradation enzyme involved in response to stressors including heavy metals and oxidative stress, in bladder cancer correlates to increased grade and chemotherapy resistance. Heme oxygenase 1 depletion or inhibition restored chemotherapy responsiveness in bladder cancer cells [9,10]. Increased expression of multidrug resistance–associated proteins (e.g., MDR1 and MRP1/3) occurs in bladder cancer with correlation to poor outcome and chemotherapy resistance [11,12]. In cisplatin-resistance models, raised GSH and GSH S-transferase expression occurred and GSH depletion or GSH S-transferase inhibition allowed resensitization [13]. Other resistance models exhibited high thioredoxin levels with resensitization on thioredoxin depletion [14]. Despite data linking clinical or experimental cisplatin resistance, or poor bladder cancer clinical features, to mediators of the cellular oxidative/electrophilic stress response, evidence for a central common regulatory mechanism remains elusive.

Nuclear factor erythroid 2–related factor 2 (Nrf2) is a transcription factor master regulator of 100 to 200 target genes controlling cellular responses to oxidative/electrophilic stress. Nrf2 transcriptional targets include GSH mediators (e.g., GR and γ-glutamylcysteine synthetase), antioxidants (e.g., GSH peroxidase, superoxide dismutase, metallothionein, thioredoxin, and thioredoxin reductase), and efflux pumps (e.g., MDR1 and MRP1/3). Other targets include growth factors, transcription factors, chaperones, protein-degradation mediators, and phase I and II detoxification enzymes. Therefore, Nrf2 is a critical regulator of many diverse cellular mediators of oxidative/electrophilic response linked to cisplatin resistance in bladder cancer but has not been investigated in this disease [15].

Nuclear Nrf2 binds antioxidant response elements (ARE) within target gene promoters [15]. Activation and nuclear translocation following oxidative/electrophilic stress are partially understood. Kelchlike ECH-associated protein 1 (Keap1) is a critical negative regulator of Nrf2 and facilitates Nrf2 ubiquitylation and proteosomal degradation by the cullin 3/Rbx1 ubiquitin ligase complex. Oxidative/electrophilic stress stabilizes Nrf2 partly through inhibitory oxidation or covalent modification of Keap1. Other influences on Nrf2 activation include nuclear export signal modulation and kinase activation.

Nrf2 activation appears beneficial for carcinogen detoxification in normal cells. However, in some cancers it may induce chemoresistance [16–18]. Nrf2-activating mutations occur in some cancers; however, none were observed in 29 bladder cancer samples [15,19]. Keap1-inactivating mutations (e.g., lung, biliary tract, and breast cancers) [17,20,21] and epigenetic suppression through promoter hypermethylation (e.g., lung cancer) also occur [22]. Neither has been investigated in bladder cancer. In lung cancer cells, Nrf2 forced activation-induced chemoresistance, whereas depletion, or Keap1 overexpression, enhanced sensitivity [17,18].

We hypothesized that aberrant Nrf2 activation in bladder cancer might drive a unified mechanism for cisplatin resistance. We present data to indicate increased Nrf2 protein expression in bladder cancer associated with cisplatin resistance.

2. Materials and methods

2.1. Reagents and cell lines

Antibodies (goat polyclonal anti-Nrf2, goat polyclonal anti-Keap1, rabbit polyclonal anti-GR-1, rabbit polyclonal antimetallothionein, and mouse monoclonal anti-HSC70) were obtained from Santa Cruz Biotechnology (Texas, US). Horseradish peroxidase–conjugated secondary antibodies were obtained from GE Healthcare (UK). Wild-type and mutant ARE luciferase plasmids and cisplatin-resistant RT112 subline (RT112-CP) cells were contributed by Jeffrey Johnson, University of Wisconsin, and John Masters, University College London, respectively [23]. Cell lines (Health Protection Agency Culture Collections, UK) were maintained in Dulbecco's Modified Eagle Medium or Roswell Park Memorial Institute medium (RPMI; Lonza Group Ltd, Switzerland) with 10% (v/v) fetal calf serum (FCS; PAA Laboratories, UK), 1 mM of L-glutamine, and penicillin/streptomycin.

2.2. Cell proliferation assays

Relative cell number percentage vs. controls was determined as previously described [24] using CellTiter 96 AQueous One Solution Reagent (Promega, UK) following the manufacturer's instructions.

2.3. Immunohistochemistry

Nrf2 immunohistochemical analyses used a tissue microarray of archival bladder cancer radical cystectomy samples from the Department of Histopathology, Southampton University Hospitals NHS Trust, United Kingdom. This research had UK National Research Ethics Service committee's approval.

Sections were deparaffinized in xylene substitute and rehydrated through graded alcohols. Peroxidase activity was blocked (0.1 ml of 0.5% hydrogen peroxide in 5.9-ml methanol, 10 min) and washed (water, 5 min). Antigen retrieval was performed (microwave, 25 min, 0.21% (w/v) sodium citrate buffer to pH 6.0) and washed (water, then 3 times Tris-buffered saline). Sections were blocked with

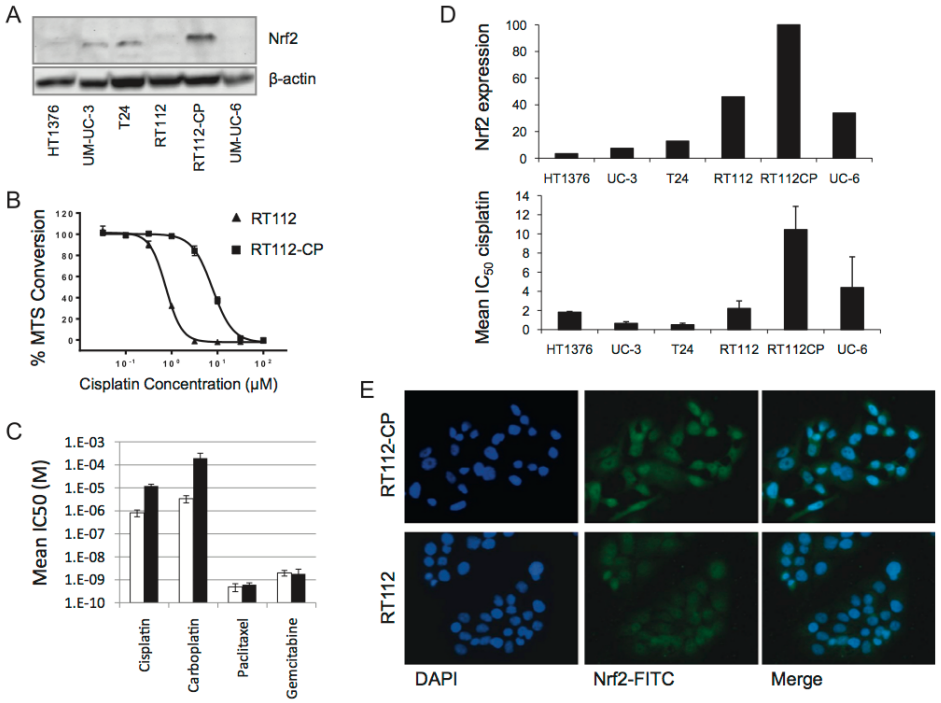

Fig. 1. Nrf2 expression and platinum sensitivity in bladder cancer cell lines. (A) Immunoblot analysis of Nrf2 and β-actin (loading control) expression in bladder cancer cell lines. (B) Representative cell proliferation assay following exposure to the indicated concentrations of cisplatin in RT112 and RT112-CP cells for 4 days. Data points are means of triplicate determinations ± standard error of the mean (SEM). (C) IC_{50} values for RT112 (white bars) and RT112-CP (black bars) cells in response to the indicated drugs. Results are mean values ± SEM from 3 separate experiments (D) Nrf2 protein expression and mean IC_{50} for cisplatin effect in cell proliferation assays in a panel of bladder cancer cell lines. Data are representative of 3 independent experiments. (E) Immunofluorescence microscopy for Nrf2 expression in RT112 and RT112-CP cells and nuclear costaining with DAPI. DAPI = 4′,6-diamidino-2-phenylindole. (Color version of figure is available online.)

avidin-blocking solution, biotin-blocking solution (Vector Laboratories), and culture media containing 5% bovine serum albumin (BSA)/10% FCS. Primary Nrf2 antibody was applied (1 in 200, overnight, 4°C), washed 3 times, and incubated with biotinylated secondary antibody (DakoCytomation, Denmark) for 30 minutes. After 3 washes, slides were incubated with streptavidin-biotin peroxidase (DakoCytomation, 30 min), washed 3 times, and then incubated with DAB substrate (Launch Diagnostics, UK, 5 min). Sections were washed (Tris-buffered saline and then water) and counterstained (Mayer hematoxylin). Sections were stained blue (running tap water, 10 min), dehydrated (graded alcohol), cleared (xylene substitute), and mounted (dinbutyl phthalate/xylene (DPX)-mounting medium). Nrf2 staining was evaluated by histopathologists (G.J.T. and M.S.).

2.4. Fluorescence microscopy

Cells were plated overnight on coverslips, fixed (phosphate buffered saline [PBS], 4% formaldehyde), made permeable (PBS, 0.1% Triton X), and blocked (RPMI, 10% FCS, 3% BSA, 30 min) before Nrf2 antibody incubation (1 in 50, PBS, 0.6% BSA, overnight, 4°C, darkness). After washing PBS, fluorescein isothiocyanate (FITC)-conjugated secondary rabbit anti–goat antibody (DakoCytomation; 1 in 50, PBS, 0.6% BSA) was applied (1 h, room temperature) followed by a nuclear counterstain (4′,6-diamidino-2-phenylindole, 1 in 1000, PBS, 0.6% BSA, 10 min). Cells were washed (PBS and then dH_2O) before mounting in fluorescence mounting medium (Dako, Cambridgeshire, UK; incubation 1 h, room temperature, darkness). Samples were then imaged (Olympus IX81 microscope, xcellence software [Olympus, UK]).

