BIOMARKER AND TREATMENT TARGET DEVELOPMENT IN MUSCLE INVASIVE BLADDER CANCER

A thesis submitted to the University of Manchester for the degree of Doctor of Medicine in the Faculty of Medical and Human Sciences

2014

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School of Medicine
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<th>Full Form</th>
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<tbody>
<tr>
<td>5-ALA</td>
<td>5-Aminolevulinic acid</td>
</tr>
<tr>
<td>AIA</td>
<td>Automated image analysis</td>
</tr>
<tr>
<td>AKT</td>
<td>Synonymous with: Protein kinase B</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>Arf</td>
<td>Adenosine deaminase binding protein ribosylation factor</td>
</tr>
<tr>
<td>ASCO</td>
<td>American Society of Clinical Oncology</td>
</tr>
<tr>
<td>ASR</td>
<td>Age standardised rate</td>
</tr>
<tr>
<td>AZ</td>
<td>Astra Zeneca</td>
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<tr>
<td>BCG</td>
<td>Bacillus Calmette-Guérin</td>
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<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
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<tr>
<td>CIS</td>
<td>Carcinoma in situ</td>
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<td>CRUK</td>
<td>Cancer Research U.K.</td>
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<tr>
<td>CT</td>
<td>Computerised tomography</td>
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<tr>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
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<tr>
<td>CYR61</td>
<td>Cysteine rich angiogenic inducer 61</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3-Diaminobenzidine</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>E2F</td>
<td>A family of transcription factors</td>
</tr>
<tr>
<td>EAU</td>
<td>European Association of Urology</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>EORTC</td>
<td>European Organisation for Research and Treatment of Cancer</td>
</tr>
<tr>
<td>ERBB</td>
<td>Avian erythroblastic leukaemia viral oncogene</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridisation</td>
</tr>
<tr>
<td>FOXP3</td>
<td>Forkhead box P3</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<td>---------------------------------------------------------------------------</td>
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<tr>
<td>GSMT1</td>
<td>Glutathione S-transferase 1</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HAL</td>
<td>Hexaminolevulinate</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>Her-2</td>
<td>Synonymous with: EGFR2, ERBB2 and Neu</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HGFR</td>
<td>Hepatocyte growth factor receptor</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>ICC</td>
<td>Intra-class correlation</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>ILK</td>
<td>Integrin linked kinase</td>
</tr>
<tr>
<td>IVU</td>
<td>Intravenous urography</td>
</tr>
<tr>
<td>KRT20</td>
<td>Keratin 20, synonymous with CK20</td>
</tr>
<tr>
<td>LGUC</td>
<td>Low grade urothelial carcinoma</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of heterozygosity</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAPKK</td>
<td>Mitogen activated protein kinase kinase</td>
</tr>
<tr>
<td>MCRC</td>
<td>Manchester Cancer Research Centre</td>
</tr>
<tr>
<td>MDM2</td>
<td>Murine double minute 2 oncogene</td>
</tr>
<tr>
<td>MEK</td>
<td>Extracellular signal regulated kinase kinase, synonymous with MAPKK</td>
</tr>
<tr>
<td>MIBC</td>
<td>Muscle invasive bladder cancer</td>
</tr>
<tr>
<td>MR</td>
<td>Magnetic resonance</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>NAT2</td>
<td>N-acetyltransferase 2</td>
</tr>
<tr>
<td>NMIBC</td>
<td>Non-muscle invasive bladder cancer</td>
</tr>
<tr>
<td>NMP22</td>
<td>Nuclear matrix protein 22</td>
</tr>
<tr>
<td>NRG1</td>
<td>Neuregulin 1</td>
</tr>
<tr>
<td>PAK</td>
<td>p21 activated kinase</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>pRB</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PUNLMP</td>
<td>Papillary urothelial neoplasm of low malignant potential</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>RPA2</td>
<td>Replication protein A2</td>
</tr>
<tr>
<td>SCC</td>
<td>Squamous cell carcinoma</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin ribonucleic acid</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering ribonucleic acid</td>
</tr>
<tr>
<td>SWOG</td>
<td>Southwest Oncology Group, USA</td>
</tr>
<tr>
<td>TCC</td>
<td>Transitional cell carcinoma</td>
</tr>
<tr>
<td>TCP</td>
<td>Tissue culture plastic</td>
</tr>
<tr>
<td>TMA</td>
<td>Tissue microarray</td>
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<tr>
<td>TSC1</td>
<td>Tuberous sclerosis protein 1</td>
</tr>
<tr>
<td>TUR</td>
<td>Transurethral resection</td>
</tr>
<tr>
<td>TURBT</td>
<td>Transurethral resection of bladder tumour</td>
</tr>
<tr>
<td>UICC</td>
<td>International Union Against Cancer</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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Abstract

Biomarker and treatment target development in muscle invasive bladder cancer

A thesis submitted to the University of Manchester for the degree of Doctor of Medicine

Richard Robinson – 2014

Introduction: The outcomes following radical treatment for bladder cancer (BC) remain poor, with 5 year overall survival (OS) rates of approximately 50% and over 5000 deaths per year in the U.K. There has been paucity of significant therapeutic developments since the introduction of cisplatinum based chemotherapy in the 1970’s. The aim of this study was to identify putative drug targets for the treatment of this aggressive form of cancer.

Methods: A tissue microarray (TMA) was constructed from the cystectomy specimens of 497 BC patients and 70 controls, linked to a clinical database with extended follow up. The online database Oncomine® was interrogated to identify putative treatment targets which were subsequently evaluated using in-vitro models of high grade invasive bladder cancer (using the J82 and T24 cell lines). In-vitro modelling was conducted using siRNA target knockdown during proliferation, chemo-sensitivity, migration and Matrigel™ invasion assays. Expression of the putative targets was then correlated with tumour characteristics and patient outcomes, by IHC and automated image analysis of the TMA.

Results: The proteins CYR61 and CTGF were selected from Oncomine® and studied in conjunction with the HGF/MET axis, on the basis of known interactions in other cancer types. siRNA knockdown of both proteins abrogated HGF induced Matrigel™ invasion in both cell lines. CYR61 knockdown significantly reduced HGF induced cell migration and foetal calf serum (FCS) induced Matrigel™ invasion in both cell lines. Knockdown of both proteins also significantly increased the sensitivity of both cell lines to cisplatinum. CYR61 expression was significantly increased in BC samples compared to normal controls and an independent predictor of OS at 6 years (HR 1.493, p=0.030). In contrast, loss of CTGF expression was significantly associated with increasing tumour stage and worse OS. MET expression was reduced in BC compared to controls and not predictive of survival following cystectomy.

Conclusions: The in-vitro findings for CTGF as a treatment target were encouraging, although these findings were not supported by the TMA data. CYR61 promotes an aggressive bladder cancer phenotype and knockdown reverses features of EMT and increases chemo-sensitivity. Clinical cohort correlation confirms CYR61 to be a promising treatment target in bladder cancer.
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Dedication

This thesis is dedicated to my wife Claire, for her unwavering support and understanding.
1 Introduction

1.1 Epidemiology

Urinary bladder cancer is the second most frequently occurring malignancy of the urinary tract, after prostate cancer. It is the seventh commonest cancer in the U.K with 10 399 new cases diagnosed in 2011. Bladder cancer is the fourth commonest cancer in men and the thirteenth in women, with 5242 deaths attributable to bladder cancer in 2012 [1]. There is a wide variation in both the global incidence and histological subtype of bladder cancer that occurs. The highest rates occur in developed countries with age standardised incidence rates (ASR) of 19.5 and 4.2 per 100 000 for men and women respectively, compared to 5.3 and 1.6 for in less developed areas of the world [2].

There are a number of factors that may falsely give the impression that all of the differences in reported incidence of bladder cancer throughout the world are attributable to true differences between these populations:

- Bladder cancer is rare under the age of 50, with the peak ASR occurring in men aged 75-79 and in women over the age of 85 in the U.K. As a consequence, in the developing world where life expectancy is lower, this age of peak incidence may not be reached.

- The data used in examining the epidemiology of cancer is obtained from national cancer registries, for which there is significant recording bias, leading to geographical variation [3]. Even throughout the developed world there is wide variation in the reporting and registration of bladder cancer, particularly with respect to the variable inclusion of CIS and stage Ta transitional cell carcinoma (TCC) in registries [4].
Taking these factors into consideration it is still considered that there remains a true difference in the incidence of bladder cancer, largely attributable to variations in exposure to risk factors for the disease [2].

In the U.K. there has been a progressive decline in bladder cancer incidence rates since a peak in the early 1990’s, with overall ASR falling from 18.4 per 100 000 in 1993 to 11.5 in 2008, although this decline is far more pronounced in men than women. This trend in the U.K. corresponds to declining incidence and mortality rates in the European Union (EU) since the early 1990’s and in USA since the 1970’s [5-7]. Whilst incidence and mortality rates have declined in most of Europe, in the eastern European countries of Bulgaria, Poland and Romania, mortality rates have continued to increase with the highest mortality rates recorded in Poland, Spain, Latvia and Lithuania in 2005-2008 [5].

In stark contrast to declining rates in most developed countries the incidence of bladder cancer is predicted to rise in less developed areas of the world as smoking rates increase in these countries. Tobacco use, a significant risk factor for developing bladder cancer, has increased by 16.1, 8.7 and 6.5% in Africa / Middle East, Eastern Europe / former Soviet Union and Asia / Australia between 1998 and 2008 respectively [2].

In developing regions of the world where schistosomiasis is endemic, squamous cell carcinoma is the predominant histological subtype of bladder cancer. This contrasts the predominance of transitional cell carcinoma (TCC) observed in developed countries, although this balance may be changing. In Egypt, where schistosomiasis is endemic, there has been a reported decline in the proportion of SCC histology bladder cancer from 78% in 1980 to 27% in 2005, mirrored by an increase in TCC. This change is attributed to a reduction in infection rates with Schistosoma haematobium and increasing smoking prevalence, a known risk factor of TCC [8].
1.2 Aetiology

Occupational exposure and cigarette smoking are the most well defined risk factors for the development of bladder cancer, other than male gender and increasing age.

The identification of a link between occupational exposure and bladder cancer was first described in 1895 by Rhen [9], who identified an association between bladder cancer and aniline dye production in Germany. In 1954 Case and Hosker described in an epidemiological study the association between bladder cancer and occupational exposure to aromatic amines in the rubber industry in England and Wales [10]. Following this study a great number of studies were published linking occupational to exposure arylamines with an increased risk of bladder cancer, in a number of industries including: rubber workers, dye manufacture, printing and textiles, reviewed by Vineis [11]. In historical series in developed countries, up to 25% of bladder cancer cases are attributable to occupational exposure. The compound 2-naphthylamine was banned along with a number of other aromatic amines in the U.K. in 1967 and was subsequently banned in the majority of developed countries, although exposure to these agents continues to be of concern in a number of countries.

Large scale epidemiological studies of cancer risk continue to identify high risk occupations for the development of bladder cancer, which appear to centre around occupational exposure to bladder carcinogens and smoking, including: tobacco workers, hairdressers, printers, chemical process workers [12, 13]. The exact causation in these populations may not relate directly to their occupational exposure as smoking remains confounding factor. This is exemplified by the work of Pukkala [12] in which waiters and tobacco workers were found to have a high risk of bladder cancer but were also were the highest risk groups for lung cancer, indicating that smoking probably assigns the risk for bladder cancer in these groups rather than occupational exposure.
A number of publications have investigated the link between the use of hair dyes and bladder cancer risk, in part due to epidemiological evidence and the finding of a number of potentially carcinogenic aromatic amine and derivative compounds in hair dyes in the 1970’s [14]. The evidence to date is contradictory, as published in a meta-analysis by Rollinson et al. in 2006 [15], although it appears that the time and duration of exposure coupled to genetic susceptibility may be important. Recent work by Koutros et al. [16] found no overall association between hair dye use and bladder cancer risk, although there was an increased risk amongst women users of hair dye who were NAT2 slow acetylators. Bladder cancer has been linked to a number of other agents, including diesel exhaust pollution [17] and coffee consumption to although again the confounding factor with coffee appears to be smoking status [18].

The process of aluminium smelting results in the exposure to a number of polycyclic aromatic hydrocarbons and a large number of studies, predominantly from the USA and Canada, have demonstrated an increased risk of bladder cancer amongst aluminium smelters, although the exact compounds responsible for this risk remain uncertain [19].

N-acetyltransferase 2 (NAT2) is an enzyme involved in the metabolism of aromatic amines. The status of an individual is designated as either a ‘slow or fast acetylator’ and has been implicated in bladder cancer risk. The slow acetylator genotype is associated with a 40% increased risk of bladder cancer. Glutathione S-transferase 1 (GSMT1) status, involved in the metabolism of polycyclic hydrocarbons and detoxification of reactive oxygen species, is also a significant risk factor with the null genotype conferring a 50% increased risk of bladder cancer, although this association is only strong in non smokers [20].

Smoking remains the most significant preventable cause of bladder cancer with up to 30% of female and 50% of male bladder cancers attributable to tobacco smoking [21]. Smokers have a 2-4 times increased risk of bladder
cancer compared to those who have never smoked. The risk reduces with stopping smoking, but never down to the risk of those who have never smoked. [22]. Tobacco smoke contains reactive oxygen species and a number of important carcinogenic compounds including: polycyclic aromatic hydrocarbons, aromatic amines, heterocyclic amines and N-nitrosamines that cause the DNA damage that drives carcinogenesis [23].
1.3 Pathology and Grading

1.3.1 Grading

In developed countries TCC contributes the majority of bladder cancer cases at around 90% with the remaining cases attributable to SCC, adenocarcinoma, small cell carcinoma and other rare forms, in decreasing order of frequency [7] The terms transitional cell carcinoma and urothelial carcinoma are synonymous and used interchangeably in published literature.

In 1973 the WHO introduced a classification system for urothelial / transitional tumours of the human urinary bladder. This categorised tumours into four groups, comprising: urothelial papillomas and carcinomas of grade 1, 2 or 3. In 1998 an updated version of the classification system was proposed by the WHO and International Union Against Cancer (UICC) and subsequently published in 2004 by the WHO [24].