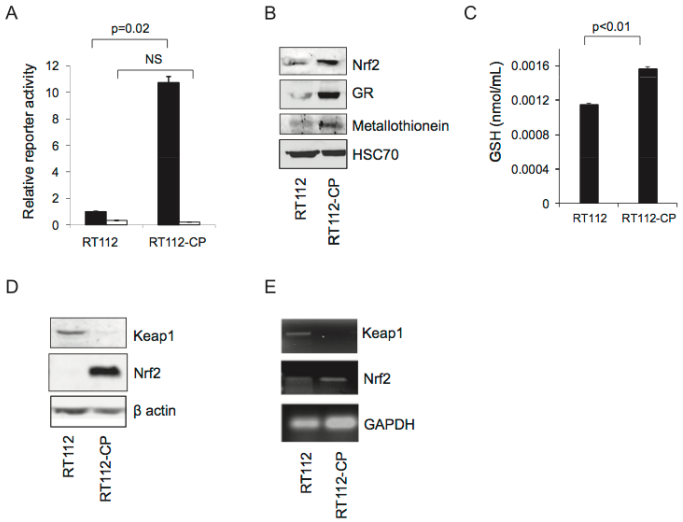

Fig. 2. Comparison of Nrf2 activity in RT112-CP and parental RT112 cells. (A) RT112 and RT112-CP cells were transfected with wild-type (black bars) or mutant (white bars) ARE luciferase constructs. Normalized luciferase activity was then determined at 24 hours relative to RT112 cells transfected with the wild-type ARE construct set at 1.0. Presented data are means of duplicate determinations ± sd. (B) Immunoblot analysis of Nrf2, GR, metallothionein, and HSC70 (loading control) in RT112 and RT112-CP cells. (C) Total cellular GSH content of RT112 and RT112-CP cells. Data are means of duplicate determinations ± sd. (D) Immunoblot and (E) RT-PCR analyses of Keap1 and Nrf2 protein and mRNA expression, respectively, in RT112 and RT112-CP cells (β-actin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH)—loading controls). All data are representative of a minimum of 2 independent experiments. mRNA = messenger RNA; RT = reverse transcription; sd = standard deviation.

2.5. Immunoblotting

Immunoblotting was performed as previously described [25].

2.6. siRNA reverse transfection

INTERFERin transfection reagent of 5 µl was incubated (15 min, room temperature, with mixing) with 50 nM of

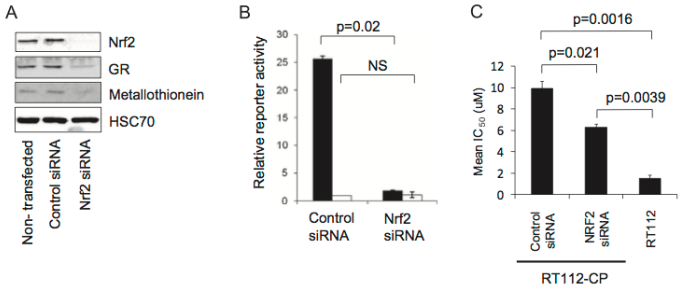

Fig. 3. Effects of Nrf2 depletion in RT112-CP cells. (A) RT112-CP cells were transfected with Nrf2-specific or control siRNAs. Expression of Nrf2, GR, metallothionein, and HSC70 (loading control) was analyzed by immunoblotting after 24 hours. Data are representative of 2 independent experiments. (B) RT112-CP cells were transfected with wild-type (black bars) or mutant (open bars) ARE luciferase reporter constructs and either Nrf2-specific or control siRNAs as indicated. Normalized luciferase activity was determined at 24 hours. Data are means of duplicate determinations ± sd. Luciferase values in cells transfected with the mutant ARE reporter construct and control siRNA are set to 1.0. (C) RT112-CP cells were transfected with Nrf2-specific or control siRNAs. After 24 hours, cells were treated with various concentrations of cisplatin for 4 days, and then cell proliferation assays were undertaken to determine IC_{50} values. Parallel assays were also performed using untransfected RT112 cells. Data shown are mean IC_{50} values ± sd from 3 independent experiments. sd = standard deviation.

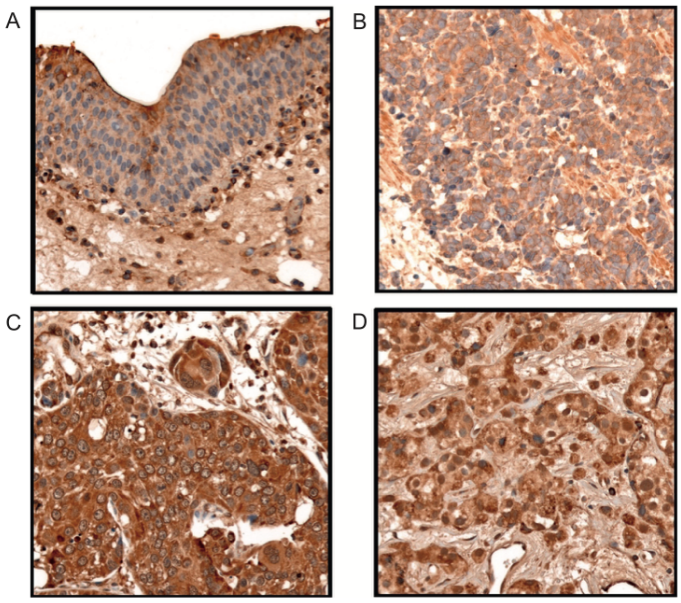

Fig. 4. Nrf2 expression in bladder cancer. (A–D) Representative Nrf2 expression patterns by immunohistochemical analysis in clinical bladder cancer samples: (A) normal bladder tissue, (B) bladder cancer (transitional cell carcinoma) with low Nrf2 expression, (C) bladder cancer with predominant cytoplasmic Nrf2 expression, and (D) bladder cancer with predominant nuclear Nrf2 expression. (Color version of figure is available online.)

Nrf2 or scrambled control small interfering RNA (siRNA) (Dharmacon, UK) in 750-μl serum-free RPMI. We plated 1.5×10^5 cells/35-mm well (750-μl RPMI, 20% FCS) before adding 750-μl transfection mix for 24 hours before subsequent assays.

2.7. Luciferase assays

Luciferase assays were performed as previously described [26]. ARE's luciferase activity was determined (Dual-Glo Luciferase Assay System [Promega]), following the manufacturer's instructions.

2.8. GSH assays

Cellular GSH (10^8 cells) was quantified against a reduced GSH standard curve (Glutathione Assay Kit, Sigma-Aldrich, UK) following the manufacturer's instructions.

2.9. Reverse transcription–polymerase chain reaction

RNA was extracted (RNeasy Mini Kit, Qiagen, UK) following the manufacturer's instructions. RNA quantification and complementary DNA synthesis used M-MLV reverse transcription (Promega, UK) following the manufacturer's instructions. Polymerase chain reaction (PCR) samples comprised 10-μl SYBR green PCR Precision MasterMix (Primerdesign, UK), 1-μl primer, 4-μl water, and 5-μl complementary DNA. PCR involved denaturing (95°C, 10 min) and 40 cycles of 95°C/15 seconds and 60°C/60 seconds (7500 Fast Real-Time PCR Machine, Applied Biosystems, New York, US).

2.10. Statistics

The Student *t* test was used to compare differences, and the Fisher exact test was used for contingency table analysis. Survival outcomes were analyzed using the Kaplan-Meier method and the log-rank tests. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Nrf2 expression is associated with platinum sensitivity in bladder cancer cells

We first assessed Nrf2 expression by immunoblotting bladder cancer cell lines, which was detected at variable levels (Fig. 1A). It is noteworthy that Nrf2 expression was

Table 1
Clinical characteristics of patients within the radical cystectomy tissue microarray (TMA) cohort

	Frequencies (n = 225)
Age at cystectomy	
Median (range)	72 (33–87)
≤70	98 (44%)
>70	127 (56%)
Sex	
Female	54 (24%)
Male	171 (76%)
Histology	
TCC/mixed histology with predominant TCC	203 (90%)
Adenocarcinoma	3 (1%)
Squamous cell carcinoma	15 (7%)
Sarcomatoid carcinoma	2 (1%)
Small cell carcinoma	2 (1%)
Grade	
3	186 (83%)
≤2	15 (7%)
Unknown	24 (11%)
T category	
0	1 (<1%)
1	35 (15%)
2	105 (47%)
3	34 (15%)
4	8 (4%)
a	12 (5%)
is	7 (3%)
Unknown	23 (10%)
N category	
0	190 (84%)
1	7 (3%)
2	1 (<1%)
Unknown	27 (12%)
Use of neoadjuvant chemotherapy	
Yes	71 (32%)
No	154 (68%)

TCC = transitional cell carcinoma.

particularly high in RT112-CP [23] with approximately 10-fold less growth inhibitory sensitivity to cisplatin (Fig. 1B). Similar sensitivity difference was also seen to carboplatin (~10-fold IC_{50}) but not to nonplatinum agents (paclitaxel and gemcitabine; Fig. 1C), each of which is used in bladder cancer. IC_{50} values for cisplatin in other cell lines broadly correlated with Nrf2 expression (Fig. 1D). Nrf2 cellular localization in RT112-CP cells by immunofluorescence microscopy was increased and predominantly nuclear compared with that in RT112 cells (Fig. 1E).

3.2. Nrf2 activation in RT112-CP cells

We used RT112 and RT112-CP cells as a model to investigate functional consequences of Nrf2 expression in bladder cancer. To investigate transcriptional activity, we transfected cells with wild-type or mutant ARE luciferase reporters. Wild-type ARE reporters were ~10-fold more active in RT112-CP cells than they were in RT112 cells, whereas mutant ARE activity did not differ (Fig. 2A). Compared with RT112 cells, RT122-CP cells expressed higher levels of the Nrf2 targets GR and metallothionein (Fig. 2B), contained higher total GSH (Fig. 2C), and had reduced expression of Keap1 protein and *Keap1* RNA (Fig. 2D and E).

3.3. Nrf2 contributes to cisplatin resistance

We depleted Nrf2 by siRNA in RT112-CP cells and confirmed reduced Nrf2 transcriptional activation in terms of decreased GR and metallothionein expression (Fig. 3A) and ARE reporter activity (Fig. 3B). In growth inhibition assays, after Nrf2 depletion, we found partial restoration of cisplatin sensitivity with respect to IC_{50} value, although it did not reach that of RT112 cells (Fig. 3C).

3.4. Nrf2 expression in bladder cancer and survival after chemotherapy

We performed immunohistochemical analysis on archival samples to assess Nrf2 expression in bladder cancer. Within malignant cells, both nuclear and cytoplasmic staining patterns were observed (Fig. 4). No association existed between cytoplasmic and nuclear staining (the Fisher exact test, P = 0.088). To assess the relevance of Nrf2 expression in this disease, we used a tissue microarray of 225 radical cystectomy specimens with available clinical data. A total of 71 (32%) received prior neoadjuvant cisplatin and gemcitabine chemotherapy. Clinical characteristics are shown in Table 1. We dichotomized Nrf2 expression within the cancer as high (3+) staining ("positive"), in which either cytoplasmic or nuclear staining was considered positive, and none ("negative"). A total of 177 samples had cancer present and were evaluable for Nrf2 staining, of which 57 (32%) were Nrf2 positive. Of these, only 3 cases had predominant nuclear staining and the rest had predominant cytoplasmic staining. In patients treated with radical cystectomy alone, we found no difference in overall, bladder cancer–specific, or recurrence-free survival with respect to Nrf2 expression. However, in those treated with chemotherapy also, these outcomes were favorable if Nrf2 expression was negative (Fig. 5 and Table 2).

4. Discussion

Cisplatin-based chemotherapy is the central treatment for metastatic bladder cancer and is also used perioperatively to improve cure rates [1]. However, cisplatin resistance is a common clinical problem. For example, in a randomized trial of cisplatin-based regimens for advanced disease, 17% had primary refractory disease, and by 3 years only 13% were alive and progression free [4,27]. Platinum-resistant

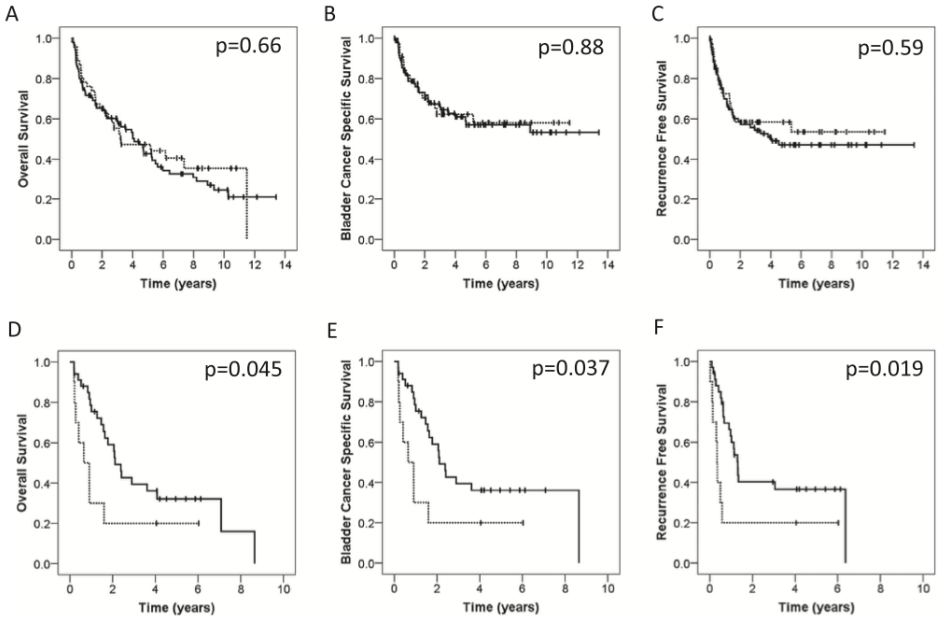

Fig. 5. Clinical outcomes following radical cystectomy. Kaplan-Meier curves for patients treated by radical cystectomy for bladder cancer who either did not (A–C) or did (D–F) receive prior neoadjuvant cisplatin and gemcitabine chemotherapy to show overall (A and D), bladder cancer–specific (B and E), and recurrence-free (C and F) survival with respect to Nrf2 expression (broken line positive, solid line negative).

disease is swiftly lethal with progression-free survival of 2 to 4 months with second-line chemotherapy [1].

Cisplatin-resistance mechanisms are complex and multifactorial, including enhanced cellular detoxification (e.g., increased thiols including GSH and metallothioneins), deranged drug transporter activity (e.g., reduced copper transporter 1 expression), altered DNA repair (e.g., through ERCC1 activation and loss of mismatch repair), altered DNA damage tolerance (e.g., p53 mutation), and apoptotic cell death mechanisms (e.g., abnormal Bcl-2 and Bcl-xL expression) [28]. However, a cohesive mechanistic understanding of cisplatin sensitivity/resistance is lacking. We noted that various proposed mediators of cisplatin resistance, or determinants of bladder cancer prognosis, are Nrf2 transcriptional targets, and therefore we tested its role as a putative master–regulating factor (acknowledging that not all potential resistance mechanisms are accounted for).

A key finding is of partial reversibility of cisplatin resistance in cell line models through Nrf2 protein depletion. This reduction in cell proliferation IC_{50}, by around half, is of similar magnitude to that published for cisplatin in Nrf2-null vs. $Nrf2^{+/+}$ fibroblasts [29]. Although it is a modest dose-effect change, if differences in drug sensitivity

Table 2
Outcomes following surgery in the radical cystectomy cohort

Neoadjuvant chemotherapy	Nrf2 expression	*n*	Overall survival			Bladder cancer–specific survival			Recurrence-free survival		
			Median, y	At 3 y	*P*	Median, y	At 3 y	*P*	Median, y	At 3 y	*P*
No	–	86	4.11	58.9%	0.66	NR	65.9%	0.88	4.03	54.3%	0.59
	+	47	3.24	55.4%		NR	62.2%			58.4%	
Yes	–	34	2.11	39.4%	0.04	2.11	39.4%	0.03	1.31	40.3%	0.01
	+	10	0.65	20.0%	5	0.65	20.0%	7		20.0%	9

NR = not reached.

of this magnitude were confirmed clinically, it would likely be significant in view of the narrow therapeutic window for efficacy/toxicity outcomes with this drug. As discussed, other cellular mechanisms are also likely to determine cisplatin sensitivity aside from Nrf2 regulation. This raises a pressing need for clinical validation of predictive biomarkers for cisplatin response, and Nrf2 axis members warrant inclusion.

Variable Nrf2 protein expression was seen across different bladder cancer cell lines with raised Nrf2 expression in a cisplatin-resistant bladder cancer cell line. Using this model of cisplatin resistance, we confirmed that this basal increase in Nrf2 expression is functionally active and reversible on Nrf2 depletion. The exact nature of the raised expression and functional activation of Nrf2 in bladder cancer requires elucidation. In the cell model used, we found inverse correlation with Keap1 expression. This, alongside data indicating that Nrf2 mutation is not a feature of transitional cell carcinoma [19], is consistent with a model whereby Keap1 repression drives increased Nrf2 activity. Our finding of low expression of both Keap1 protein and messenger RNA in RT112-CP cells implies that this might be through other mechanisms other than Keap1 mutation, for example, promoter methylation as in lung cancer [22]. Downstream factors affecting nuclear localization may also be relevant to functional pathway activation.

In view of our cell line findings, we assessed clinical Nrf2 expression and confirmed that Nrf2 is indeed expressed in a subset of bladder cancers. We have also provided preliminary data associating Nrf2 expression with poor survival outcomes in patients who have received neoadjuvant chemotherapy before radical surgery but not in those patients proceeding to surgery alone. These findings require external validation. However, they suggest that Nrf2 activity in bladder cancer is important to platinum chemotherapy response and thus warrant prospective evaluation. The predominant finding of cytoplasmic vs. nuclear staining for Nrf2 is of interest. Our sample size was not adequate to differentiate whether this distinction might be relevant to prognosis, which warrants future investigation.

Some limitations in our work warrant acknowledgment. Firstly, the tissue microarray and immunohistochemical analysis used retrospective tissue and clinical data collection. Both are potential sources of bias, and so, our outcomes data, with respect to Nrf2 status, should be viewed as requiring external validation. Secondly, our findings, with respect to outcomes on the basis of prior use of chemotherapy, were not a randomized comparison. As such, they should be viewed as hypothesis generating. Finally, our cell line work used model systems for cisplatin resistance. As we have noted, cisplatin resistance is undoubtedly multifactorial in its potential causes and so our models may not encapsulate all of the potential mediators of this important clinical complication.

5. Conclusion

We have demonstrated functional Nrf2 overexpression in bladder cancer associated with clinically relevant cisplatin resistance that is experimentally reversible.