Grignon in 2009 [24, 25] stated that this reclassification was felt necessary in the 1990’s for 3 main reasons:

2. With the introduction of intra-vesical therapy as standard practice there was a need to clearly identify high risk tumours.
3. The 1973 classification was imprecise and led to the many pathologists using the 3 grade system to create 5 grades of disease (1, 1-2, 2, 2-3 and 3).”

The 2004 WHO classification (Table 1.1) provided classification of flat lesions and divided non-invasive papillary lesions into papillary urothelial neoplasms of low malignant potential (tumours which have negligible risk of progression, but are not completely benign and have a tendency to recur) and low-grade or high-grade urothelial carcinomas [26]. A decade has elapsed since the
introduction of the 2004 classification system yet most studies to date have not adopted the new system, neither have most U.K. urological centres and the most recent EAU guidelines [26] on the management of bladder cancer continue to use the 1973 system. Figure 1.1 demonstrates the overlap between the 1973 and 2004 systems.

Table 1.1 - WHO grading of urothelial / transitional bladder cancer in 1973 and 2004
(Reproduced from the EAU Guidelines on the management of Non-Muscle Invasive Bladder Cancer 2013 [26])

<table>
<thead>
<tr>
<th>1973 WHO grading</th>
<th>2004 WHO grading</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urothelial papilloma</td>
<td>Flat lesions</td>
</tr>
<tr>
<td>Grade 1: well differentiated</td>
<td>Hyperplasia (flat lesion without atypia or papillary aspects)</td>
</tr>
<tr>
<td>Grade 2: moderately differentiated</td>
<td>Reactive atypia (flat lesion with atypia)</td>
</tr>
<tr>
<td>Grade 3: poorly differentiated</td>
<td>Atypia of unknown significance</td>
</tr>
<tr>
<td>Urothelial dysplasia</td>
<td>Urothelial CIS</td>
</tr>
<tr>
<td>Urothelial CIS</td>
<td>Papillary lesions</td>
</tr>
<tr>
<td>Papillary lesions</td>
<td>Urothelial papilloma (completely benign lesion)</td>
</tr>
<tr>
<td>Urothelial papilloma (completely benign lesion)</td>
<td>Papillary urothelial neoplasm of low malignant potential (PUNLMP)</td>
</tr>
<tr>
<td>Low-grade papillary urothelial carcinoma</td>
<td>High-grade papillary urothelial carcinoma</td>
</tr>
</tbody>
</table>

Figure 1.1 - Comparison of the 1973 and 2004 WHO grading system of urinary bladder TCC
(Reproduced from Maclennan et al. E Urology 2007 [24])
1.3.2 Staging

Bladder cancer is staged using the UICC TNM Classification of Malignant Tumours system, first developed by Pierre Denoix in France between 1943 and 1952, described in the latest edition of the UICC TNM classification in 2009 [27]. In this classification system, T denotes the tumour stage, N denotes the lymph node status and M denotes the distant metastatic status (Table 1.2). The prefixes c and p denote whether the status is based upon clinical findings, or pathological examination respectively.

Approximately 75% of patients with TCC present with non-muscle invasive bladder cancer (NMIBC) comprising Ta, Tis (CIS) and T1 tumours with the remaining 25% presenting with muscle invasive bladder cancer (MIBC) ≥ T2 with or without lymph node involvement or distant metastases.
### T - Primary tumour

<table>
<thead>
<tr>
<th>Code</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TX</td>
<td>Primary tumour cannot be assessed</td>
</tr>
<tr>
<td>T0</td>
<td>No evidence of primary tumour</td>
</tr>
<tr>
<td>Ta</td>
<td>Non-invasive papillary carcinoma</td>
</tr>
<tr>
<td>Tis</td>
<td>Carcinoma in situ: ‘flat tumour’</td>
</tr>
<tr>
<td>T1</td>
<td>Tumour invades sub-epithelial connective tissue</td>
</tr>
<tr>
<td>T2</td>
<td>Tumour invades muscle</td>
</tr>
<tr>
<td>T2a</td>
<td>Tumour invades superficial muscle (inner half)</td>
</tr>
<tr>
<td>T2b</td>
<td>Tumour invades deep muscle (outer half)</td>
</tr>
<tr>
<td>T3</td>
<td>Tumour invades perivesical tissue:</td>
</tr>
<tr>
<td>T3a</td>
<td>Microscopically</td>
</tr>
<tr>
<td>T3b</td>
<td>Macroscopically (extravesical mass)</td>
</tr>
<tr>
<td>T4</td>
<td>Tumour invades any of the following: prostate, uterus, vagina, pelvic wall, abdominal wall</td>
</tr>
<tr>
<td>T4a</td>
<td>Tumour invades prostate, uterus or vagina</td>
</tr>
<tr>
<td>T4b</td>
<td>Tumour invades pelvic wall or abdominal wall</td>
</tr>
</tbody>
</table>

### N - Lymph nodes

<table>
<thead>
<tr>
<th>Code</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NX</td>
<td>Regional lymph nodes cannot be assessed</td>
</tr>
<tr>
<td>N0</td>
<td>No regional lymph node metastasis</td>
</tr>
<tr>
<td>N1</td>
<td>Metastasis in a single lymph node in the true pelvis (hypogastric, obturator, external iliac, or presacral)</td>
</tr>
<tr>
<td>N2</td>
<td>Metastasis in multiple lymph nodes in the true pelvis (hypogastric, obturator, external iliac, or presacral)</td>
</tr>
<tr>
<td>N3</td>
<td>Metastasis in common iliac lymph node(s)</td>
</tr>
</tbody>
</table>

### M - Distant metastasis

<table>
<thead>
<tr>
<th>Code</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MX</td>
<td>Distant metastasis cannot be assessed</td>
</tr>
<tr>
<td>M0</td>
<td>No distant metastasis</td>
</tr>
<tr>
<td>M1</td>
<td>Distant metastasis</td>
</tr>
</tbody>
</table>

**Table 1.2 - TNM Classification of Bladder Cancer**

(Reproduced from the EAU Guidelines on the management of Non-Muscle Invasive Bladder Cancer 2013 [26])
1.4 Molecular Biology and Biomarkers

There is a distinction in the clinical management of bladder cancer between NMIBC (Ta-T1 and CIS) and MIBC (T2-T4). Those patients with stage ≥T2 mandate a radical approach to treatment, where possible, with curative intent by radical cystectomy or radical radiation therapy. For patients with NMIBC the mainstay of treatment is endoscopic resection of tumours with the addition of intra-vesical chemotherapy or immunotherapy in those at high risk of recurrence of progression. This distinction separates bladder cancer into two almost separate diseases on a clinical basis and this distinction is supported in part when the molecular characteristics of these two groups are examined. This is an over simplification and there are exceptions. High grade T1 tumours are at high risk of progression, with a poor prognosis compared with Ta tumours, particularly when additional risk factors including tumour multiplicity, tumour size and the presence of co-existent CIS are considered [28-30]. CIS, which is non-invasive by virtue of its histological appearance, is at high risk of recurrence and progression [26]. CIS is less well characterised at a molecular level than papillary and solid tumours, due to the limited amounts of tumour tissue available to work with. This is due in part to the fact that the urothelial cells involved in CIS are only loosely adherent to the underlying basement membrane and as such are often lost from pathological specimens. The evidence to is somewhat contradictory, but indicates that most T1 tumours and CIS do not form part of the same molecular sub-group as Ta tumours [31, 32].

1.4.1 Chromosomal Changes

Chromosomal alterations in bladder cancer are well characterised. Deletion or allelic loss at “2q, 3p, 4p, 5p, 8p, 9p, 9q, 10q, 11p, 13q, 17p and 18q” [33] are common events in transitional cell carcinoma. Chromosome 9 alterations appear to be an early event in the development of bladder cancer but do not
appear to be associated with cancer behaviour. This is in contrast to 3p changes that appear to be a late event associated with advanced stage and grade and alteration in chromosomes 8p, 17p and 13q that are associated with a more aggressive phenotype [33].

1.4.2 p53

The TP53 gene, the first tumour suppressor gene identified in 1979, located on chromosome 17p13, encodes the protein p53. This protein forms the central basis for the tumour suppressive mechanisms within cells. It acts as a transcription regulator of a great number of genes by binding to gene promoter regions. In response to a number of signals, including: oncogene activation, cell stress and in particular DNA damage, p53 exerts a number of effects including:

- **Cell cycle arrest and DNA repair**
  Through the up-regulation of p21, a number of cyclin dependent kinases are inhibited leading to arrest of the cell cycle at the G1-S and G2-M checkpoints. p21 also activates DNA repair mechanisms through PCNA (proliferating cell nuclear antigen).

- **Apoptosis**
  p53 exerts control over a number of cell apoptosis factors, enabling the direction of a cell down a pathway of apoptosis, rather than replication and propagation of DNA damage, preserving the genome.

The control of p53 in the normal cell is via the ubiquitin ligase MDM2, rather than regulation of the TP53 gene. MDM2 is itself transcriptionally regulated by p53, generating a regulatory feedback loop. MDM2 also binds to p53, inhibiting the transcriptional activity of p53. MDM2 therefore has the ability to inhibit p53 activity, tag the protein for degradation and is in itself dependent upon p53 for as the MDM2 gene is a transcriptional target of p53 [34].
Through this mechanism p53 acts the ‘guardian of the genome’. It is therefore not surprising that there is a high incidence of p53 pathway abnormalities within many cancers. Missense mutations of TP53 are found in approximately 50% of human cancers, with the overall percentage of cancers with abnormalities within the p53 pathway reported as higher than this figure [35].

TP53 mutation status exemplifies the genetic difference between Ta and T2 tumours. Deletion or mutation of TP53 occurs in approximately 70% of ≥T2 tumours yet is a rare event in Ta tumours [31]. Multiple studies have been published on the association between p53 status and outcome, with the majority using nuclear accumulation as p53 as a surrogate for mutation status. Missense mutations, the commonest mutation in TP53, lead to an increased half life and nuclear accumulation. Mutation status and nuclear accumulation are not entirely concordant with 51% of cases with TP53 mutations and up to 27% of wild type TP53 leading to nuclear accumulation of p53 [36]. Despite this discrepancy, p53 nuclear accumulation is widely reported to be associated with increased tumour stage and a predictor of outcome in TCC [37-43] and in SCC [41, 44] of the bladder. In 2005 a meta-analysis of the previous decade of research into p53 expression and mutation status in bladder cancer was reported [45]. This analysis included data on 10 026 patients and found a significant association between p53 status and outcome. However, the authors noted significant heterogeneity between multiple aspects of published studies and recommended that before p53 could be used in clinical practice a large multi centre prospective trial needed to be conducted using standardised laboratory methodology. It is notable, that 5 years prior to this meta-analysis Schmitz-Drager et al. conducted a meta-analysis to answer the same question and concluded that further retrospective studies would shed no further light onto the uncertainty regarding the utility of p53 in bladder cancer [46].

Although there remains uncertainty regarding the utility of p53 as a marker of disease risk in bladder cancer, a phase III trial has been conducted in which patients with p53 positive tumours following radical cystectomy, assessed by
immunohistochemistry, were randomly assigned to receive either 3 cycles of MVAC adjuvant chemotherapy or observation. Unfortunately this trial failed to confirm the prognostic value of p53 due to a high patient treatment refusal rate and the trial being stopped early by the monitoring body due to a futility of analysis [47].

1.4.3 Retinoblastoma Protein

The retinoblastoma protein (pRB) from the pocket family of proteins is encoded by the tumour suppressor gene \textit{RB1} at 13q14.1-14.2. Deletion of both alleles in early childhood leads to the development of retinoblastoma in children, although the gene is implicated in multiple cancer types outside of childhood. pRB exerts its control through the cell cycle at the G1 to S phase transition. In its hypo-phosphorylated state pRB binds the transcription factors E2F and HDAC. The phosphorylation of pRB is controlled by cyclin-D and cyclin-E, which during G1-S phase transition phosphorylate pRB leading to a loss of pRB binding to E2F and HDAC leading to increased transcription of a number of genes involved in cell cycle regulation and DNA replication [48, 49].

Abnormalities of pRB expression have been demonstrated to take one of 2 forms, either loss of expression due to heterozygosity (a common event in MIBC [50]), mutation and deletion or increased expression associated with loss of pRB function and hyper-phosphorylation of pRB [51, 52]. Alteration in the expression of pRB, assessed by immunohistochemistry, is known to be associated with increasing grade [38, 40, 53, 54] and stage [52, 55] of bladder cancer. Abnormality of pRB status is described in 31.9% of CIS, 25.5% of Ta, 42.6% of T1, 40-41% of T2, 58% of T3 and 64% of T4 tumours [40, 53, 54, 56], although these associations do not reach statistical significance in all of the published series. Shariat et al. [54] described the pRB status of the primary tumour, normal adjacent urothelium, concomitant CIS and metastatic lymph node tissue in patients who underwent cystectomy, reporting altered pRB status in 50%, 22%, 43% and 68% respectively.
In the treatment of CIS with intravesical BCG the status of: pRB, p16 and p53 for the prediction of response to treatment have been investigated. Only pRB was predictive, with normal expression predicting an improved response [57], although this study contained only 27 patients and as such the results need to be interpreted with caution. The power of pRB status to predict response to BCG has been further demonstrated in other small studies [56, 58], although subsequent research on 62 patients in 2011 by Park et al. [59] found no association with pRB and BCG response. Similar conflicting results have been published in NMIBC outside of the BCG treatment setting with studies demonstrating no association with recurrence and progression to pRB status [60, 61] and other demonstrating a predictive power of pRB status [62].

Loss of pRB expression has been demonstrated to predict improved response to radical radiation therapy [63, 64] but to date no statistically significant association between pRB loss and response to chemotherapy in MIBC has been demonstrated [38]. Conflicting results with respect to long term outcome have been obtained with a number of series demonstrating no independent predictive value for alterations in pRB status and overall survival following radical treatment of bladder cancer [38, 60], whereas other have demonstrated an independent predictive value [53] with 5 year survival rates of 64-67% for normal pRB status, contrasted with 33-43% for those with abnormal pRB status [40, 54].