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Loss of expression of the tumour suppressor gene *AIMP3* predicts survival following radiotherapy in muscle-invasive bladder cancer

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The aim of this study was to test the utility of *AIMP3*, an upstream regulator of DNA damage response following genotoxic stress, as a clinical biomarker in muscle-invasive bladder cancer (MIBC). *AIMP3* was identified from a meta-analysis of a global gene-expression dataset. AIMP3 protein expression was determined by immunohistochemistry on a customised bladder cancer tissue-microarray (TMA). The mechanism of gene silencing was probed using methylation-specific PCR. The association between *AIMP3* expression, *Tp53* transactivity and genomic stability was analysed. *In vitro* AIMP3 translocation to the nucleus in response to ionising radiation was demonstrated using immunofluorescence. Radiosensitisation effects of siRNA-mediated *AIMP3*-knockdown were measured using colony forming assays. TMAs derived from patients enrolled in BCON, a Phase III multicentre radiotherapy trial in bladder cancer (ISRCTN45938399) were used to evaluate the association between AIMP3 expression and survival. The prognostic value of AIMP3 expression was determined in a TMA derived from patients treated by radical cystectomy. Loss of AIMP3 expression was frequent in MIBC and associated with impaired Tp53 transactivity and genomic instability. *AIMP3*-knockdown was associated with an increase in radioresistance. Loss of AIMP3 expression was associated with survival in MIBC patients following radiotherapy (HR = 0.53; 95% CI: 0.36 to 0.78, $p = 0.002$) but was not prognostic in the cystectomy set. In conclusion, AIMP3 expression is lost in a subset of bladder cancers and is significantly predictive of survival following radiotherapy in MIBC patients.

Bladder cancer is the ninth most frequently diagnosed cancer worldwide, accounting for an estimated 386,000 new diagnoses and 150,000 deaths each year.[1] At diagnosis, approximately 30% of cases are classified as muscle-invasive bladder cancers (MIBC), which in most cases that are organ-confined, are treated by cystectomy or radiotherapy. Radical

Key words: AIMP3, bladder cancer, biomarker, organ-preservation, radiotherapy
Abbreviations: AIMP3: Aminoacyl-tRNA synthetase (ARS)-interacting multifunctional protein 3; DDR: DNA damage response; DNA: deoxyriboneucleic acid; HR: hazard ratio; IR: ionising radiation; MIBC: muscle-invasive bladder cancer; OS: overall survival; PCR: polymerase chain reaction; RCT: randomised control trial; TMA: tissue microarray
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What's new?

Radical cystectomy in the treatment of muscle-invasive bladder cancer (MIBC) is associated with significant morbidity and mortality, calling for organ-preservation strategies such as radical radiotherapy with radiosensitisation and chemo-radiotherapy. This paradigm change will be accelerated with the identification of biomarkers that can reliably select patients who will respond to radiotherapy. This study tested *AIMP3*, an upstream regulator of DNA damage response following genotoxic stress, as a potential clinical biomarker. *AIMP3* loss of expression was associated with a decrease in genomic-stability, p53-transactivity, and radiosensitivity *in vitro* and was predictive of overall-survival in a phase-III randomised control trial of radiotherapy in MIBC.

cystectomy with bilateral pelvic node dissection is considered the standard of care for organ-confined MIBC with an overall 5-year survival of 60–70%.[2–6] However, following cystectomy, about one-third of patients will develop local recurrence or distant metastasis within 2 to 3 years and radical surgery is associated with significant morbidity and a 90-day mortality of approximately 5%.[7–9] Consequently, radiotherapy or other forms of organ-preservation treatments potentially avoid the morbidity associated with radical cystectomy. Radiosensitisation strategies show promise but require optimisation.[10,11]

One challenge is to identify biomarkers that can reliably predict response to treatment by identifying patients who will respond to radiotherapy and for whom bladder preservation may be a viable alternative option. A biomarker-driven approach could improve outcomes for patients with MIBC and help to resolve some of the issues surrounding clinical equipoise and difficulties in conducting trials of selective organ preservation.[12] Genome-wide surveys cataloguing global transcriptome and copy number variations have refined the molecular basis of bladder cancer and helped define molecular subtypes, predict clinical outcome and identify biomarkers that can potentially improve bladder cancer management.[13–18]

While MIBC share some common molecular features such as the dysregulation of the tumour suppressor genes, p53 and pRb, there is genetic divergence across tumours.[17–20] The accumulation of genetic aberrations is characteristic of increasing stage and grade progression in MIBC and the development of genetic instability, which is a characteristic of advanced disease, is often associated with the dysregulation of DNA Damage Response (DDR) genes. These genes include the serine/threonine kinases ataxia telangiectasia mutated (ATM) and ATM and Rad3-related (ATR) as well as other cell cycle checkpoint, DNA repair and surveillance genes.[15,21] Radiotherapy, like many cytotoxic chemotherapeutic agents, targets tumour cells by damaging DNA. Many of these DDR genes have been targeted as modulators of tumour response to ionising radiation (IR).[22]

In this study, we investigated the role of a tumour suppressor, *AIMP3* (Aminoacyl-tRNA synthetase (ARS)-interacting multifunctional protein 3), in bladder cancer and tested its utility as a clinical biomarker. Through a meta-analysis of previously published gene-expression data, we identified the loss of expression of *AIMP3* as a frequent feature in MIBC. AIMP3 is an auxiliary component of the macromolecular multisynthetase complex that mediates protein translation but it has also been implicated as an upstream activator of p53 in response to DNA damage or oncogenic stimuli.[23,24] *AIMP3*-homozygous mice are embryonic lethal, while *AIMP3*-heterozygous mice spontaneously develop tumours.[23]

Reduced AIMP3 expression has been reported in some cancers but not in bladder cancer.[23,25] We investigated the mechanism of loss of expression as well as the association with Tp53 transactivation and genomic stability in a subset of tumours. As *AIMP3* has been associated with various DDR genes, we measured the effect of *in vitro* modulation of *AIMP3* expression on radiosensitivity. Subsequently, we tested if *AIMP3* expression was predictive of response to radical radiotherapy in 217 patients who were recruited for the BCON (bladder carbogen nicotinamide) trial that compared radical radiotherapy with and without carbogen and nicotinamide in organ-confined MIBC.[11]

Material and Methods

Bladder cancer specimens and tissue microarrays

University of Cambridge cohort. Tumour samples from 109 primary bladder cancers were collected at cystectomy or transurethral resection of bladder tumour (TURBT) and snap-frozen in liquid nitrogen. Use of tissue for this study was approved by the Cambridgeshire Local Research Ethics Committee (Ref: 03/018). A total of 30 sections of 30 μm of fresh-frozen tissue were homogenised for DNA and RNA extraction. Parallel H&E sections were assessed for cellularity and grade by a reference uro-histopathologist. Tumours were staged and graded according to the American Joint Committee on Cancer and World Health Organization/International Society of Urologic Pathology classifications. Samples displaying tumour cellularity of less than 70% or significant inflammatory cell contamination were excluded. An independent cohort of 123 formalin-fixed, paraffin-embedded tissue samples of primary bladder cancer were obtained from the pathology archives of Addenbrookes Hospital, Cambridge University Trust. Tumours were representative of stages and grades. Carcinoma *in situ* samples were included. Normal bladder urothelium were obtained from non-cancer tissues. A TMA was constructed and protein expression was scored as previously described.[17,26]

BCON cohort. Detailed description of the characteristics of patients enrolled in the BCON trial and the trial protocol have been described previously.[11] In brief, eligible patients with organ-confined MIBC were assigned randomly to treatment with either radical radiotherapy alone or radical radiotherapy supplemented with carbogen and nicotinamide.

Of 333 patients enrolled in the trial, pre-treatment TURBT specimens incorporated into formalin-fixed paraffin-embedded TMA blocks that were amenable to analysis were available for 217 patients. Clinical demographic details of the 217 patients are outlined in Table 1.

Radical cystectomy cohort. Transurethral biopsies from 151 patients undergoing radical cystectomy between 2000 and 2011 at Southampton University were obtained with local ethics approval (Ref: 10/H0504/32). One millimetre cores of tumour tissue were organized in triplicates on a TMA. Supporting Information Table 1 summarizes the clinical demographic details of this cohort.

RNA extraction and Reverse Transcriptase-Polymerase Chain Reaction Analysis

Total RNA was extracted using Tri-Reagent following the manufacturer's protocol. RNEasy Minikit (Qiagen, Hilden, Germany), including a DNase step, was used to optimize RNA purity. Isolated RNA was analysed using RNA 6000 NanoLabChip (Agilent Technologies, CA). The NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, MA) was used to measure the concentration and quality at 260/280 absorbance ratio. Five micrograms of total RNA was reverse-transcribed in a final volume of 40 μl using a Superscript kit (Invitrogen, CA). *AIMP3* and *TP53TG1* transcript levels were analysed and *SDH*, *UBC*, *GAPDH* and *ACTB* were used as house-keeping genes. RT-qPCR assays were performed on the ABI Prism 7700 following manufacturer's recommendation (Applied Biosystems, CA). Each sample was analysed in triplicate.