### 1.4.4 Fibroblast Growth Factor Receptors

The fibroblast growth factor receptor (FGFR) family comprises a group of 4 highly conserved transmembrane receptor tyrosine kinases, FGFR 1-4, whose ligands constitute the 22 fibroblast growth factors (FGF) [65]. Activation of FGFR’s by their ligands leads to the activation of pathways involved in cell proliferation, differentiation and tumourigenesis [66] including MAPK, PI3K and protein kinase C [67]. In 1999 Cappellen et al. initially
sparked interest in FGF3 and bladder cancer, identifying activating mutations of FGFR3 in 9 of 26 (35%) bladder cancer cases [68]. It is thought that activating mutations of FGFR3 may drive tumourigenesis by overcoming the normal mechanisms of cell-cell contact inhibition [67].

Studies to date have identified mutations in the FGFR3 gene in: 0% CIS, 64-74% of Ta, 21% pT1, 16% pT2-4 tumours, comprising 84-88% of grade 1 tumours, 55-62% grade 2 and 7-16% of grade 3 [69-73]. Over-expression of FGFR3 is found in 49-76.6% of pTa, 34-72% pT1 and 15-49% pT2 tumours [74-76]. A highly significant correlation between protein expression and mutation status has been described [72], and in those patients with high FGFR3 expression 38.5% of pT2 tumours were wild-type versus 7.7% mutant and for pTa tumours 13.4% were wild-type versus 56.7% mutant.

Analysis of the mutation status of areas of normal appearing urothelium from patients with mutant FGFR3 tumours [77] has revealed no evidence of FGFR3 mutations, indicating that FGFR3 mutations are not the earliest event in bladder cancer carcinogenesis. Although mutations are observed in flat urothelial hyperplasia, believed to be the precursor of papillary tumours, indicating that FGFR3 mutation must occur relatively early in the development of bladder cancer via this pathway [78].

There is a lower recurrence rate of Ta/T1 tumours that were FGFR3+/p53- (with respect to protein expression) compared with FGFR3+/p53+, FGFR3-/p53+ and FGFR3-/p53- tumours [75]. In PUNLMP’s a statistically significant difference between recurrent and non-recurrent tumours is described with 56.4% and 80.5% staining strongly for FGFR3 respectively [79].

In 2003 van Rhijn examined the significance of a number of markers, including FGFR3 mutation status, and identified in multivariate analysis that FGFR3 mutation status was an independent predictor of outcome, with the conclusion that FGFR3 mutation represented a more favourable pathway of bladder cancer [71]. This favourable prognosis associated with FGFR3 mutation has been replicated in retrospective studies of pTa [69] and pT1.
tumours [80] but not demonstrated in a tissue microarray (TMA) based study of pTa tumours [81]. The only prospective study to date [82] which aimed to investigate the prognostic potential of 3 molecular markers, including FGFR3 mutation, and both the WHO 1973 and 2004 grading systems in NMIBC revealed that overall none of the markers or systems could predict recurrence. Subsequent work by van Rhijn et al. [83] classified non-muscle invasive tumours into one of 3 molecular grades based upon FGFR3 mutation status and MIB-1 expression. They concluded that the addition of this molecular grade to the EORTC scores (described below) increases predictive accuracy for progression from 74.9% to 81.7% (p<0.001) and that molecular grade was more reproducible than pathological grade.

FGFR3 has subsequently been investigated as a target for therapy in cancer. Monoclonal antibodies to FGFR3 have demonstrated an anti-proliferative effect in the high FGFR3 expressing RT-112 cell line [74, 84]. The anti-human FGFR3 IgG1 monoclonal antibody, MFGR1877A, has completed preclinical pharmacokinetic studies and is due to commence the Phase 1 stage in multiple myeloma, the other notable human cancer with high rates of FGFR3 mutation [85]. Tyrosine kinase inhibitors (TKI) targeting FGFR3 are under investigation. Cell lines harbouring mutant FGF3 but at low levels of expression are unresponsive to TKI treatment in vitro, compared to those lines over-expressing either mutant or wild type FGFR3 which are sensitive [66]. This pattern has also been demonstrated in mouse xenograft models [86].

FGFR1 is far less well characterised in bladder cancer, although research interest has increased in recent years. TMA analysis by FISH of FGFR1 (8p12) has demonstrated an association between gene amplification or deletion and higher tumour grade and stage, but no association with FGFR1 protein expression assessed by immunohistochemistry [87]. Tomlinson et al. [88] demonstrated that over-expression of FGF1 in normal urothelial cells led to increased proliferation, survival and reduced apoptosis with downstream activation of the MAPK pathway. The same study demonstrated a variety of consequences from shRNA knockdown in a number of FGFR1 expressing
bladder cancer cell lines but no association between FGFR1 expression and tumour grade or stage. This indicates that it may be potential target for therapy but that its significance or role has yet to be determined. The same group have also examined the significance of splice variants of FGFR1. This is on the background of a known association between increases in the FGFR1β:FGFR1α ratio with worse prognosis in astrocytoma, breast and pancreatic cancer. Tomlinson et al. have shown that this ratio is significantly increased in association with tumour stage and grade and further, that FGFR1β is expressed at higher levels in most bladder cancer cell lines and has a higher affinity for FGF1 leading to increased proliferation [89].

Recent work supports the differing roles of FGFR 1 and 3 in the subtypes of bladder cancer. FGFR3 appears to be responsible for cell proliferation in “epithelial’ cell types, and FGFR1 responsible for invasion and metastasis formation in “mesenchymal” tumours [90]. The authors argue that it is the EMT status of the tumour in conjunction with FGFR status that will determine response to FGFR targeting therapy.

1.4.5 Ras

The Ras oncoproteins are GTPases, from a much larger superfamily of proteins that includes: Ras, Rho, Rab, Ran and Arf; and play a central role in the transduction of extracellular signals across the cytoplasm to the nucleus. They exist in either an active form when bound to GTP or in an inactive form when bound to GDP and form 3 groups K-, N-, and H-Ras. The GTPase-activating proteins (GAP’s) promote the conversion from active to inactive Ras. The most notable and well characterised activity of Ras is in the downstream activation of Raf with subsequent activation of the MAPK pathway in response to receptor tyrosine kinase EGFR signalling [91]. Mutation of the Ras gene has a frequency of 5-13% in bladder cancer with a decreasing incidence in higher grades and stages [73, 92-94]. Ras gene and FGFR3 mutation appear to be mutually exclusive events [92, 93, 95].
1.4.6 Phosphatidylinositol 3-kinase pathway

Abnormalities of PI3K pathway activation are believed to occur in bladder cancer by either mutation of: PIK3CA, TSC1, AKT or through loss of PTEN or PTEN function by LOH, deletion or mutation [31, 96]. Mutations in the PIK3CA gene have been described in 13-25% of bladder tumours, with a trend towards lower rates of mutation in higher grades and stage of tumour but without statistical significance in all studies [73, 93-95]. The TSC1 gene is located at 9q34, with chromosome 9 loss being a common event in bladder cancer, in addition the frequency of mutation of the TSC1 gene is up to 14.5% of bladder cancer [31]. LOH of chromosome 10 has been described 23-39% of bladder tumours [97-99] with higher rates in muscle invasive tumours of 24% compared with 6.6% in non-muscle invasive tumours [97, 99]. Although LOH for the PTEN locus, 10q23, is described in only 12% of bladder tumours, with no significant relationship to grade or stage [95].

Mutations are described with a reported frequency of 6-17% [98-100] and the frequency of abnormal PTEN protein expression is much higher at 49-63.8%, with loss of expression associated with increasing grade [59, 95, 101, 102]. In a single study, containing all grades and stages of bladder cancer PTEN expression was predictive of survival, more significantly when combined with p53 status, however the authors did not explain the multivariate analysis and this prediction may be explained by stage and grade alone [101], particularly as a this predictive power has not been confirmed by other authors [59, 102, 103]. AKT1 mutations have been described in high grade tumours but at a very low frequency and have been shown to occur in combination with FGFR3 mutations but not in association with PIK3CA mutations [73].

1.4.7 Epidermal Growth Factor Receptor Family

Interest in the EGFR family of transmembrane receptor tyrosine kinases in many cancers, including bladder cancer, has in part been prompted by the advances that have been made in treating patients with breast cancer whose tumours are positive for Her-2. The EGFR family includes EGFR
(ERBB1/Her-1), Her-2 (EGFR2/ERBB2/Neu), EGFR3 (ERBB3/Her-3) and EGFR4 (ERBB4/Her-4). Her-2 is an orphan receptor and is activated by dimerization with ligand activated adjacent receptors such as EGFR3, leading to auto-phosphorylation and activation of the MAPK pathway via Ras [104, 105]. Breast carcinomas that over-express Her-2 display a more aggressive phenotype and are associated with a worse prognosis. Trastuzumab, a monoclonal antibody directed against Her-2, has been extensively proven to improve the prognosis of breast cancer patients with both early and advanced Her-2 positive breast cancer [106].

The over-expression of Her-2 in bladder cancer is reported at between 8.5 and 81%, dependent upon patient group and the methodology used to assess expression [104, 107, 108], with increased expression significantly associated with increased stage [105]. In breast cancer there is evidence that response to trastuzumab is increased in those with higher HER2 copy numbers, indicating that IHC alone may be insufficient to guide treatment decisions [109]. In the largest series published to date, Lae et al. assessed Her-2 expression by IHC and gene amplification by FISH in 1005 invasive urothelial carcinomas. The authors reported IHC positivity in 9.2% of cases but protein over-expression and gene amplification in only 5.1%, indicating the number of patients with bladder cancer who would benefit for Her-2 targeted therapy may be small [108].

Chakravarti et al. [110] pooled patients from 4 neo-adjuvant chemoradiotherapy trials and examined Her-2 over-expression, revealing a statistically significant reduced complete response rate to chemotherapy in Her-2 positive patients. Bolenz et al. [111] reported a cohort of 198 patients undergoing radical cystectomy, positive Her-2 status assessed by protein expression by IHC was independently associated, on multivariate analysis, with recurrence and mortality with hazard ratios of 1.96 and 2.07 respectively [112]. Subsequently Fleischmann et al. in 2011 [113] described protein expression by IHC and gene amplification by FISH in a cohort of patient with node positive disease following cystectomy, using standardised criteria [112]. Higher rates of gene amplification in 13.8% of nodal metastases compared to
7% in the primary tumour were identified, with poor concordance between IHC and FISH with 38.5% of amplified tumours being negative on IHC. Only Her-2 status in the primary tumour was predictive of outcome.

Phase 2 clinical trials using agents to target EGFR’s in bladder cancer have overall been disappointing. The use of trastuzumab in combination with chemotherapy for patients with metastatic TCC in a phase 2 trial, Hussain et al. [107], demonstrated feasibility but the reported median survival of 14.1 months is very similar to that seen in patients treated with chemotherapy alone in other trials [114-116]. As single agent neo-adjuvant therapy there is some evidence of tumour down staging with erlotinib prior to cystectomy [117]. In the metastatic setting, gefitinib in combination with chemotherapy in both the chemotherapy naïve and those who had received prior chemotherapy has failed to yield an improved response, reviewed by Mooso et al. [117].

Targeting EGFR’s in bladder cancer has to date failed to yield responses that are likely to change current management. One explanation for this may be poor patient selection. In the majority of trials conducted to date, with the exception of Hussain and co-workers, protein expression and gene copy number alterations have not formed part of trial inclusion criteria. It is therefore not unsurprising that the responses observed are disappointing, given the low level of Her-2 copy number alterations observed in bladder cancer [108] and the importance of Her-2 status in predicting response to Her-2 targeting therapy in breast cancer.

1.4.8 **MET**

Activation of the receptor tyrosine kinase MET by its only known ligand, hepatocyte growth factor (HGF/scatter factor) is known to induce angiogenesis, cell proliferation, motility and anti-apoptotic responses. Although, the exact response is cell type dependent with certain epithelial cells undergoing EMT and developing a more invasive phenotype [118].
Downstream effectors involved in MET signalling include MAPK, PI3K, AKT, MEK, FAK and PAK 1-7 [119]. In cancer the ligand HGF is predominantly expressed and secreted by tumour associated stromal cells [120, 121], although ligand independent activation through phosphorylation of MET by EGFR and increased expression of MET in response to EGFR signalling has been demonstrated in lung cancer [122, 123].

There is a body of evidence in a number of tumour types, including sarcoma, breast, lung, gastric and renal that MET pathway alterations are associated with a more aggressive tumour phenotype and a poor prognosis. Analysis of a number of components including plasma HGF and tissue expression of total MET, phosphorylated MET, HGF and downstream effectors has been reported in multiple tumour types. What is clear from published studies is that although individual components, including total MET, do not reach significance for the prediction of outcome in all cases, the pathway is clearly implicated in cancer progression [124-128].

The publications to date on the prognostic significance of MET in bladder cancer are contradictory. Three studies have described a significant relationship between high MET expression and poor survival in multivariate analysis, Yeh et al. and Cheng et al. [129, 130], and univariate analysis, Sanchez-Carbayo et al. [131]. However, Cheung et al. published a further manuscript in 2005 based upon an increased cohort, where MET failed to yield prognostic power in relation to survival in multivariate analysis [132], indicating that the initial study was underpowered. Three subsequent studies [133-135] have failed to demonstrate a significant association between survival and MET expression, although one did demonstrate that phosphorylation of was highly prognostic of survival [133]. The most recent publication in the field by Kluth et al. described IHC analysis of MET expression and FISH analysis of MET amplification in 560 and 504 cases of bladder cancer respectively [134]. High expression was found in 45.4% and 17.3% of G1pTa and G3pT2-4 tumours respectively with loss of expression being significantly associated with increasing stage of tumour. They found no association between MET expression and survival and a very low rate of
MET amplification in tumours of only 0.8%. Significantly increased levels of plasma HGF have been recorded in patients with MIBC compared to NMIBC [120], although the significance of this finding in relation to prognosis remains unknown.

Encouraging results have been demonstrated using MET inhibitors in vitro and in mouse models of cancers including prostate [136, 137], sarcoma [138], lung [139] and breast [140]. Several phase I and II clinical trials [141] of small molecule and monoclonal antibody inhibitors of HGF and MET are ongoing or have reported, with smaller numbers of phase III trials [142]. Unfortunately the recent COMET-1 trial of carbozantinib, a TKI targeting MET and VEGEF2, failed to meet its primary endpoint of increasing overall survival in metastatic prostate cancer [143]. There have been no clinical trails conducted targeting MET in bladder cancer.