DNA extraction, bisulphite modification methylation-specific PCR assays

DNA from bladder cancers from the University of Cambridge cohort as well as normal bladder controls from patients without cancer were used in the study. Up to 1 μg of DNA was subjected to bisulphite modification using a commercially available kit (Zymo Research, CA). This reaction selectively deaminates unmethylated cytosine residues, resulting in a conversion to uracil, while 5-methyl cytosine residues are not modified. The modified DNA was eluted in 20 μl Tris-HCl (1 mM, pH 8.0), then stored at −80°C. Analyte quantification was performed by real-time MSP assays, which consisted of parallel amplification/quantification processes using specific primers (AIMP3 F:5′-TGTTTTGTAGGTTTCGCGTTT-3′, R:5′-CTCCTTCTCACC TATCGCTCG-3′ and ACTB F:5′-TAGGGAGTATATA GGTTGGGGAAGTT-3′, 5′ AACACACAATAACAAACA CAAATTCAC-3′) and Molecular Beacon® assay (AIMP3: 5′-CGACATGCACCGCCGCCATCTTCCGACCGCATGTCG-3′ and ACTB: 5′-CGACTGCGTGTGGGGTGGTGATGGAGG AGGTTTAGGCAGTCG-3′) on an ABI Prism® 7900HT instrument (Applied Biosystems). The MSP results were generated using the SDS 2.2 software (Applied Biosystems), exported as Ct (cycle threshold) values, and then used to calculate copy numbers based on a linear regression of the values plotted on a standard curve of $20^{-2} \times 10^{6}$ gene copy equivalents, using plasmid DNA containing the bisulfite modified sequence of interest. Cell lines with known methylation status were included in each run as positive and negative controls, and entered in the procedure at the DNA extraction step. A run was considered valid when the following five criteria were met: (*i*) slopes of each standard curve above −4 corresponding to a PCR efficiency > 77.8%; (*ii*) R2 of at least four relevant data points ≥ 0.99; (*iii*) routinely included non-template controls (NTC) were not amplified; (*iv*) 10% of a 1 μg conversion reaction of a positive methylation control cell line had to give a methylated signal; and (*v*) 10% of a 1 μg conversion reaction of a negative control cell line had to give no signal. A tissue sample was classified as valid when at least 10 copies of the *ACTB* gene were measured in the genomic DNA isolated from the sample. Only valid samples were checked for the AIMP3 promoter methylation status. The results were expressed as ratios (AIMP3 copy number/ACTB copy number X1000).

Tissue MICROARRAY IMMUNOHISTOCHEMISTRY (TMA-IHC)

Immunohistochemistry was performed using the Bond Polymer Refine Detection kit with Bond-III automated immunostaining system (Leica Microsystems, Milton Keynes, UK) following the manufacturer's instructions. In brief, the TMA slides were deparaffinised, rehydrated, washed and endogenous peroxidase was blocked using Bond-III "*Dewax Protocol D*" following the manufacturer's instructions (Leica Biosystems, Newcastle, UK).

Epitope retrieval was achieved using Bond-III "*Protocol H1(30)*" (Leica). The slides were incubated with antibodies against AIMP3 (1:25) (Atlas Antibodies, Sigma-Aldrich, UK) at room temperature for 1 hr. Antibody binding was detected using diaminobenzidine (DAB) with haemotoxylin counterstaining following Bond-max and Bond-x "IHC protocol F" (Leica). External controls were non-cancer colon tissue (positive) and non-cancer liver tissue (negative). The cores were examined under a light microscope at 400× magnification, standardising the scoring according to the reference control cores. The reference material was assigned a staining intensity of 2, graded on a scale of 0 to 3, against which the bladder cancer cores were compared. Three independent investigators, blinded to the clinical data, assessed the cores with the primary investigator scoring the staining a second time to assess intra-observer variance. At least 5 areas of each core were viewed and the proportion of cells in each core staining positively was assigned a proportion score (0 if 0%, 0.1 if 1% to 9%, 0.5 if 10% to 49%, and 1 if 50% to 100%), as previously described.[27] A semi-quantitative histopathology (H) score was obtained by multiplying the staining intensity with the

Table 1. BCON trial patients and tumour characteristics stratified by AIMP3 expression

Demographics	All patients (n = 217)	AIMP3 negative (n = 106)	AIMP3 positive (n = 111)	p
Number (percent)				
Age, yr				
Median age	74	75	74	0.06[1]
Range	51–90	53–88	51–90	
Sex				
Male	174 (80)	83 (78)	91 (82)	0.497[2]
Female	43 (20)	23 (22)	20 (18)	
Stage				0.997[2]
1	22 (10)	11 (10)	11 (10)	
2	144 (66)	70 (66)	74 (67)	
3	41 (19)	20 (19)	21 (19)	
4	10 (5)	5 (5)	5 (4)	
Grade				
2	33 (15)	19 (18)	14 (13)	0.345[2]
3	184 (85)	87 (82)	97 (87)	
Preceding tumour				
No	186 (86)	87 (82)	99 (89)	
Yes	31 (14)	19 (18)	12 (11)	0.174[2]
Preceding tumour treatment				
TURBT				
Complete	90 (42)	35 (33)	55 (50)	0.405[2]
Partial	66 (30)	36 (34)	30 (27)	
Biopsy only	53 (24)	28 (26)	25 (22)	
Unknown	8 (4)	7 (7)	1 (1)	
BCON randomisation				
RT alone	113 (52)	58 (55)	55 (50)	0.446[2]
RT + CON	104 (48)	48 (45)	56 (50)	
Hb, g/dL				
Median	13.6	13.5	13.9	0.08[1]
Range	9.3–17.2	9.3–17.2	9.8–17.0	

[1]Mann-Whitney U test.
[2]Fisher's Exact Test.
Abbreviations: TURBT, transurethral resection of bladder tumour; RT, radiotherapy; CON, carbogen and nicotinamide; Hb, hemoglobin.

proportion score. The median value of all H scores was defined as the cut-off value to categorize antibody staining in the cores as either positive or negative. Cores with discordant scores, resulting in H values that altered the staining status stratification were assessed by a third investigator to reach a consensus.

AIMP3 copy number and fraction of genome altered (FGA) annotation

The frequency of *AIMP3* locus deletions was identified using data previously obtained from whole genome CNV profiling of the University of Cambridge cohort by a 1-Mb BAC clone aCGH as previously reported.[17] DNA copy number was confirmed with centrosome fluorescent *in situ* hybridization (FISH). As an estimate of genomic instability of each tumour, we determined the FGA as previously described.[20]

Cell lines and cell culture

Bladder cancer cell lines (T24, 253J, RT112, RT4) and HeLa (cervical cancer cell line; as control) were grown in monolayers in RPMI1640 medium (Gibco Invitrogen, Paisley, UK) supplemented with 10% FBS (fetal bovine serum) (Flow Laboratories, Irvine, UK) and 2 mM L-glutamine (Gibco

Table 2. List of non-redundant GOs of potentially methylated low-expressing genes in bladder cancer

Gene Ontology	Example of genes
Biological process	
Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	*C20orf100,RBM15B, MTERF,NPAS2,RARB,*
RNA biosynthetic process	*C20orf100,MTERF,NPAS2, RARB,TCEA3,*
Intracellular transport	*IMMP2L,MRPL32,TRAPPC1, BNIP1,STARD3*
Protein modification	*STK11,PRKCD,CHM,ARD1A, MINK1,CASK*
Translation	*PELO,PABPC4,MRPL13,AIMP3, MRPL32*
Cell death	*TPT1,IFNB1,BNIP1,SCIN,AIMP3*
Phosphorylation	*MINK1,CASK,STK11,PRKCD*
Blood coagulation	*ENTPD2,PABPC4,TBXAS1,FGA*
DNA packaging	*CHAF1B,L3MBTL2,ARD1A*
Cellular secretion	*BNIP1,SCIN,TRAPPC1*
Molecular function	
Zinc ion binding	*L3MBTL2,ZNF138,APOBEC3G, PRKCD,TCEA3*
Protein kinase activity	*CASK,PRKCD,STK11,MINK1*
ATP binding	*STK11,MINK1,CASK,PRKCD*
Pyrophosphatase activity	*RRAS,ENTPD2*
Symporter activity	*SLC16A4,SLC25A18*
Iron ion binding	*SC5DL,TBXAS1*
Double-stranded DNA binding	*MTERF*
Phosphatidylinositol binding	*SCIN*
Chloride transporter activity	*SLC26A1*
Phosphatidylinositol-4, 5-bisphosphate binding	*SCIN*
Cellular component	
Intracellular organelle	*PRKCD,TCEA3,ZZZ3,NPAS2, NUP37,IMMP2L*
Intrinsic to membrane	*JAM2,RNF133,LIME1,SLC25A36, GRIK3*
Cytoplasm	*SC5DL,CHAF1B,STARD3,AIMP3, SCIN*
Plasma membrane part	*SLC16A4,PELO,FLRT3,AQP4, JAM2,CASK*
Ribonucleoprotein complex	*MRPL13,MRPL32*
Synaptosome	*CASK*
Aminoacyl-tRNA synthetase multienzyme complex	*AIMP3*
Outer membrane	*AQP4*
SNARE complex	*BNIP1*
Basal lamina	*ENTPD2*

Invitrogen). All cells were maintained in a 5% CO_2 humidified incubator at 37°C and sub-cultured when confluent.

Irradiation

Cells in logarithmic growth (40–80% confluence) were irradiated using a CP320 Bipolar X-ray machine (Gulmay, Kent, UK). Doses were adjusted by varying the duration of irradiation (2Gy/min) while the voltage (250kV), current (12.5mA), X-ray filter (Sn, Cu, Al) and distance from X-ray source (30cm) remained constant.

Colony forming assays

Single-cell suspensions (n = 200, 200, 750, 2,000, 1,000, respectively for T24, 253J, RT112, RT4, and HeLa) were plated in 60mm Nunc petri dishes (Thermo Scientific, Nottingham, UK) and maintained in culture medium. Medium was changed at day 7. Cells were fixed at day 14 in 70% industrial methylated spirit (IMS) and stained with 1% crystal violet. Colonies consisting of more than 50 cells were counted using a phase-contrast microscope.

Western Blot

Cells were lysed with RIPA buffer. Thirty micrograms of protein were loaded onto 14% sodium dodecyl sulphate (SDS)-polyacrylamide gels and run in a Mini PROTEAN III Electrophoresis System (Bio Rad, CA). After electrotransfer onto a Polyvinylidene fluoride (PVDF) membrane (Millipore, Sigma-Aldrich, MO), the membrane was blocked with 5% non-fat milk in PBS-T (0.1% Tween-20 in PBS), incubated overnight at 4°C in 1:10,000 anti-AIMP3 antibody (Abcam, Cambridge, UK) in 1% non-fat milk in PBST-T followed by incubation for 1 hr at room temperature in horseradish peroxidase (HRP)-conjugated secondary antibody diluted in 1% non-fat milk in PBS-T. Immunoblot image intensities were measured using ImageJ software (http://rsb.info.nih.gov/ij). Blot intensities for each lane were quantitated as a ratio of the corresponding β actin intensity.