1.4.9 Separate Pathways of Development

It has been shown that there is a distinction between the molecular events that occur in high grade invasive and low grade non invasive papillary bladder cancer [figure 1.2]. The current theories regarding the pathways of development of bladder cancer are that non-invasive papillary tumours develop from areas of flat urothelial hyperplasia with a high incidence of FGFR3 and Ras abnormalities. In contrast solid and invasive tumours develop from areas of severe dysplasia and CIS with high rates of p53, pRb and pTEN abnormalities with genetic instability [31, 144]. In addition when the epigenetic events that occur in bladder cancer are considered, such as gene methylation and miRNA profiles, very similar phenotypic groups to those defined by the above molecular events continue to be described [145-148]. Mouse models of both pathways, reviewed by McConkey [144], have been well described. In one of these models, expression of mutant h-Ras led to the development of papillary non-invasive tumours, in the other model loss of p53 led to the development of CIS, although further events were required to develop muscle invasive tumours. Further models have shown that
suppression of p53 and PTEN in combination always leads to the development of CIS and progression to muscle invasive tumours that metastasise.

These described pathways of development almost certainly represent an over simplification as it is not entirely clear exactly where T1 tumours and CIS fit within this grouping, as there have been observed differences between the events observed in isolated CIS and CIS in association with tumours [149]. It also does not explain the progression observed in a small number of patients from papillary non-invasive tumours to high grade MIBC.

**Figure 1.2 - Molecular pathways of bladder cancer development.**
(Reproduced from Goebell and Knowles 2010 [31])
In an attempt to improve the prognostic power of biomarkers in bladder cancer numerous markers, both singly and in combination, with a particular emphasis on cell cycle markers have been investigated. In addition to those studies described above, numerous studies have failed to demonstrate a significant independent predictive power of markers singly or in various combinations, with respect to factors such as recurrence and progression, in a variety of clinical situations. These include: FGFR3, p53, MDM2, pRB, PTEN, Ki-67, p16, p21, p27, cyclin-D1, cyclin-E1, TSP-1 and CD9 [56, 59-61, 150, 151]. This contrasts with studies demonstrating combinations of: p21 and p53 [38, 42], p53, p21 and pRB [40] and Bcl-2, caspase, p53 and survivin [152] to be independent predictors of outcome. This use of combination markers has been developed extensively by Shariat et al. [153] into a predictive nomogram, containing 5 markers. When used in combination with the pathological factors of T stage and lympho-vascular invasion, increased predictive accuracy with respect to cancer specific mortality following cystectomy is increased from 78.3% to 86.9%. Volkmer et al. [154], in a more novel and evolving area than the studies noted above, studied markers of cell differentiation to identify populations of tumour initiating cells within human samples and then study their tumourigenic potential in mouse xenograft models. The findings were used to correlate the expression of these markers with outcome in human series. KRT14, a marker of primitive differentiation, was identified as a highly significant predictor of outcome.

In conclusion, with respect to biomarkers in bladder cancer, there remains uncertainty, marked contradiction and a lack of translation into the clinical field. Indeed, the latest edition of the EAU guidelines on muscle invasive and metastatic bladder cancer gives a grade A recommendation that, “Currently, no biomarkers can be recommended in daily clinical practice because they have no impact on predicting outcome, treatment decisions, or monitoring therapy in muscle-invasive bladder cancer” [155].
1.4.11 Novel approaches to bladder cancer classification

It has been demonstrated in breast cancer that there is a biological taxonomy that separates the disease into distinct entities, whose response to different treatment modalities is very different. This has led to the emergence of a more tailored therapeutic approach, forming the foundations for personalised medicine [156]. This approach in bladder cancer is very much in its infancy compared to breast cancer, although there has been some encouraging data produced which may enlighten treatment decisions. Significant heterogeneity clearly exists within the MIBC population, exemplified by the divergent outcomes of patients whose disease is defined as identical using the current system of histological grading and TNM staging. There is a clear need for systems to better guide clinicians.

In 2012 Sjodahl et al. published a landmark paper in which they classified bladder cancer through hierarchical cluster analysis of 13,953 genes in 308 tumour samples [157]. This produced five subtypes, termed urobasal-A, genomically unstable, infiltrated, urobasal-B and SCC-like. The molecular subtypes did not overlap with the accepted pathological classification of tumours, demonstrated differing survival patterns and were validated in three external cohorts. The same group subsequently correctly classify tumours into these groupings using an IHC panel of 20 markers identified from their original work [158]. In the NMIBC setting the use of this classification system was able to identify a group of pT1 tumours at very high risk of progression to muscle invasion, the genomically unstable and SCC-like groups [159].

A recent publication by Choi et al. [160] utilised whole genome analysis of fresh frozen MIBC tumours and hierarchical clustering based upon gene signatures identified in breast cancer. They described three groupings of basal, luminal and “p53-like”. The basal group was associated with a worse prognosis and expression of mesenchymal and stem cell markers. Perhaps the most promising aspect of their findings was in identifying that the “p53-like” classifier was associated with a poor response to neoadjuvant chemotherapy. This finding was validated in a local validation cohort and an
external cohort, where it was also demonstrated that tumours post-chemotherapy acquired a “p53-like” signature that was not present pre-chemotherapy, indicating acquired resistance.

It remains to be seen if these approaches to classification, prognostication and determination of treatment direction will bring benefit to bladder cancer patients, as this model of studying the biology of bladder cancer has been in action for over a decade [161]. However, these models do propose a very different model of tumour classification than the WHO model of histopathological grading, which has served well but failed to deliver any significant advancement in treatment or outcome in the last four decades.
1.5 Clinical Management of Bladder Cancer

1.5.1 Presentation and Diagnosis of Bladder Cancer

At initial presentation approximately 25% of bladder cancer is muscle invasive (≥ T2) with the majority presenting as NMIBC (CIS, Ta and T1) [162]. The most common presenting symptom of bladder cancer is visible haematuria, although this must be considered in the context that visible haematuria has a population prevalence of 2.5-5% [163, 164]. Other symptoms and signs of bladder cancer include the presence of non-visible haematuria, the development of new bladder symptoms including urinary frequency or urgency and symptoms due to metastatic disease. The current standard diagnostic investigation to confirm suspected bladder cancer is to perform flexible cysto-urethroscopy under local anaesthesia. The diagnostic yield of this invasive procedure is dependent upon the context in which it is performed and it is not without morbidity, including: pain, infection and haematuria [165-168]. In the outpatient setting, the probability of detecting bladder cancer in the presence of haematuria is 7.3% and 14.7% for women and men respectively. Although, there is a significant variation with age and type of haematuria, with rates of 4.8% and 19.3% for non-visible and visible haematuria respectively [164].

Standard flexible cysto-urethroscopy utilises white light and has been shown that white light cystoscopy has a poor sensitivity for identifying flat lesions, with a sensitivity of only 60.5 - 71.4% in identifying patients with CIS [169, 170]. In an attempt to improve the diagnostic yield of cystoscopy a number of technologies including: optical coherence tomography, narrow band imaging and fluorescence cystoscopy have been investigated. A recent meta-analysis demonstrated increased sensitivity for the detection of CIS from 60.5% for white light to 92.4% for fluorescence cystoscopy using either HAL or 5-ALA [170]. Fluorescence cystoscopy has gained popularity, but its use is still far from universal.
There is a continued attempt to develop non-invasive tests that can identify patients with urothelial carcinomas in both the primary diagnostic setting and in the surveillance of those with a history of urothelial cancers managed by a bladder preserving approach. Cytological examination of voided urine has a high sensitivity for detecting high grade tumours but poor sensitivity for low grade tumours and a low overall specificity. A number of molecular tests have been developed that again utilise voided urine samples, including:

- Bladder tumour antigen.
- NMP22 BladderChek® (Matrich, Newton, MA, USA) that detects the nuclear matrix protein NMP22
- UroVysion® (Abbott Laboratories, Des Plaines, IL, USA) that utilises FISH to detect alteration in copy numbers of chromosomes 3, 7, 17 and loss of 9p21.
- ImmunoCyt (Scidmex Corp., Denville, NJ, USA) that combines cytology with immunofluorescence.

Unfortunately, a meta-analysis in 2011 [171] concluded that the currently available urinary marker tests had false positive rates and sensitivities that did not support the reduced use of cystoscopy in the diagnosis and surveillance of NMIBC. Furthermore, a prospective study into combinations of utilising cytology, NMP22 BladderChek®, UroVysion® and cystoscopy in the surveillance of NMIBC found that cystoscopy alone was the most cost effective approach. This was predominantly due to high false positive rates for the molecular tests, and that the addition of these tests did not improve the detection rate of muscle invasive tumours [172].

1.5.2 Staging and Treatment

The treatment of a patient diagnosed with a bladder tumour by outpatient flexible cystoscopy requires formal evaluation, by rigid cystoscopy, to obtain an accurate histological diagnosis and stage the tumour correctly.
1.5.2.1 TUR

Transurethral resection (TUR) of the tumour provides tissue for a histological diagnosis and in Ta and T1 tumours forms the basis of primary curative treatment. In tumours ≥ T2 tumours transurethral resection alone is insufficient, as such it is not mandatory to remove all visible tumour and indeed may be detrimental to attempt to do so. Inclusion of the underlying detrusor muscle ensures complete assessment of the T stage. At the time of TUR biopsies are taken from any abnormal appearing areas of urothelium, to ensure concomitant or isolated CIS is identified [26].

Fluorescence cystoscopy with either HAL or 5-ALA is utilised at the time of TURBT to ensure all tumours are identified and resected and that concomitant CIS is correctly identified. Despite the increased sensitivity of fluorescent cystoscopy in these respects there is still a lack of evidence that it has an effect on outcome. A recent randomised double blind control trial demonstrated increased tumour detection rates, although this did not translate into a difference in progression and recurrence rates at 12 months [173]. Current EAU guidelines recommend that fluorescence cystoscopy is restricted to those with a previous history of high grade tumour or a suspicion of high grade disease on the basis of cytology [26].

1.5.2.2 Imaging

All patients with a new diagnosis of a bladder cancer should undergo some form of excretory urography, either an intravenous urography (IVU) or preferably computerised tomography (CT), due to the 1.8% incidence [174] of synchronous upper urinary tract TCC in patient with NMIBC. In those with muscle invasive disease, in addition to the requirement for imaging to detect synchronous upper tract TCC, imaging with magnetic resonance imaging (MR) or CT to assess local tumour stage, lymph node status and for evidence of metastatic disease is required [26].
1.5.2.3 Non-Muscle Invasive Bladder Cancer (NMIBC)

NMIBC has a high propensity for recurrence despite TUR and in some patients progression of disease occurs. In 2005 the European Organisation for Research and Treatment of Cancer (EORTC) published a scoring system, based on 2,596 patients with NMIBC from a number of previous EORTC trials, to predict recurrence and progression in NMIBC [28]. The scoring system uses: number of tumours, tumour size, prior recurrence rate, tumour stage, tumour grade (WHO 1973) and the presence of CIS to place individuals into either a low, intermediate or high risk groups for recurrence and progression. The risk of recurrence and progression following treatment is up to 78% and 45% respectively at 5 years dependent upon risk group. It is this high recurrence rate that makes bladder cancer expensive to treat, despite the majority of patients not requiring radical treatment [175].

This prediction model is not intended for the management of isolated CIS, where the risk of progression, untreated, to muscle invasive disease is up to 54% [26] and despite the use of intra-vesical BCG remains at up to 45% for progression to T1 disease and 17% for progression to T2 disease at 5 years [176].
1.5.2.4 Intravesical Treatment

There is good evidence that a single immediate dose of intravesical chemotherapy (e.g. mitomycin-C or epirubicin) after primary TUR for Ta/T1 tumours significantly reduces the risk of tumour recurrence [177, 178]. This single dose is believed to have its effect in reducing recurrence by killing free floating tumour cells within the bladder, at the time of TUR, and preventing their implantation into the urothelium [179].

Adjuvant intravesical treatment beyond one immediate dose is not recommended by the EAU for Ta and T1 tumours, until recurrence occurs and even at that point it is not indicated in all patients. An assessment of risk based upon the EORTC scoring system guides those in whom further treatment would be of benefit. Although, it must be noted that there is no evidence that intravesical chemotherapy reduces disease progression. Its only effect is upon recurrence [26] and therefore only reduces the burden of disease and has no effect upon long term survival.

The primary treatment of CIS is with intravesical bacillus Calmette-Guérin (BCG) [180, 181]. Intravesical BCG is also recommended in the adjuvant treatment of patients with Ta/T1 tumours that fall into the EORTC intermediate and high risk groups [26]. There good evidence, reviewed by Ayres et al. [182], that BCG treatment reduces the risk of both recurrence and progression in these groups. The length of treatment (frequency and number of instillations) and dose (given at each instillation) is a source of much debate. Many centres use the 3 year SWOG regimen [180], however recent evidence from the EORTC indicates that 1 year of treatment for intermediate risk patients and 3 years for high risk patients is the most appropriate regime [183].
1.5.2.5 Radical Treatment

Radical cystectomy and lymphadenectomy with or without neo-adjuvant and or adjuvant chemotherapy remains the most commonly used modality globally for the treatment of MIBC [184]. The use of radical radiation therapy in place of cystectomy, in certain patient groups, is an alternative option. The indications for radical treatment are MIBC and those with NMIBC who are at high risk of progression or who have failed intravesical therapy. The indications to consider primary radical treatment in those NMIBC patients who are at high risk of progression include T1 tumours that are: high grade, multifocal, associated with CIS and > 3cm in size [181].

There is much debate regarding the optimum treatment of high grade T1 tumours with proponents of immediate cystectomy citing pathological upstaging rates at cystectomy of up to 50% for T1 tumours [185] and 10 year survival rates of 78% for immediate cystectomy versus 50-65% for those undergoing cystectomy following a failed intra-vesical therapy [30, 186, 187], with deferred cystectomy for CIS conferring the worst prognosis. The use of immediate radical treatment in high risk patients undoubtedly results in over treatment of those patients who would never have progressed. At present predicting accurately which patients will progress is not possible. Although, evidence that the outcome in those who progress is poor and that significant under-staging occurs has led many to advocate a more aggressive approach to the management of high risk NMIBC [29].

1.5.2.5.1 Radical Cystectomy

The indications for radical cystectomy include: high risk NMIBC, the primary treatment of T2-T4a N0 M0 and salvage treatment for local recurrence following radical radiation therapy. The procedure involves the surgical removal of the bladder and dissection of the regional lymph nodes. In addition the procedure involves the removal of the prostate and seminal vesicles in men and the uterus and adnexa in women, although there is
debate regarding removal of the prostate. The diversion of urine at the time of cystectomy is for the majority of patients achieved by the use of an ileal conduit to the abdominal wall forming and incontinent stoma. The construction of differing types of pouches from the intestine that are either catheterised intermittently via a stoma within the abdominal wall or more commonly anastomosed with the urethra producing and orthotopic neoblader are increasingly undertaken in younger patients [181]. Other methods of diversion exist, including ureterosigmoidostomy and cutaneous ureterostomy, however they are rarely used in modern urological practice [188].

The peri-operative mortality from cystectomy is around 2.5%, at 30 days following surgery, with overall 5 and 10 year survival figures of 59-66% and 37-43% respectively [188-191]. Survival directly correlates with increasing stage of disease with only 23-31% and 21-23% of node positive patients alive at 5 and 10 years following cystectomy [192].

1.5.2.5.2 Radical Radiation Therapy

Bladder preservation using a multimodality approach (TURBT, chemotherapy and radiation therapy) is gaining popularity as an alternative to cystectomy [193], although cystectomy remains the main treatment modality throughout the world [194]. Combining radiation therapy with chemotherapy has been demonstrated to be superior to radiation therapy alone [194-196], although level 1 evidence to date has demonstrated only a significant improvement in loco-regional control and not overall survival. The chemotherapeutic agents with level 1 evidence include cisplatinum, 5-fluorouracil (5FU) and mitomycin-C. Gemcitabine has evidence of efficacy as a radiosensitising agent in phase 2 trials [197] and there is increasing interest in the use of hypoxia modifying therapy in conjunction with radiotherapy with level 1 evidence for carbogen and nicotinamide [194, 198, 199].

The outcomes following radical radiation therapy alone were demonstrated in a Cochrane review in 2002 [200] to be inferior to surgery. This review is
however over a decade old and even at the time of publication the authors acknowledged that improvements in both treatments had occurred since the initiation of the included trials. The EAU does not support the position of radiation therapy alone as a primary treatment of bladder cancer and recommend its use only when “the patient is unfit for a cystectomy or a multimodality bladder-preserving approach” [155]. This position by the EAU is based upon the data presented in the Cochrane review of 2002 and is slightly controversial, given the changes in radiotherapy techniques since that time and the absence of an oncologist on the EAU MIBC guideline panel.

The reported overall survival rates following multimodal bladder preserving treatment, with appropriate use of salvage cystectomy are comparable to the outcomes reported for primary cystectomy [195, 197, 201-204]. However, radiation therapy has been shown to be less effective in treating G3pT1 tumours [205, 206] and its use is not advocated for the treatment of CIS. Although the data regarding CIS and radiation therapy is somewhat historic and based upon series of patients treated with radiation therapy alone rather than multi-modal therapy. Attempts have been made to define which patients are most likely to benefit from multi-modal therapy [193]. The proposed ideal parameters include: maximal TURBT, early stage T2, no associated hydronephrosis, no extensive CIS and no invasion into the prostatic stroma. There are no prospective randomised trials of radiation therapy and surgery, and it seems unlikely that such trials will ever be conducted.

Due to the lack of level 1 evidence comparing radiation therapy and cystectomy, the reported equivalence has been evaluated, mostly retrospectively, by comparing outcomes in reportedly similar patients treated by different modalities, with a huge potential for bias [207]. However, this bias likely favours cystectomy, due to the well described differences between clinical and pathological staging and the pathological up-staging that occurs at cystectomy.
1.5.2.5.3 Chemotherapy

A significant change in the management of bladder cancer in recent years has been the incorporation of neo-adjuvant chemotherapy into treatment regimens. In 2003 [208], updated in 2005 [209], Vale et al. on behalf of the Advanced Bladder Cancer (ABC) group, published a seminal meta-analysis on neo-adjuvant chemotherapy in radical therapy for MIBC. Using data on around 2800 patients from 11 randomised trials a 5% overall survival benefit at 5 years from neo-adjuvant platinum based chemotherapy was demonstrated. This data did not include newer regimens that combine gemcitabine with cisplatin (GC) although there is data that demonstrates equivalence [210]. The survival advantage of neo-adjuvant chemotherapy was confirmed in the subsequent BA06 30894 trial [211], which randomised 947 patients to no neo-adjuvant chemotherapy or 3 cycles of cisplatinum, methotrexate and vinblastine prior to cystectomy or radiation therapy. Despite this evidence, only around half of patients in the U.K. receive neo-adjuvant chemotherapy [212]. This is felt to be due to a combination of associated co-morbidities, concerns regarding the over treatment of lower stage patients, or disease progression in others during chemotherapy resulting in a worse outcome following radical treatment [213].

The role of adjuvant chemotherapy is less well defined and the evidence to support its use is at present lacking. A meta-analysis published in 2006 [214], again by the ABC group, on the role of adjuvant chemotherapy in MIBC concluded “that there is insufficient evidence on which to reliably base treatment decisions”. The EORTC-30994 trial comparing immediate versus delayed chemotherapy following radical cystectomy for T3/4 and/or N+M0 patients reported 5 year follow up at the 2014 ASCO meeting [215]. The trial demonstrated a significant reduction in progression free survival, but not overall survival, from immediate versus deferred cisplatinum based chemotherapy. It seems likely that adjuvant chemotherapy has a role, although this has yet to be fully defined. Irrespective of trials indicating a potential benefit from adjuvant treatment not all patients will benefit, as
around 30% of patients are unable to receive chemotherapy following cystectomy, due to the complications of surgery [213].

Chemotherapy does have a definite role in the treatment of bladder cancer that is metastatic at diagnosis or in those who develop metastatic relapse after radical treatment. In this context it is palliative in nature with long term survival being a very rare event. Initial response rates are variable at 40-70% although median survival is poor at around 15 months with 5 year survival rates of 20% [114-116]. Of those who go on to receive second line chemotherapy response rates are much worse at around 20% with a median survival of less than 9 months [213, 216]. This poor outcome is confounded further in that large numbers of patients are ineligible to receive chemotherapy in this setting due to poor performance status and renal function [217].

Evaluation of tumour levels of ERCC1, involved in the removal of cisplatinum induced DNA adducts and cisplatin resistance, has demonstrated predictive power in determining bladder cancer response to chemotherapy [218, 219]. Research is ongoing in this setting in an attempt to define predictive biomarkers of response [220] with ERCC1 and Bcl-2 highlighted as putative candidates [221].
1.5.2.6 Targeted Therapies

A number of more novel targeted agents are currently being evaluated in the field of bladder cancer. None have been developed specifically for the disease, and a defined population in whom they should be utilised is lacking. Many appear to be under investigation following gains made in the management of other cancer types rather than on the basis of good evidence regarding the molecular biology of bladder cancer. Targeting EGFR signalling with trastuzumab, gefitinib and erlotinib has been disappointing [described in section 1.4.7] and VEGFR therapy has been even less promising. A recent phase 2 trial conducted in locally advanced and metastatic bladder cancer comparing sorafenib versus placebo in combination with cisplatinum and gemcitabine failed to demonstrate a difference in progression free or overall survival [222]. It could be argued that the setting in which these novel agents are being tested makes it more likely that they will fail to shown significant advantage. The outcome in patients with metastatic bladder cancer is so poor and survival so short that unless these agents are able to cause major alterations in disease the observed change in outcome is likely to be small. Testing them in the earlier stages of the disease may prove more enlightening, although the ethical considerations are more complex making this more difficult to achieve. A new direction in therapy is urgently required.
1.6 Summary – Biomarkers and Targets

Decades of research into the molecular biology of bladder cancer has started to unravel the mechanisms involved in the carcinogenesis of this heterogeneous disease and the field may be moving towards a classification that incorporates molecular biology. Although this molecular model is gaining popularity, the use of molecular biomarkers for prognostication have yielded only very modest improvements over what can be achieved using a more traditional pathological assessment of MIBC, which is somewhat disappointing [153]. As a consequence none have subsequently fulfilled their promise and been incorporated into clinical management schedules.

Bladder cancer outcomes have changed very little in the last 3 decades, with paucity in significant discovery relating to the pharmacological management of MIBC. This contrasts starkly with other urological cancers, in particular renal cell cancer where the introduction of tyrosine kinase inhibitors into therapeutic regimes have been met with increasingly encouraging clinical results. The most notable advances in MIBC treatment include improvements in surgical technique and peri-operative care, leading to reduced treatment associated death with cystectomy [190] and the incorporation of chemotherapy into radiation therapy regimens [195].

The National Institute for Health defines a biomarker as “A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” [223]. The driving force behind future investigation into the molecular basis of bladder cancer should be to identify potential targets for therapy and to develop biomarkers that define which populations of patients will benefit from combinations of these targeted therapies. The field does not need biomarkers that simply attempt to prognosticate outcome without subsequent intervention.
1.7 Tissue microarrays and IHC staining analysis

1.7.1 Tissue microarrays in biomarker research

The first description of tissue microarray methodology was in 1987 by Wan et al. [224]. The process involves taking cores of tissue, typically 0.6-1mm in diameter, from a ‘donor’ formalin fixed paraffin embedded (FFPE) tissue block and then embedding the cores into a ‘recipient’ paraffin wax block. The cores are arranged asymmetrically in the ‘recipient’ block, with the location and contents of each core recorded. This allows the core to be easily be linked to a clinical database. Cores are taken from areas within the ‘donor’ block that are determined by the pathologist marking up sections from the blocks. This ensures that the cores contain the tissue required and are representative of the ‘donor’ tissue. In this way thousands of cores of tissue can be placed in a single paraffin wax block. This allows for the analysis of the tissue from multiple patients by IHC or genomic hybridisation on a single section cut from the ‘recipient’ block. In practice increasing the number of cores to more than 100 cores of 1mm diameter increases the risk of the block fracturing during sectioning and is therefore generally avoided [225].

Tumour heterogeneity is the main concern regarding the use of TMA methodology in biomarker research. In an attempt to reduce the effects of this TMA construction includes multiple cores of tissue from the same patient/tumour. Despite this, tumour heterogeneity has resulted in publications that have failed to demonstrate a good correlation between markers expression in TMA cores and whole tissue sections [81, 226]. This is contrasted with multiple studies demonstrating that two cores per patient (≥ 0.6 mm diameter) are sufficient to ensure a high level of correlation, in around 95% of cases, between whole tissue sections and TMA cores in [227-230]. However, there is no doubt that due to tumour heterogeneity and under sampling the higher the number of cores taken the higher the degree of concordance [231]. This appears to be a particular problem when looking at markers of tissue hypoxia [230, 232], due to the high level of intra-tumour
variation in hypoxia. It is therefore recommended that cores be taken from areas of the tumour that are 3-dimensionally distant from each other and are representative of the heterogeneity visualised by the pathologist marking up the donor blocks.

There are multiple advantages and disadvantages of utilising a TMA based approach to biomarker research. These are outlined very clearly in the NCRI publication: ‘Guidelines and considerations for conducting experiments using tissue microarrays’ [225] and in a paper by Camp et al. from 2008 discussing the first decade of research using TMA based technology [233]. The key advantages and disadvantages described are been summarised in table 1.3.

The advantages described have led to the widespread utilisation of TMA based technology, with over 700 publication’s per year in the late 2000’s, comprising around 10% of all IHC biomarker based research [233]. Bladder cancer is no exception, with multiple studies published in a number of areas of the disease [234], including studies examining genetic susceptibility to bladder cancer [235], recurrence and progression of NMIBC [75, 236], response to chemotherapy [237] and survival [153, 238-242].
### Advantages of TMA methodology

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<th>Disadvantages of TMA methodology</th>
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<tr>
<td>High throughput process – analysing in excess of 100 tissue cores per slide allows large cohorts to be studied in a more resource and time efficient way.</td>
<td>Construction costs – TMA construction is a lengthy process, highly labour intensive, expensive.</td>
</tr>
<tr>
<td>Scale – the high throughput nature of the TMA staining and analysis process means cohorts of increased size can be analysed, increasing the statistical power of the data generated.</td>
<td>TMA handling – block are easily damaged and sectioning is a process that required highly skilled technicians compared to the use of whole sections.</td>
</tr>
<tr>
<td>Optimal utilisation of precious human tissue samples – multiple replicate TMA’s can be produced from the same cohort of patients, increasing the research potential of the tissue samples.</td>
<td>Wide spectrum of sample age – TMA construction often uses archival samples over many decades, increasing the probability that important differences in tissue handling and fixation will exist.</td>
</tr>
<tr>
<td>Consistency of staining - synchronously staining all samples at the same time reduces variability. Contrasted with the prior need to stain hundreds of sections over many days with the potential for variation in staining due to minor alterations at any point in the multi-step IHC process</td>
<td>Donor tissue heterogeneity – due to the heterogeneous nature of tumours the TMA cores may not represent the biology of the overall tumour. This factor is reduced by increasing the number of cores taken.</td>
</tr>
<tr>
<td>Image digitisation – stained sections can easily be scanned into digital format to enable AIA and</td>
<td>Core heterogeneity – the content of the core may change as repeatedly sectioned due to heterogeneity throughout its length</td>
</tr>
<tr>
<td>Simplification of scoring process - as the content of each core is pre-determined by a pathologist the scoring of staining can be undertaken by individuals following very simple training, rather than requiring a pathologist.</td>
<td>Methodology – TMA’s are not usually constructed for a single specific study, as such the TMA may not be have been constructed in a way that is applicable to all of the research for which it is ultimately used.</td>
</tr>
<tr>
<td>Control cores – control cores (both positive and negative) can be included the TMA blocks.</td>
<td>Core loss – up to 15% of sectioned cores can be expected to be lost or unusable due to distortion.</td>
</tr>
</tbody>
</table>

### Table 1.3 - Advantages and disadvantages of TMA technology

This table represents a summary of the publication by Camp et al. in 2008 outlining the findings of the first decade of TMA based research [233] and the NCRI publication: Guidelines and considerations for conducting experiments using tissue microarrays [225].
1.7.2 IHC staining analysis

The use of automated image analysis (AIA) software is increasingly being utilised to analyse the expression of proteins using IHC staining [243]. Prior to the development of AIA software the only method of quantifying protein expression was the application of a semi-quantitative scoring systems by human eye. In an attempt to improve the reproducibility of IHC scoring a number of systems have been developed. The majority of these systems rely upon the estimation of staining intensity (usually graded as 0, 1+, 2+ and 3+, indicating negative, weak, moderate and intense) combined with an estimation of the percentage of cells staining at each intensity level. In some scoring systems this is also combined with other factors, for example the completeness of membrane staining in Her-2 scoring in breast cancer [244]. Two commonly employed semi-quantitative systems are the H-score [245] and Allred score [246].

\[
H \text{ score} = (0 \times \% \text{ at 0}) + (1 \times \% \text{ at 1+}) + (2 \times \% \text{ at 2+}) + (3 \times \% \text{ at 3+})
\]

The Allred score is obtained from the sum of the intensity score (IS) and the proportion score (PS), giving a range of 0-8.