Immunofluorescence

Single-cell suspensions were incubated on 22 mm × 22 mm coverslips (BDH Laboratory supplies, Poole, UK) placed in six-well plates (Nunc, Roskilde, Denmark). After fixing with formaldehyde (BDH Laboratory), the cells were treated with a modified Permeabilizing Buffer (0.5% Triton X, 1% BSA (bovine serum albumin), PBS) for 10 min and incubated overnight at 4°C with 1:500 anti-AIMP3 antibody diluted in Permeabilizing Buffer. The cells were subsequently incubated with 1:500 FITC (Fluorescein Isothiocyante) (Sigma-Aldrich, Dorset, UK) and then 1:500 TRITC (Tetramethyl Rhodamine Isothiocyanate) (Sigma-Aldrich), both diluted in Permeabilising Buffer and at room temperature for 1 hour. The nuclei were stained by incubating briefly with DAPI (4′,6-diamidino-2-phenylindole) (Invitrogen, OR). Images were acquired using a Leica DMRE confocal microscope with a TCSSP scanhead (Leica, Newcastle, UK). Colocalisation was measured on ImageJ as previously described.[28]

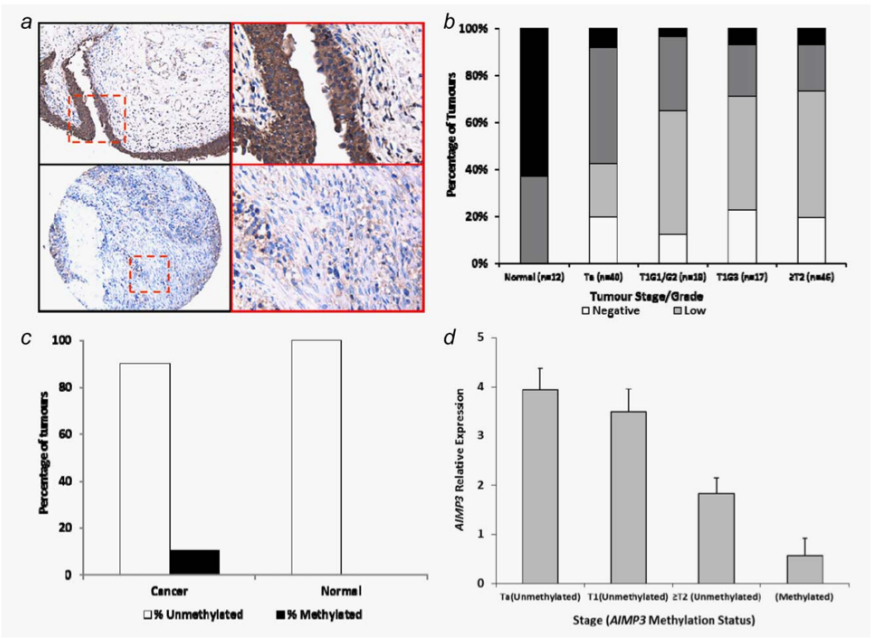

Figure 1. Loss of AIMP3 is a feature in bladder cancer and *AIMP3* promoter methylation is cancer-specific. (*a*) AIMP3 TMA-IHC. Top: Normal urothelium demonstrating high levels of cytoplasmic AIMP3 expression (L ×20; R ×100). Bottom: AIMP3 is not expressed in an invasive T2G3 tumour (MIBC) (L ×20; R ×100). In A, the dashed square indicates the area at a higher magnification (×100) in the photomicrographs on the right (R). (*b*) Distribution of AIMP3 expression levels across tumours of different stages and grades and normal tissues. There is a reduction in AIMP3 expression in cancer as compared to normal bladder tissue. A downward-shift in the percentage of moderate/high expressing tumours exists with stage/grade progression. (*c*) Promoter methylation of *AIMP3* was detected in about 10% of bladder tumours (both MIBC and NMIBC) but was undetected in normal tissues. (*d*) Relative *AIMP3* mRNA expression stratified by tumour stage/grade and methylation status. Methylated tumours demonstrated a significant loss in expression, while a marked difference was also observed between Ta/T1 and T2/higher tumours ($p < 0.001$). Bars, SE. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Cancer Therapy

siRNA transfection

Sub-confluent (60–80%) cells were incubated overnight with 5 nM siRNA to *AIMP3* (sequence: GGGUAUAACUUUACAUUAG) (Thermo Fischer Scientific, CO) in serum-free Optimem medium (Gibco Invitrogen) mixed with DharmaFECT transfection reagent (Thermo Fischer Scientific). Non-targeting, scrambled siRNA (Thermo Fischer Scientific), *GAPDH* siRNA (Thermo Fischer Scientific) and siRNA-free media were used as controls. Transfection media was replaced with serum-supplemented RPMI 24 hrs post-transfection.

Statistical analysis

Statistical analyses were performed using SPSS, version 17.0 (SPSS Inc, IL). For *in vitro* work, the differences in means between the groups (at least three independent experiments) were measured using two-tailed Student's *t*-test. For the BCON dataset, agreement in the scoring of the stained TMA cores (inter- and intra-observer agreement) was measured using Kappa statistics. Overall survival (OS) was the primary outcome measure, estimated using the Kaplan-Meier method. The effect of AIMP3 by staining status was measured using a Cox proportional hazards model adjusted for important covariates such as age, gender, tumour grade (G2 or G3), tumour stage (T1, T2, T3 or T4), randomised treatment received (radical radiotherapy alone or radical radiotherapy with carbogen and nicotinamide), and hemoglobin (Hb) status (Hb <13.5 g/dL or Hb ≥ 13.5 g/dL). Hazard ratio (HR) and confidence interval (CI) calculations were based on the Cox model; *p* values of <0.05 were used to denote statistical significance.

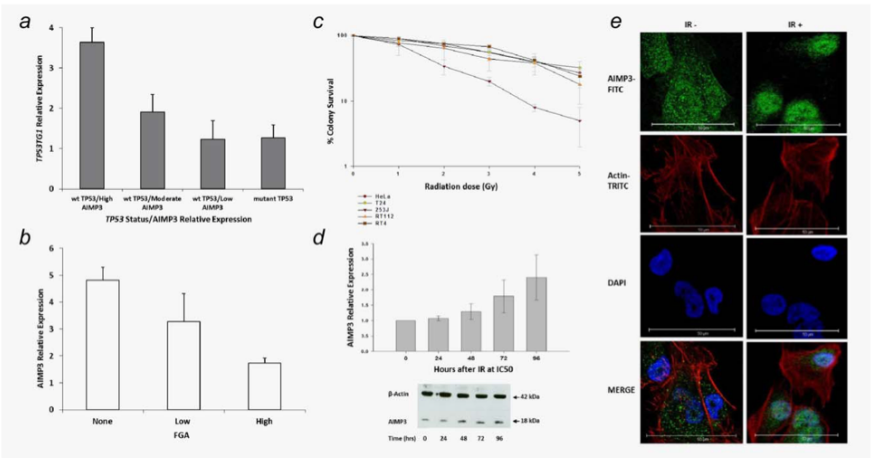

Figure 2. Association between AIMP3 expression, Tp53 transactivity and genomic stability. (*a*) Relative *TP53TG1* mRNA expression stratified by *AIMP3* expression levels and *TP53* mutation status. Low expressing *AIMP3* wt *TP53* tumours presented with a loss of *TP53TG1* expression comparable to that of mutant Tp53 tumours. Bars, SE. wt, wild-type. (*b*) Relative AIMP3 expression stratified by FGA. FGA of all tumours were defined as a quantitative expression of genomic instability, assessed by 1-Mb aCGH. Tumours with high levels of *AIMP3* exhibited a lower fraction of genome altered (FGA) ($p < 0.001$). Bars, SE. (*c*) Dose–response curves of cell lines HeLa, T24, 253J, RT112 and RT4 irradiation (0–5Gy). Bars, SD. (*d*) AIMP3 protein expression increased with time upon exposure to IR as assessed by western blots and normalised with β actin. Bars, SD (*e*) Confocal immunofluorescence imaging showing subcellular localisation of AIMP3 in RT4 cells without irradiation (IR−) and with irradiation. (IR+) (×600) (*AIMP3*-FITC (green-yellow); Actin-TRITC (red-pink); Nuclear DAPI staining (blue); and merged images). Following IR, there is increased nuclear localisation of AIMP3 (Manders coefficient: 0.362 without irradiation compared to 0.778 with irradiation). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Results

Reduced expression of the tumour suppressor gene *AIMP3* in bladder cancers

Table 2 lists the gene ontologies that represent the identified targets including the molecular function of the gene products, their role in multi-step biological processes, and their localisation to cellular compartments. One of the targets identified was *AIMP3*. No studies to date have reported on *AIMP3* expression in bladder cancer. To confirm the transcript loss of *AIMP3* in bladder cancer, we assessed the protein expression of AIMP3 on an independent TMA that covered the spectrum of stages and grades in bladder cancer. Staining for AIMP3 was predominantly cytoplasmic in normal urothelium (Fig. 1*a*). Stromal expression was limited. The intensity of expression was higher in the normal urothelium than in bladder cancer tissue specimens. AIMP3 was expressed at moderate and high levels in all normal urothelium tissues (Fig. 1*b*). However, only 56% of tumours expressed AIMP3 at moderate and high levels. Loss of AIMP3 expression was more pronounced in late stage (≥T2) tumours, where less than 30% of these tumours expressed AIMP3 at moderate and high levels.