IS
0 = none
1 = weak staining
2 = intermediate staining
3 = strong staining

PS
0 = 1/100 cells stained
1 = 1/10 cells stained
2 = 1/3 cells stained
3 = 2/3 cells stained
4 = all cells stained

Correlation between both systems is poor. For example, a sample where 100% of the cells stain weakly for a protein gives an Allred score of 5 (range
0-8) and an H-score of 100 (range 0-300). Those against the Allred system argue that the inherent weakness is that it apportions equal weighting to both intensity and proportion [246]. In Her-2 scoring in breast cancer the percentage of cells staining has a fixed threshold of 10% with the score assigned based upon the intensity and completeness of staining at this threshold [247].

Her-2 score 0 (negative) =

No staining observed

or

Membrane staining that is incomplete and is faint/barely perceptible and within ≤ 10% of tumour cells.

Her-2 score 1+(negative) =

Incomplete membrane staining that is faint/barely perceptible within >10% of tumour cells.

Her-2 score 2+ (equivocal) =

Circumferential membrane staining that is incomplete and/or weak/moderate and within >10% of tumour cells

or

Complete and circumferential membrane staining that is intense and within ≤ 10% of tumour cells.

Her-2 score 3+ (positive) =

Circumferential membrane staining that is complete, intense, and within >10% of tumour cells.

When scoring oestrogen receptor (ER) status in breast cancer, used to determine if anti-oestrogen therapy is likely to be of benefit, and progesterone receptor (PR) status a very low threshold of 1% is used [248]. Tumours are considered as positive if >1% of tumour cells stain positively at any intensity. However, there is ongoing debate regarding the optimal
threshold to determine ER positivity in relation to clinical outcome [249] and significant intra-observer variation when determining the percentage of cells staining positive in the 0-5% range and when deciding between negative or weak positivity [250].

AIA has the potential to remove a number of the issues that surround scoring of protein expression by the human eye. It has proven ability in identifying tissue types within a sample (tumour, stroma, vessels etc.), quantifying the staining intensity in each cell on a continuous scale and quantifying the characteristics of the staining pattern [244, 246, 251-256]. This allows the generation of true quantitative data and can analyse the expression of multiple proteins on a single tissue section using a multiplexed approach [245]. From this raw data, H-scores, Allred scores and Her-2 scores can all be generated. AIA’s ability to quantitatively analyse the staining intensity in each cell means that expression can be measured on a continuous scale rather than the categorical classifiers used in human scoring (e.g. negative, weak, intermediate and intense). The most simple of these is the calculation of the mean staining intensity within a field of interest, which has been demonstrated to correlate well with H-scoring by the human eye [246]. It is noted in the literature that when analysing such continuous data in relation to outcome, dichotomising at an optimal cut point based upon the lowest p-value should be avoided as this risks significant bias [257]. It is recommended that thresholds are defined in a test cohort and then applied to the overall cohort [258].

AIA by its nature should reduce variability in scoring as it is based upon algorithms systematically applied by a computer, thereby reducing the intra-observer variability seen with traditional methods [251]. The current gold standard remains scoring by an experienced pathologist [253]. Although, there are an increasing number of publications indicating that AIA can produce results that are equivalent to image interpretation by a pathologist with respect to both scoring and in predicting patient outcome [244, 254].
1.8 REMARK Guidelines

In 2005 the US National Cancer Institute (NCI) and the European Organisation for Research and Treatment of Cancer (EORTC) published guidelines for reporting of studies into tumour markers [259]. The REporting recommendations for tumour MARKer prognostic studies (REMARK) guidelines included a summary table of the key elements that should be described and included in any study into prognostic tumour markers. The guidelines are broken down into sections that correspond to the sections included in a typical manuscript. The contents of that table are reproduced below as they have clear relevance to the project undertaken.

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. randomised 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 end point.
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 employed. 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 end points 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 and 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 tumour marker, including numbers of missing values.
Analysis and presentation

14. Show the relation of the marker to standard prognostic variables.
15. Present univariate 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 analysed. For the effect of a tumour 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 significance.
18. If done, report results of further investigations, such as checking assumptions, sensitivity analyses, internal validation.

Discussion

19. Interpret the results in the context of the prespecified hypotheses and other relevant studies; include a discussion of limitations of the study.
20. Discuss implications for future research and clinical value.
1.9 Experimental rationale

The Christie is one of the largest MIBC treatment centres in the UK and over the last 40 years has amassed one of the largest intact pathology archives of this condition. This tissue archive is allied to a database of all cases undergoing cystectomy from 1969 to the current date. The archive contains tissue from all cases of bladder cancer treated by cystectomy as a primary treatment or as salvage therapy following relapse after radical radiation therapy. Advances in the pharmacological treatment of bladder cancer have been lacking and it seems unlikely that further refinement in surgical technique will deliver the improvements in survival that are desperately required for high risk NMIBC and MIBC. This tissue archive provided a notable opportunity, in combination with in vitro models of bladder cancer, to investigate the role of putative treatment targets in bladder cancer.

1.9.1 Aims

1. To generate a bladder cancer TMA using the Christie Urology / Manchester Cancer Research Centre biobank repositories and link it to an accurate clinical database including outcome data.

2. To identify and characterise putative treatment targets and biomarkers in muscle invasive bladder cancer; specifically using in-vitro models to evaluate their role in:
   - cell motility
   - invasive potential
   - chemo-sensitivity

3. To utilise the TMA to validate the treatment targets and biomarkers evaluated by in vitro modelling; correlating expression with:
   - tumour characteristics
   - known prognostic parameters
   - clinical outcomes
2 Materials and Methods

2.1 Materials

2.1.1 Media and cell culture

<table>
<thead>
<tr>
<th>Material</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium – high glucose. AQmedia™, with 4500 mg/L glucose, L-alanyl-glutamine, NaHCO₃ and without sodium pyruvate. Sigma-Aldrich (Poole, U.K.).</td>
</tr>
<tr>
<td>EMEM</td>
<td>Minimum Essential Medium Eagle, with Earle’s salts, L-glutamine and NaHCO₃. Sigma-Aldrich (Poole, U.K.).</td>
</tr>
<tr>
<td>FAF BSA</td>
<td>Albumin from bovine serum, fatty acid free. Sigma-Aldrich (Poole, U.K.).</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Bovine Serum. PAA Laboratories (Pasching, Austria)</td>
</tr>
<tr>
<td>HEPES</td>
<td>HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer solution, 1M. PAA Laboratories (Pasching, Austria)</td>
</tr>
<tr>
<td>L-Glut</td>
<td>L-Glutamine solution 200mM. Sigma-Aldrich (Poole, U.K.).</td>
</tr>
<tr>
<td>Matrigel</td>
<td>BD Matrigel™ Basement Membrane Matrix. BD Biosciences (NJ, USA)</td>
</tr>
</tbody>
</table>
McCoy's 5a Modified Media, with 2.2g/L NaHCO₃ and without L-glutamine. Sigma-Aldrich (Poole, U.K.).

NEAA MEM Non-Essential Amino Acids 100x solution. Gibco® Life Technologies (Carlsbad, CA, USA)

RPMI RPMI 1640, with 2.0g/L NaHCO₃ and without L-glutamine. Sigma-Aldrich (Poole, U.K.).

Trypsin Trypsin-EDTA solution. 0.05% procine trypsin and 0.02% EDTA in PBS. PAA Laboratories (Pasching, Austria)

2.1.2 Antibodies

2.1.2.1 Primary antibodies

β-Actin Mouse monoclonal anti actin antibody. Clone ID: C4. Millipore (MA, USA)

CTGF Goat polyclonal anti human CTGF antibody. Clone ID: L-20. Santa Cruz Biotechnology (Santa Cruz, CA, USA)

CTGF Rabbit polyclonal anti human CTGF antibody. Sigma-Aldrich (Poole, U.K.).

CTGF Goat polyclonal anti CTGF antibody. Abcam (Cambridge, U.K.)

c-MET Mouse monoclonal anti- c-MET antibody. Clone ID: 3D4. Invitrogen™ Life Technologies (Carlsbad, CA, USA)
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Description</th>
<th>Clone ID</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-MET</td>
<td>Mouse monoclonal anti-human HGF R/c-MET antibody.</td>
<td>95308</td>
<td>R&amp;D Systems (Abingdon, U.K.)</td>
</tr>
<tr>
<td>CYR61</td>
<td>Rabbit polyclonal anti human CYR61 antibody.</td>
<td>H-78</td>
<td>Santa Cruz Biotechnology (Santa Cruz, CA, USA)</td>
</tr>
<tr>
<td>CYR61</td>
<td>Rabbit polyclonal anti CYR61 antibody.</td>
<td></td>
<td>Abcam (Cambridge, U.K.)</td>
</tr>
<tr>
<td>E-Cad</td>
<td>Rabbit monoclonal anti E-cadherin antibody.</td>
<td>24E10</td>
<td>Cell Signalling Technologies (Beverly, MA, USA)</td>
</tr>
<tr>
<td>FGFR1</td>
<td>Rabbit monoclonal anti FGF Receptor 1 (C-term) antibody.</td>
<td>EPR806Y</td>
<td>Epitomics (Burlingame, CA, USA)</td>
</tr>
<tr>
<td>FGFR3</td>
<td>Mouse monoclonal anti human FGF Receptor 3 antibody: sc-13121.</td>
<td>B-9</td>
<td>Santa Cruz Biotechnology (Santa Cruz, CA, USA)</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Rabbit polyclonal anti GAPDH antibody.</td>
<td></td>
<td>Abcam (Cambridge, U.K.)</td>
</tr>
<tr>
<td>N-Cadherin</td>
<td>Mouse monoclonal anti N-Cadherin antibody.</td>
<td>32/N-Cadherin</td>
<td>BD Biosciences (NJ, USA)</td>
</tr>
<tr>
<td>pan-CK</td>
<td>Mouse monoclonal anti-pan-cytokeratin pan antibody.</td>
<td>C-11</td>
<td>Sigma-Aldrich (Poole, U.K.).</td>
</tr>
<tr>
<td>p53</td>
<td>Mouse monoclonal anti-human p53 protein antibody.</td>
<td>DO-7</td>
<td>Dako (Ely, Cambridgeshire, U.K.)</td>
</tr>
</tbody>
</table>
P-Tyr Mouse monoclonal anti phospho-tyrosine antibody (P-Tyr-100). Cell Signalling Technologies (Beverly, MA, USA)


2.1.2.2 Negative control antibodies

Rb Control Rabbit immunoglobulin fraction (Normal)
Dako (Ely, Cambridgeshire, U.K.)

Ms Control Mouse IgG1 Isotype Control, monoclonal mouse IgG1.

Gt Control Normal goat IgG control (polyclonal).
R&D Systems (Abingdon, U.K.)

2.1.2.3 Secondary antibodies

Anti-Gt HRP Mouse anti-goat IgG antibody, HRP conjugate. Millipore (MA, USA)

Anti-Ms HRP Rabbit anti-mouse IgG antibody, HRP conjugate. Millipore (MA, USA)

Anti-Rb HRP Goat anti-rabbit IgG antibody, HRP conjugate. Millipore (MA, USA)

RAGBO Polyclonal rabbit ant-goat immunoglobulins/biotinylated.
Dako (Ely, Cambridgeshire, U.K.)
<table>
<thead>
<tr>
<th>RAMBO</th>
<th>Polyclonal rabbit anti-mouse immunoglobulins/biotinylated rabbit F(ab')2. Dako (Ely, Cambridgeshire, U.K.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SARBO</td>
<td>Polyclonal swine anti-rabbit immunoglobulins/biotinylated. Dako (Ely, Cambridgeshire, U.K.)</td>
</tr>
</tbody>
</table>

### 2.1.3 Reagents and solutions

- Avidin/Biotin Blocking kit. Vector Laboratories (Peterborough, U.K.)
- Bond™ Polymer Refine Detection System, Leica Microsystems (Milton Keynes, U.K.)
- Bond™ primary antibody diluant, Leica Microsystems (Milton Keynes, U.K.)
- Candor Block The blocking solution. CANDOR Bioscience GmbH (Wangen, Germany)
- DAB 3,3′-Diaminobenzidine. SIGMAFAST™ 3,3′-diaminobenzidine tablets. Sigma-Aldrich (Poole, U.K.).
- DMSO Dimethyl sulfoxide. Sigma-Aldrich (Poole, U.K.).
- High pH epitope Target retrieval solution (10x). 500 ml citrate buffer, pH 9, 10x concentrated. Dako (Ely, Cambridgeshire, U.K.)
Loading buffer 80% v/v 4x loading buffer (Glycerol 4ml, SDS 0.8g, 1M TRIS/HCL pH6.8 2.5ml, Bromophenol Blue 0.08ml, made up to 8mls with H$_2$O), 20% v/v β-mercapto-ethanol.

Low pH epitope Target retrieval solution (10x). 500 ml citrate buffer, pH 6, 10x concentrated. Dako (Ely, Cambridgeshire, U.K.)

Luminata™Forte Luminata™ Forte Western HRP substrate.
Millipore (MA, USA)

Lysis buffer 60.5% v/v H$_2$O, 2% v/v 0.5M EDTA pH 8.0, 25% v/v 20% SDS, 12.5% v/v TRIS 0.5M pH 6.8

Milk 2.5 g Marvel® made up to 50mls with TBST

PBS Phosphate buffered saline

ProLong® Gold Antifade Reagent with DAPI Life Technologies (Paisley, U.K.)

RNAiMAX Lipofectamine® RNAiMAX transfection reagent.
Life Technologies (Paisley, U.K.)

Running buffer 450mls Milli-Q water, 50mls 10x running buffer (30.4g TRUS base, 144.2g glysine, 50mls 20% SDS, make up to 1000mls with Milli-Q water).

siRNA buffer 5 x siRNA buffer. Thermo Scientific (PA, USA)

SRB Sulforhodamine B acid chloride. Sigma-Aldrich (Poole, U.K.).
<table>
<thead>
<tr>
<th>Buffer</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBS</td>
<td>900 mls Milli-Q water, 100 mls of 10 x TBS (87.6 g NaCl, 12.1 g TRIS base, made up to 1000mls with Milli-Q water at pH 7.4).</td>
</tr>
<tr>
<td>TBST</td>
<td>1000 mls TBS, 700μl TWEEN</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>Transfer buffer</td>
<td>800mls Milli-Q water, 3.03g TRIS base, 14.4g glysine, 200mls methanol.</td>
</tr>
</tbody>
</table>

Vectastain® ABC kit. Vector Laboratories (Peterborough, U.K.)
2.1.4 siRNA’s

ON-TARGETplus siRNA – SMART pool, Thermo Scientific (PA, USA), were used in all experiments described. The individual SMARTpool siRNA’s used are described below.

siNT  ON-TARGETplus Non-targeting Control Pool

siMET ON-TARGETplus Human MET (4233) siRNA – SMARTpool

J-003156-13, MET
Target sequence: GAACUGUGUCCCCGAUAU
J-003156-14, MET
Target sequence: GAACAGCGAGCUAAAUAUA
J-003156-15, MET
Target sequence: GAGCCAGCCUGAAUGAUGA
J-003156-16, MET
Target sequence: GUAAGUGCCCCGAUGUGAA

siCYR61 ON-TARGETplus Human CYR61 (3491) siRNA – SMARTpool

J-004263-07, CYR61
Target sequence: GAUGUUUGAGUGUGAAGAA
J-004263-08, CYR61
Target sequence: GGUCAAAGUUACCGGGCAG
J-004263-09, CYR61
Target sequence: CCAGAAAUGUAUUGUUCAA
J-004263-10, CYR61
Target sequence: GCAGCAAGACCAAGAAAUC

siCTGF ON-TARGETplus Human CTGF (1490) siRNA – SMARTpool

J-012633-10, CTGF
Target sequence: ACAAUGACACUCUUUGAUC
J-012633-10, CTGF
Target sequence: AGGAAGAUGUACCGGAGACA
J-012633-10, CTGF
Target sequence: CGAUUAGACUGGAGACGCUU
J-012633-10, CTGF
Target sequence: GAGAGCAUUAACUCAUUA
### 2.1.5 Cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Source of cell line</th>
<th>Origin of cell line</th>
<th>Authentication</th>
<th>Standard culture media</th>
<th>Trypsin time at 37°C (s)</th>
<th>Sub-culture/passage split</th>
</tr>
</thead>
<tbody>
<tr>
<td>5637</td>
<td>TRB group PICR</td>
<td>G2 Primary tumour TCC</td>
<td>PICR molecular biology core facility</td>
<td>McCoy's 1% L-glutamine v/v 10% FCS v/v</td>
<td>400</td>
<td>1:10</td>
</tr>
<tr>
<td>RT-112</td>
<td>ECACC</td>
<td>G2 papillary Primary tumour untreated TCC</td>
<td>PICR molecular biology core facility</td>
<td>EMEM 1% NEAA v/v 10% FCS v/v</td>
<td>300</td>
<td>1:10</td>
</tr>
<tr>
<td>UM-UC-3</td>
<td>TRB group PICR</td>
<td>TCC</td>
<td>PICR molecular biology core facility</td>
<td>DMEM 10% FCS v/v</td>
<td>60</td>
<td>1:10</td>
</tr>
<tr>
<td>HT-1376</td>
<td>TRB group PICR</td>
<td>G3 invasive Primary tumour untreated TCC</td>
<td>PICR molecular biology core facility</td>
<td>DMEM 10% FCS v/v</td>
<td>60</td>
<td>1:10</td>
</tr>
<tr>
<td>T24</td>
<td>TRB group PICR</td>
<td>G3 Primary tumour untreated TCC</td>
<td>PICR molecular biology core facility</td>
<td>RPMI 10% FCS v/v 1% L-glutamine v/v</td>
<td>60</td>
<td>1:10</td>
</tr>
<tr>
<td>J82</td>
<td>TRB group PICR</td>
<td>G3 T3 Primary tumour treated TCC</td>
<td>PICR molecular biology core facility</td>
<td>EMEM 10% FCS v/v</td>
<td>60</td>
<td>1:10</td>
</tr>
</tbody>
</table>

Table 2.1 - Cell Lines, culture media and trypsin times
2.1.6 Western blotting gels

<table>
<thead>
<tr>
<th>Running gel</th>
<th>7.5% acrylamide</th>
<th>10% acrylamide</th>
<th>12% acrylamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>4.290ml</td>
<td>3.545ml</td>
<td>2.950ml</td>
</tr>
<tr>
<td>Tris 1.5 M pH 8.8</td>
<td>2.240ml</td>
<td>2.240ml</td>
<td>2.240ml</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>2.240ml</td>
<td>2.985ml</td>
<td>3.582ml</td>
</tr>
<tr>
<td>SDS 10 %</td>
<td>0.185ml</td>
<td>0.185ml</td>
<td>0.185ml</td>
</tr>
<tr>
<td>APS 10 %</td>
<td>0.036ml</td>
<td>0.036ml</td>
<td>0.036ml</td>
</tr>
<tr>
<td>Temed</td>
<td>0.018ml</td>
<td>0.018ml</td>
<td>0.018ml</td>
</tr>
</tbody>
</table>

Table 2.2 - Composition of running gels used in western blotting

<table>
<thead>
<tr>
<th>Stacking gel</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>2.300ml</td>
</tr>
<tr>
<td>Tris 0.5 M pH 6.8</td>
<td>1.000ml</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>0.665ml</td>
</tr>
<tr>
<td>SDS 10 %</td>
<td>0.034ml</td>
</tr>
<tr>
<td>APS 10 %</td>
<td>0.016ml</td>
</tr>
<tr>
<td>Temed</td>
<td>0.008ml</td>
</tr>
</tbody>
</table>

Table 2.3 - Composition of the stacking gel used in western blotting
2.2 Methods

2.2.1 Cell culture

2.2.1.1 Maintenance of Cell lines

Cell lines were maintained and subjected to the following protocol for the purposes of all experiments described, except where otherwise stated. Cells were cultured for three passages following thawing from liquid nitrogen storage prior to being subjected to any form of experimentation. Passage numbers were kept within 20 passages of baseline.

Cells were grown in uncoated T75 plastic cell culture flasks (BD Falcon™ Cell Culture Flask, 75cm$^2$, 250ml, BD Biosciences, NJ, USA), and maintained sub-confluent in standard media appropriate for each cell line (except where specified otherwise), as shown in table 2.1.

2.2.1.1.1 Sub-culturing / passage of cell lines

The cell monolayer appearances were checked by phase contrast microscopy prior to any passage. The media was removed and discarded, following which the monolayer was washed twice with 10mls of PBS. To the T75 flask, 2mls of Trypsin solution was added, the flask was then sealed and placed in an incubator at 37°C, for the times indicated in table 2.1. Following the specified time the flask was removed from the incubator, agitated for 2-3 seconds and then examined to ensure the cells had appropriately detached from the base of the flask. If insufficient cells had detached the flask was returned to incubator for a further 30 seconds, following which the process of agitation and microscopy was repeated. Following these steps 8mls of standard media was added to the flask cells and the resulting cell suspension removed. A manual cell count was performed at this point using a
haemocytometer, if required, to obtain specific cell suspensions for further experiments or to seed a flask of a predetermined density. To a new T75 flask, containing 18mls of fresh standard media at 37°C, the predetermined volume of cell suspension was added. For a standard passage for maintenance of a cell line, all cell lines were subjected to 1:10 split. The flask was then charged with 5% CO₂ in air, through a 10 μm filter, sealed and incubated at 37°C. Cell lines were subjected to passage once every 3-4 days to maintain sub-confluence.

### 2.2.1.1.2 Long Term Cell Line Storage

All long term cell line storage was undertaken by maintaining cells frozen in liquid nitrogen. To prepare cells for freezing down, T75 cell culture flasks containing a sub-confluent monolayer of cells were trypsinised as previously described and re-suspended to a total volume of 10mls per flask of cells, in standard media. The cells were then centrifuged for 5 minutes at 400g, brake rate 3 (Mistral 2000, MSE, Leicestershire, U.K.). The supernatant was removed and the cells re-suspended in a solution of FCS + 10% DMSO v/v. 1ml aliquots in freezing tubes were suspended in the vapour phase of nitrogen for 1 hour before being immersed in liquid nitrogen for long term storage.

Cell vials were thawed by placing in a water bath at 37°C for 2 minutes. The thawed cell suspension was then gently added to 9mls of standard media at 37°C and centrifuged for 5 minutes at 400g, brake rate 3. The supernatant was discarded and the cell pellet re-suspended in 1ml of standard media before being added to a T75 cell culture flask containing 19mls of standard media. The flask was charged with 5% CO₂ in air, through a 10μm filter, sealed and placed in a hot room maintained at 37°C.
2.2.1.2 siRNA transfection of cells

Cells were seeded in 2.5mls of standard media with 10% FCS into 6 well plates at the required density and placed in an humidified incubator for 24 hours at 37°C with 5% CO₂ in air. Stocks of siRNA solutions at 20μM concentration were stored at -80°C following reconstitution in siRNA buffer (4:1 RNase free water: 5x siRNA buffer) according to the manufacturers instructions. For each siRNA target, 3.75μl of 20μM stock siRNA solution and 7.5μl RNAiMAX, per well, were combined in 238.75μl of EMEM for 5 minutes at room temperature. 1ml of media was removed from each well and discarded. 250μl of the siRNA/RNAiMAX/MEM mixture was then combined with 750μl of standard media (containing 10% FCS), per well, following which 1ml added to each well. The plated were then returned to the incubator. Subsequent treatment of the plates varied by experiment, as described in each section.

2.2.1.3 Boyden chamber Matrigel™ cell invasion assay

Sub-confluent cells, growing as a monolayer on plastic, were washed 2x with PBS and placed in serum free media (RPMI 1640, 1% L-Glutamine v/v) for 24 hours at 37°C with 5% CO₂ in air. In knockdown experiments using cells transfected with siRNA, this step was undertaken after 24 hours of incubation in the presence of siRNA/RNAiMAX, as described in section 2.2.1.2.

PET (polyethylene terephthalate) track-etched membrane cell culture inserts (8.0μm pore size, growth area 0.3cm³, BD Falcon™, NJ, USA) were placed into a 24 well plate. Un-used wells were filled with 1ml of sterile water. To each insert 100μl of 1:25 Matrigel™, defrosted to 0°C on ice, was added prior to incubation at 37°C for 2 hours. From the surface of the ‘set’ Matrigel™, 50μl of solution was removed from each insert, without disturbing the Matrigel™. Into the chamber beneath each insert, 1ml of invasion assay
media (RPMI 1640, 10% FAF BSA v/v, 1% L-glutamine v/v) was added, either with or without the required chemo-attractant.

The cell monolayer was trypsinised as previously described, except that PBS was added to each flask containing the cells and trypsin, not standard media. The PBS cell suspension was then centrifuged for 5 minutes at 400g, brake rate 3. The supernatant was then discarded and the cell pellet re-suspended in 5mls of invasion assay media. Cell were counted using a manual haemocytometer and diluted, using invasion assay media, to the required concentration (typically 1x10^5 cells per insert).

The inserts were gently seeded with 250ul of the cell suspension, without disturbing the Matrigel™ and incubated at 37°C with 5% CO₂ in air for 18 hours.

Inserts were washed gently in PBS and the Matrigel™ gently removed from the upper surface of the membrane using a PBS moistened cotton swab/Q-tip. Inserts were stained with 1ml of crystal violet for 10 minutes. The inserts were then washed gently 4x in tap water and then air dried in a 33°C hot room for 2 hours.

Using a bright field microscope and graticule, the number of cells stained with crystal violet present within a 10mm x 10mm graticule field were counted at 100x magnification.

2.2.1.4 SRB proliferation and cytotoxicity assay

2.2.1.4.1 Proliferation assay

Cell proliferation was determined using a standard SRB assay as described. Briefly, 200μl of cells at various concentrations in standard media were seeded in sextriplet in 96 well flat bottomed plates. In knockdown
experiments using cells transfected with siRNA, this step was undertaken after 48 hours of incubation in the presence of siRNA/RNAiMAX, as described in section 2.2.1.2. All experiments included a day1 control plate that included replicates in sextriplet of the experimental conditions used in the experiment. Plates were then placed in an humidified incubator at 37°C with 5% CO₂ in air. After 2 hours the day 1 plates were removed and fixed. At the specified time points further plates were removed from the incubator and fixed. Proliferation was calculated as fold change in relation to the day 1 plate.

2.2.1.4.2 Fixing and staining with SRB

The media was removed from wells, discarded and the wells washed 2x with 100μl PBS. Cells were fixed in 100μl TCA (10% w/v dd H₂O) for 1 hr at 4°C. The TCA was then discarded and the wells washed 2x with 100μl PBS before drying at room temperature for 2 hours. 100μl of SRB solution (0.4% w/v dd H₂O) was added to each well for 15 minutes at room temperature. Wells were then washed with acetic acid (1% w/v dd H₂O) until the acid ran clear. Plates were then dried at room temperature for 2 hours. The SRB was then re-suspend by adding 100μl Tris-HCl (1.5M; pH 8.8) and agitating on a shaker for 5 mins. 100μl of Tris-HCl (1.5M; pH 8.8) was also added to a row of 6 empty (control) wells. The plate was then immediately read on colorimetric plate reader at 490nm absorbance (BioTeck, Winooski, WT, USA). The absorbance for each well was calculated by subtracting the mean absorbance of the 6 control wells from the read for each well.