Real-time methylation-specific PCR (MSP) assays of the promoter region of *AIMP3* were performed on DNA extracted from normal and tumour tissues. Promoter methylation of *AIMP3* was detected in about 10% of bladder cancer but not in normal tissue (Fig. 1*c*). Using RT-qPCR, relative *AIMP3* mRNA expression was compared between tumours of different stages and grades and methylation status (Fig. 1*d*). Methylated tumours demonstrated a significant reduction in *AIMP3* expression when compared to unmethylated tumours across all stages. Interestingly, when only the unmethlylated cases were compared, a significant reduction in AIMP3 expression was also observed between Ta/T1 and T2/higher tumours ($p < 0.001$) (Fig. 1*d*).

Reduced AIMP3 accompanies impaired Tp53 transactivity, genomic instability and radioresistance

To investigate the biological significance of the loss of *AIMP3* on p53 transactivation in p53 wild-type bladder tumours, we compared the mRNA levels of *AIMP3* and TP53 Target Gene 1 (*TP53TG1*) in 60 p53-wild-type and 10 mutant-p53 bladder cancer samples. Independent of stage and grade, wild-type p53 tumours that expressed low levels of *AIMP3* exhibited low levels of *TP53TG1* in agreement with the reported tumour suppressor role of *AIMP3* ($p < 0.001$). The level of expression of *TP53TG1* in these tumours was comparable to that of mutant p53 tumours

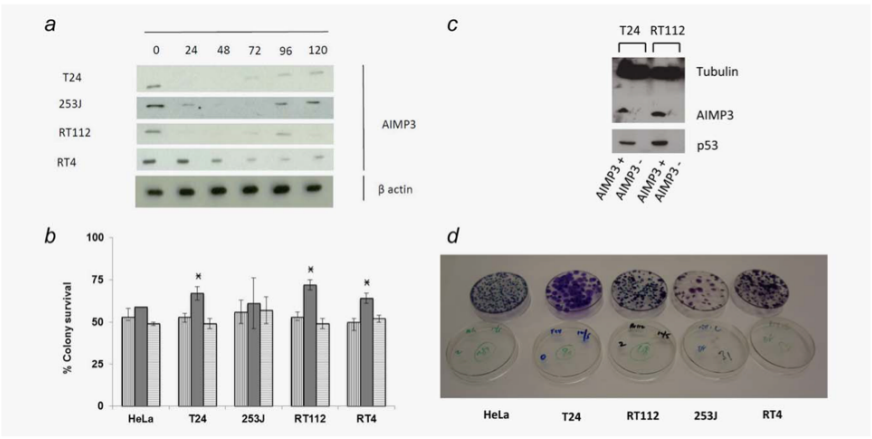

Figure 3. Reduction of *AIMP3 in vitro* is associated with increased radioresistance. (*a*) Western Blot demonstrating significant siRNA-mediated knockdown of AIMP3 between 24 and 96 hrs post-transfection. (*b*) AIMP3-knockdown cells demonstrate a significant increase in clonogenic survival following irradiation ($p < 0.05$*). Bars, SD. Relative to cells exposed to IR at IC50 (vertical-banded bars) and those with just transfection-medium exposure with IR at IC50 (horizontal-banded bars), those with AIMP3-knockdown and exposed to IR at IC50 (grey, unbanded bars) have a higher clonogenic survival. Untreated cells were used as reference (100%) for relative changes in clonogenic survival (bars not shown). (*c*) Clonogenic assay images demonstrating stained and counted colonies in the respective cell lines. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(Fig. 2*a*). When the relative *AIMP3* expression by RT-qPCR was compared across tumours that were stratified according to the fraction of genome altered (FGA), we found *AIMP3* to be downregulated in tumours with high FGA ($p < 0.001$) (Fig. 2*b*).

To confirm the upregulation of AIMP3 in response to genotoxic stress, we examined the effect of irradiation (IR) on AIMP3 expression in bladder cancer cells *in vitro*. Western blot analyses confirmed AIMP3 expression in the different bladder cancer cell lines (T24, 253J, RT112, RT4) as well as HeLa cells (Supporting Information Fig. 1). There were no significant differences in AIMP3 expression levels between these cell lines. Colony forming assays following a range of IR doses was conducted on these cell lines (Fig. 2*c*). With the exception of 253J, the radiosensitive patterns were similar across the different cell lines. The IC50 values for all the cell lines were above 2Gy (RT4: 4.6Gy, T24: 3.9Gy, RT112: 2.9Gy, 253J: 2.7Gy, HeLa: 3.4Gy).

Western blot analysis revealed an upregulation of AIMP3 expression in T24 cells by about 48 hrs following IR, supporting the postulated role of AIMP3 as an upstream mediator of response to genotoxic stress (Fig. 2*d*). When the bladder cancer cell lines were irradiated at IC50 doses, increased localisation of AIMP3 from the cytoplasm into the nucleus was observed by immunofluorescence (Fig. 2*e* and Supporting Information Fig. 2).

Reduction of AIMP3 increases the resistance of bladder cancer cells to ionising radiation and is a predictive marker of response to radiotherapy

Efficient *AIMP3* knockdown was achieved at 48 hrs post-transfection with 5 nM siRNA (Fig. 3*a* and Supporting Information Fig. 3). *AIMP3*-knockdown cells were treated with IR at their IC50 doses at 48 hours post-transfection and their clonogenic survival was measured after 14 days (Figs. 3*b* and 3*d*). Clonogenic survival increased in *AIMP3*-knockdown cells (Fig. 3*b*). The increase in survival was significant in T24, RT112 and RT4 cells ($p < 0.05$) but not in 253J or HeLa cells. Reduction in AIMP3 expression, by siRNA knockdown, was noted to downregulate p53 expression in T24 and RT112 cells. Interestingly, we did not observe a correlation between AIMP3 expression and p53 expression in the tissue samples and, while our observations support a role for AIMP3-mediated radiosensitisation via a p53-dependent mechanism, further studies are warranted (Fig. 3*c*).

To test our hypothesis that AIMP3 status might be predictive to radiotherapy outcome, we interrogated the BCON TMA stratified by AIMP3 staining in the cores (Fig. 4*a*). There were no significant demographic differences between the AIMP3-positive and AIMP3-negative groups. There was good intra-observer (Kappa = 0.7 ± 0.1, $p < 0.001$) and inter-observer (Kappa = 0.5 ± 0.1, $p < 0.001$) agreement in the scoring methodology. The median overall survival (OS) of the cohort was 40.8 ± 9.4 months (95% CI: 22.3 to 59.3 months).

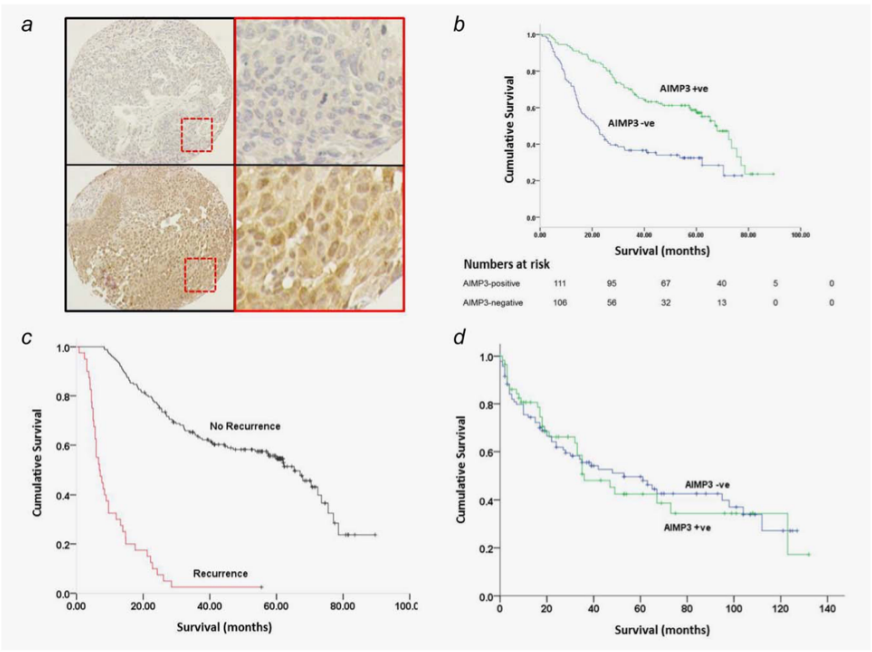

Figure 4. Reduced expression of AIMP3 is associated with reduced survival following radiotherapy and higher risk of recurrence. (*a*) AIMP3 expression in the BCON TMA. Example of AIMP3-negative (top) and AIMP3-positive (bottom) tumours that were treated with either radical radiotherapy alone or radical radiotherapy supplemented with carbogen and nicotinamide. (*b*) Kaplan-Meier plot illustrates that the adjusted HR for death in AIMP3-positive group was half the HR for death in the AIMP3-negative group in the BCON cohort. (*c*) Kaplan Meier plot depicts the HR for death in patients with tumour recurrence following radiotherapy was 8.8 relative to patients without recurrence. (*d*) AIMP3 expression was evaluated on a radical cystectomy TMA set. Kaplan Meier plot demonstrates no significant differences in the HR for death between the AIMP3-positive and negative groups. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

For those with AIMP3-negative tumours, median OS was estimated at 21.5 ± 3.0 months (95% CI: 15.7 to 27.3 months). The median OS of patients with AIMP3-positive tumours was greater at 67.9 ± 5.0 months (95% CI: 22.3 to 59.3 months). This difference in OS between the AIMP3-negative and AIMP3-positive groups was significant (Fig. 4*b*); the hazard ratio was 0.53 (95% CI: 0.36 to 0.78, $p = 0.002$) indicating a 47% lower risk of death in the AIMP3- positive group as compared to the AIMP3-negative group. There was no significant interaction effect with the BCON randomisation arms indicating that AIMP3 is not a predictor of response to hypoxia modification.

When cystoscopic tumour status following radiotherapy was considered, there were 40 (18%) recurrences. Risk of death increased significantly in those with tumour recurrence when compared to those without recurrence (HR 8.8, 95% CI: 5.5 to 14.3, $p < 0.001$) (Fig. 4*c*). Of the 40 recurrences, 30 occurred in MIBC patients with AIMP3-negative tumours pre-treatment ($p < 0.001$) (Supporting Information Table 2). To test if AIMP3 expression was prognostic for survival in MIBC, we analysed AIMP3 expression on a radical cystectomy (untreated/control) TMA set. AIMP3 was not associated with survival in this control set (HR 0.91, 95% CI: 0.56 to 1.47, $p = 0.70$). These findings indicate that AIMP3 is a predictive marker of radiotherapy response in organ-confined MIBC. In addition, we also investigated the expression of p53 in the two patient cohorts. P53 expression was neither prognostic in the control set nor predictive of radiotherapy response in the BCON set (Supporting Information Fig. 5). Furthermore, there was no significant correlation between AIMP3 and p53 protein expression in these cohorts.

Discussion

We undertook a whole genome expression profiling exercise of a cohort of bladder cancers to identify components of the regulatory and signaling pathways involved in bladder tumorigenesis.[15] In this study, we mined the global gene expression dataset to identify novel tumour suppressor genes that are downregulated in bladder cancer. We employed a modified methylated gene discovery algorithm as previously described to identify targets that were expressed at low levels in bladder cancer and shared promoter patterns with known cancer-specific methylated genes.[29,30] The identified genes were then functionally annotated based on their gene ontologies.[31] One of the targets identified was *AIMP3*, one of the non-enzymatic components of the AIMP family of macromolecular proteins involved in protein translation.[32–34]

AIMPs used to be considered as a class of housekeeping genes. In recent years, AIMPs have been shown to participate in a spectrum of functions that are unrelated to their primary support role in protein translation. AIMPs have been implicated in splicing, inflammation, angiogenesis, cellular proliferation, DNA repair and apoptosis.[32–36] Although, the dynamic structural interactions and role in signaling cascades have not been well-delineated, there is growing evidence of ARS multi-enzyme complex participation in tumorigenesis.[34–36]

With respect to *AIMP3*, also known as eukaryotic elongation factor 1 epsilon-1 (*EEF1E1*) or elongation factor p18, there are reports suggesting that it is an important haploinsufficient tumour suppressor.[23,24] Loss of expression of AIMP3 has been reported in acute promyelocytic leukemia, chronic myelogenous leukemia, liver, gastric and colorectal cancers.[30–34] No studies to date have reported on AIMP3 expression in bladder cancers. The findings in our study, of the loss of AIMP3 in bladder cancer, corroborate the findings of loss of AIMP3 in other cancers in those studies. Furthermore, our study reports on the pattern of reduced expression of AIMP3 across different stages and grades. In our study, AIMP3 was expressed at moderate and high levels in all normal urothelium. However, only 56% of tumours expressed AIMP3 at moderate and high levels. Loss of AIMP3 expression was more pronounced in late stage (≥T2) tumours, where less than 30% of these tumours expressed AIMP3 at moderate and high levels. The pattern of expression and localisation of AIMP3 agrees with the findings in gastric and colorectal cancers, whereby loss of AIMP3 was observed in 20 to 30% of tumours.[25] In that study, the expression across different stages and grades was not reported. Although reduced AIMP3 expression was associated with higher stage and grade, AIMP3 was present in a subset of tumours of all stages and grades.

Structural analyses of AIMP3 suggest that there are molecular residues that are critical to its tumour-suppressive activity.[37] AIMP3 somatic mutations have been found in chronic myelogeneous leukaemia, but no mutations have been found in acute leukaemia, gastric, breast, colorectal or liver cancers; suggesting that point mutations are not a frequent mechanism of AIMP3 inactivation.[23,38] Hemizygous deletions at the *AIMP3* locus have been reported in cell lines but not in cancer tissues, although allelic loss in the region 6p24–25 is seen in some lymphomas and ovarian cancers.[23,34,39,40] Allelic imbalances at the terminal end of 6p have been reported in bladder cancers.[41]

When we looked for AIMP3 deletions in an aCGH analyses of a series of 98 bladder tumours, no hemi- or homozygous deletions of the *AIMP3* locus at 6p24.3 (GRCh37: 6:8,073,592-8,102,887) were detected.[17] Promoter hypermethylation of tumour suppressor genes is a reported event in bladder tumorigenesis.[42–44] Therefore, we looked into this. Although our study of promoter methylation of *AIMP3* suggests a link with AIMP3 expression in bladder cancer, it does not adequately explain the frequency of loss of expression observed in our cohort of tumours. This observation combined with the fact that the AIMP3 locus is not frequently deleted may suggest that other post-transcriptional regulatory mechanisms may contribute to the loss of *AIMP3* expression in bladder cancer. However, the potential functional importance of *AIMP3* downregulation in bladder tumorigenesis led us to further explore the link between the loss of *AIMP3* and genomic stability and, more pertinently, the potential clinical significance of *AIMP3* dysregulation.

AIMP3 is induced and translocated to the nucleus in response to DNA damage and oncogenic-mediated activation.[23,24] Low AIMP3 expressing cells have reduced p53 activity and resistance to apoptosis.[23] However, AIMP3-dependent resistance to apoptosis has also been proposed to occur in a p53-independent manner.[45] When cells are exposed to genotoxic stress, AIMP3 directly interacts with and activates the ATM and ATR tumour suppressing kinases in the nucleus, subsequently resulting in p53 upregulation. The suppression of AIMP3 accompanies the downregulation of ATM although a feedback mechanism seems to link ATM and AIMP3.[23,24,37] AIMP3 induction by Ras is evident even in ATM-deficient cells and AIMP3 target specificity is Ras-type dependent.[24] *AIMP3* heterozygous cells are susceptible to Ras and Myc induced transformation and these cells exhibit gross chromosomal instability that is associated with abrogated cell cycle controls.[24] Although *AIMP3*+/- mice demonstrate normal growth and morphology, they spontaneously develop tumours and the cancer incidence is age-dependent. Some of the tumours developed in these *AIMP3* heterozygous mice harboured invasive and metastatic abilities.[23]

Our FGA findings suggest that loss of *AIMP3* is associated with genomic instability in bladder cancer. The correlation of reduced *TP53TG1* expression in the bladder cancer specimens with high-grade/stage and loss of AIMP3 further supports this observation. *TP53TG1* expression is induced in a wild-type p53-dependent manner and is postulated to play a role in DNA damage response.[45,46] The association between low AIMP3 expression with Tp53 transactivity, genomic instability and IR resistance, coupled to the previous work

implicating this haplo-insufficient tumour suppressor as an integral player in DDR, led us to postulate that the loss of AIMP3 expression may have clinical significance. Genomic instability and the increased propensity to acquire genome-wide aberrations are associated with inherent cellular radioresistance and thus play a critical role in the failure of radiotherapy.[46,47]

As we identified an association between the loss of AIMP3 and increased genomic instability, we hypothesised that the loss of AIMP3 is biologically significant and affects cellular response to IR in bladder cancers. To test this hypothesis, we investigated the effect of AIMP3 downregulation, by siRNA transfection, on IR outcome *in vitro* using bladder cancer cell lines. Clonogenic survival was increased in the bladder cancer cell lines (not 253J or HeLa) following IC50 irradiation in the AIMP3-knockdown cells relative to those with constitutive levels of AIMP3. The mechanism of IR resistance is not adequately elucidated here; however, recent studies indicate that AIMP3 is translocated to the nuclear compartment from a cytosolic location in response to IR.[23,47,48] Our *in vitro* findings suggest that bladder cancer cell survival in response to IR is potentially influenced by AIMP3 expression levels.

We hypothesised that AIMP3 expression status may be predictive of radiotherapy outcome in bladder cancer. To answer this question, we evaluated the expression of AIMP3 in a TMA of pre-treatment TURBT specimens from 217 organ-confined MIBC patients subsequently enrolled in the BCON trial.[11] In this Phase III trial, radiotherapy alone was compared with the same regimen given with carbogen and nicotinamide. The study reported favourable outcomes with respect to overall survival, disease-control and early or late morbidity in MIBC patients receiving radical radiotherapy. Although a benefit was found in favour of the combined arm, no clinic-pathological predictors of response to CON or to radiotherapy were found. In the current study, we found that AIMP3 expression status is predictive of overall survival and tumour recurrence in the BCON trial cohort. Interrogation of the radical cystectomy cohort demonstrated that AIMP3 status was truly predictive of radiotherapy outcome rather than just being prognostic of survival in those with MIBC. In contrast, p53 expression status was not predictive of radiotherapy outcome in the BCON trial cohort and was not prognostic of survival in the radical cystectomy cohort (Supporting Information Figs. 5*a* and 5b, respectively). This was consistent with reports in the literature suggesting the lack of clinical utility of p53 status in predicting clinical outcome in patients with bladder cancer treated with radiotherapy.[49,50]

In conclusion, we report *AIMP3* as a novel tumour suppressor gene in bladder cancer. We linked the reduction of AIMP3 expression to the impairment of p53 activity, genomic instability and radioresistance. The mechanism of AIMP3-mediated radioresistance corroborates previous findings but requires further delineation. AIMP3 expression was found to be significantly predictive of both disease recurrence and overall survival following radiotherapy in localized MIBC. These findings warrant large-scale external validation and our current findings suggest that AIMP3, either alone or within a panel of other predictive DDR markers, may help stratify the management of organ-confined bladder cancer patients by facilitating the selection of those likely to respond best to radiotherapy or other organ-preservation strategies such as chemo-radiotherapy. Such a personalised strategy would maximise treatment efficacy in those receiving radiotherapy and reduce the risk of treatment-related adverse events in those unlikely to benefit from radiotherapy who could instead be stratified to undergo radical cystectomy.

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