2.2.1.4.3 Cytotoxicity assay

Cells were seeded into a flat bottomed 96 cell plate at the optimal density defined by previous SRB assay in sextriplet, and incubated at 37°C with 5% CO₂ in air; including a day 1 control plate as described in the proliferation assay. In knockdown experiments using cells transfected with siRNA, this
step was undertaken after 48 hours of incubation in the presence of siRNA/RNAiMAX, as described in section 2.2.1.2. Cytotoxic drug solutions were prepared by serial dilution, to achieve concentrations twice that of the desired concentrations. After 2 hours the plates were removed from the incubator. The day 1 plates were fixed and dried. To the control columns 100μl of standard media was added to each well. To the vehicle control columns 100μl of the drug vehicle was added to each well. To the remaining columns, 100μl per well of the drug solutions at the pre-determined concentrations were added. The plates were then returned to the incubator for 72 hours, following which they were fixed and stained using a standard SRB assay, as described in section 2.2.1.4.2.

2.2.1.5 Migration assay

Six well plates were seeded with cells and incubated at 37°C with 5% CO₂ in air in an humidified incubator until 85% confluent. In knockdown experiments using cells transfected with siRNA, this time point corresponds to the end of 24 hours incubation in the presence of siRNA/RNAiMAX, as described in section 2.2.1.2. The wells were washed 2x with PBS and placed in serum free media (RPMI 1640, 1% L-Glutamine v/v) for 24 hours at 37°C with 5% CO₂ in air. Scratch ‘wounds’ were then made by scoring a hash symbol (#) through the monolayer using a p1000 pipette tip. The wells were washed x2 with PBS and fresh media added; the composition of this media varied for each experimental condition. Each of the four intersections of the ‘wound’ were then photographed at 40x magnification using phase contrast microscopy. The plates were then returned to the incubator at 37°C with 5% CO₂ for 14-24 hours.

At set time points the plates were removed from the incubator, the wells were washed x2 with PBS and the plate re-photographed at x40 magnification.

The images were then analysed using Image J software (National Institute of Health, USA, www.info.nih.gov/ij). The width of the scratch ‘wound’ was
measured at two identical points, at each of the 4 intersections, before and after 14-24 hours incubation. This measurement was made in units specific to the programme. The difference between the widths of the ‘wound’ at the two time points was calculated. The mean of the 16 values (2 values per intersection, 4 intersections per well and 2 wells per cell line for each experiment) was calculated and assigned as the migration index for that cell line under each experimental condition.

2.2.2 HGF stimulation assay

Cells were cultured in 6 well plates in standard media with 10% FCS in an humidified incubator at 37°C with 5% CO₂ in air until 80% confluent. The media was removed and discarded, the wells washed 2x with PBS and 2.5mls of RPMI + 1% L-glutamine v/v added to each well followed by a further 24 hours incubation. From each well 1000µl was media was then removed and discarded and 1000µl of fresh media (RPMI + 1% L-glutamine v/v) containing HGF at a concentration of 25ng/ml added to each well, to give a final concentration of 10ng/ml. The plates were then returned to the incubator and at set time points lysates produced from the wells by discarding the media, washing 2x with PBS and then lysing the monolayer of cells with 100µl of lysis buffer. Lysates were stored at -20°C.

2.2.3 Western blotting

2.2.3.1 Preparation of lysates for western blotting

Cell lysates were produced for western blotting by either direct lysis of cell monolayers in culture, or by lysis of cells obtained from the cell suspensions used in invasion assay experiments.
For monolayers in culture the media was removed and the cells washed 2x with PBS. Any remaining PBS was removed, an appropriate volume of lysis buffer added (for a 6 well plate this was 75-100µl), and the lysate removed with a cell scraper and pipette.

Cells in suspension were centrifuged for 5 minutes at 400g, brake rate 3 and then re-suspended in 500µl of PBS. This process was repeated, the PBS discarded, an appropriate volume of lysis buffer added and the cells lysed by mixing with a p200 pipette.

Following cell lysis, DNA shearing was performed using a 25G needle and protein concentration measured using a colorimetric DC® Protein Assay, used according to the manufacturers instructions and read on a plate reader at 630nm absorbance (BioTeck, Winooski, WT, USA). The required volume of lysate, to add a predetermined quantity of protein to each well in the electrophoresis gel (typically 5-20µg), was mixed with loading buffer at a ratio of 3:1 and heated to 99°C for 10 minutes and cooled prior to adding to the wells in the gel.

2.2.3.2 Protein electrophoresis and blotting

Gels were cast, 1.5mm thickness, with the lower ¾ composed of a running gel, of predetermined acrylamide concentration, and the upper ¼ composed of a stacking gel, both detailed in tables 2.2 and 2.3. Protein electrophoresis was performed in a running tank (Bio-Rad Laboratories, Hemel Hempstead, U.K.) containing 500mls of running buffer, initially at 90V (max. 200mA) to the limit of the stacking gel followed by 110V (max. 200mA), at room temperature. Protein blotting onto a nitrocellulose membrane was performed in a transfer tank on ice (Bio-Rad Laboratories, Hemel Hempstead, U.K.) containing 800mls of chilled transfer buffer at 110V (max. 300mA) for 70 minutes. Protein transfer was confirmed by staining the membrane with Ponceau Red followed by washing the membrane 4x for 15 minutes in TBST.
2.2.3.3 Antibody incubation and membrane exposure

The membrane was blocked by incubation in milk for 1 hour on an agitator followed by a single 60 second wash in TBST. Incubation with the primary antibody, diluted in TBS, was performed overnight at 4°C on an agitator. The membrane was washed for 15 minutes 4x in TBST and incubated with the appropriate HRP conjugated secondary antibody, diluted in milk, for 1 hour at room temperature followed by 4 final 15 minutes washes in TBST. Following the addition of Luminata® Forte solution the membrane was exposed using the chemiluminescent BioSpectrum® Imaging System in conjunction with VisionWorks®LS Image Acquisition and Analysis Software (Ultra-Violet Products Ltd., Cambridge, U.K.), producing a digital image of the exposed membrane.

2.2.3.4 Quantitative analysis of protein expression

Protein expression was quantified by densiometric analysis of each band using Image J software (National Institute of Health, USA, www.info.nih.gov/ij). In the time lapse experiments all changes were represented as fold change from baseline. Statistical comparisons were conducted using either a paired or unpaired T-test.
2.2.4 Immunohistochemistry

2.2.4.1 Antibody dilutions

All antibodies were stored at either 4°C or -20°C as per the manufacturer's instructions. Antibodies were diluted to the required concentrations for staining of sections for all experiments in either 1% BSA in PBS w/v or Bond® primary antibody dilutant.

2.2.4.2 Bench top manual immunohistochemistry staining

All bench top staining was performed with the slides in an humidified light proof IHC chamber. All steps were undertaken at room temperature unless otherwise specified.

2.2.4.2.1 DAB based chromogenic staining

Slides were de-waxed by sequential 5 minute immersions in three separate xylene baths. The sections were re-hydrated by sequentially dipping 6x in three 100%, one 90% and one 70% ethanol bath and immersed in water.

If required, epitope retrieval was performed by immersion in diluted low or high pH epitope retrieval solution (25mls target retrieval solution 10x, diluted in 225mls dd H₂O) and heating to 125°C for 1 minute and holding at 90°C for 10 minutes in a Pascal pressurised heating chamber (Dako, Ely, Cambridgeshire, U.K.). Slides were cooled in running tap water.

The tissue sections were encircled using a wax pen. 150μl of 0.3% H₂O₂ in PBS v/v was added for 20 minutes and the slides washed 2x with TBST. 150μl of blocking solution was added (1%BSA and 10% serum of the host of the secondary antibody in PBS) for 30 minutes at room temperature and the
blocking solution tapped off the slide.

For certain antibodies an avidin/biotin block was added that this stage. Two drops of avidin block for was added for 20 mins, the solution was then tapped off, 2 drops of biotin block for was added for 20 mins and the solution tapped off.

The primary antibody was then added for the specified incubation time and incubation temperature. The slides were washed 2x with TBST. 150ul of biotinylated secondary antibody (diluted 1:400) was added for 40 minutes and the slides were washed 2x with TBST. 150μl of Vectastain ABComplex (2% solution A and 2% solution B in PBS v/v) was added slide for 20 minutes and the slides washed 2x with TBST. 150μl of DAB solution (one gold and silver SIGMAFAST™ tablet in 5 mls of dd H2O) was added to each slide for 10 minutes. The slides were washed by immersion in a bath of PBS and placed in a water bath. The slides were counterstained with haematoxylin, dehydrated by sequentially dipping the slides 6x in one 70%, one 90% and three 100% ethanol baths followed by immersion in xylene. The sections were then cover-slipped.

### 2.2.4.2.2 Immunofluorescence staining

Slides were de-waxed, rehydrated and subjected to epitope retrival, if required, as described for DAB based chromogenic staining in section 2.2.4.2.1. The tissue section was then encircled using a wax pen and 150ul of blocking solution was added (1%BSA and 10% serum of the host of the secondary antibody in PBS) for 30 minutes at room temperature. The blocking solution was tapped off and the primary antibody added for the specified incubation time and incubation temperature. The primary antibody was tapped off the slide, the slides were washed 2x in PBS and the fluorophore conjugated secondary antibody added followed by incubation for 1 hour at room temperature. The secondary antibody was tapped off the slide and the slides washed 4x in PBS prior to mounting coverslips, previously
washed in 100% ethanol, with ProLong® Gold Antifade Reagent with DAPI. The slides were then stored in the dark at 4°C, for no longer than 48 hours, prior to being imaged using a MIRAX SCAN 150 slide scanner (Carl Zeiss Ltd. Cambridge, U.K.).
2.2.4.3 Automated staining

Automated staining was performed using the Leica BOND-MAX™ (Leica Microsystems, Milton Keynes, U.K.), a robotic system that undertakes automated: de-waxing, epitope retrieval, IHC DAB staining and haematoxylin counterstaining. Slides, blocking solutions, primary and secondary antibodies (diluted manually to a predetermined concentration) were loaded onto the robot at the start of each staining ‘run’. Automated dewaxing was performed followed by epitope retrieval at 98°C in a pH buffered solution. The post primary steps of the IHC protocol were undertaken using either the Bond™ Polymer Refine Detection System or the Bond™ Open Secondary Detection System (Leica Microsystems, Milton Keynes, U.K.). The Bond™ Polymer Refine Detection System is a biotin-free, polymeric HRP-linked antibody conjugate system for the detection of mouse and rabbit IgG. The Bond™ Open Secondary Detection System required the addition of a biotinylated antibody against the primary antibody (eg. RAGBO) and is used with non-mouse or non-rabbit primary antibodies. Both systems utilise a DAB based chromogenic staining step and haematoxylin counterstaining. Following these automated steps the slides were dehydrated and cover-slipped as for bench top staining in section 2.2.4.2.1.
2.2.4.4 Antibody optimisation

All antibodies were optimised by DAB staining sequential sections of either normal bladder and bladder cancer tissue or prostate and renal tissue samples (as positive controls). For each antibody protocol a negative control was also conducted by substituting the primary antibody for both an IgG1 isotype control or TBS. During the optimisation process a number of different steps were varied and directly compared, including:

- pH of epitope retrieval
- Epitope retrieval time
- The use of either a serum or serum free block
- The length of time the block was applied
- The primary antibody used
- Primary antibody concentration
- Primary antibody incubation time
- The use of an avidin and biotin block
- Secondary antibody concentration
2.2.5 Database and TMA construction

2.2.5.1 Database Updating

A cystectomy database existed containing information on all patients that had undergone a cystectomy at the Christie Hospital, for bladder and non-bladder malignancies, from 1969 to the current date. The existing cystectomy database was examined systematically, on a patient by patient basis, to ensure that all patients had contemporary follow up data recorded and to identify missing and conflicting data fields.

Documented follow up / patient contact was updated for each patient in July 2013. For patients recorded as being alive beyond 10 years following cystectomy, no further attempts were made to update their follow up information beyond what was already recorded.

The long term follow up of patients within the database was updated using two processes. Firstly a manual search of the Christie hospital electronic patient record (Medway) was undertaken on a patient by patient basis. For a number of patients, this database failed to provide sufficient information on the date and cause of death. For those patients where this data remained missing, date and cause of death data was obtained from the North West Cancer Intelligence Service (NWCIS). Data obtained by the NWCIS was derived from regional and national cancer registry data that includes information obtained from death certificates.
2.2.5.2 Case identification and TMA construction

2.2.5.2.1 Identification of tissue for inclusion

All patients within the updated cystectomy database prior to the introduction of the Human Tissue Act on the 1st September 2006 were identified. The original H&E slides, from the time of cystectomy, were retrieved from the Christie Pathology archive for review. Where the slides were not available the FFPE tissue blocks were retrieved, new 4μm sections were cut, mounted on to slides and stained with H&E. All slides were then reviewed by the author (RR), to identify tissue blocks of interest which contained areas of tumour, normal appearing urothelium and CIS.

Following initial screening, the identified FFPE blocks of interest were retrieved from the archive. Blocks not mounted into modern cassettes were melted down and the tissue re-embedded in paraffin wax in modern cassettes, prior to sectioning. A single 4μm tissue section was cut, mounted on a slide and stained with H&E. All of the new slides were reviewed by two pathologists (Dr C Womack, Honorary Professor of Pathology, Manchester University and Dr S Verma, Consultant Pathologist, Salford Royal Hospital), who identified areas of tumour, normal appearing urothelium and CIS. The pathologists individually marked these areas on the slides. For the non-bladder cancer cases, included as normal controls, areas of normal urothelium were identified and marked by the author (RR).

Cases were excluded for the following reasons:

- Patient in database not identified within the pathology records.
- FFPE blocks not present within archive.
- No urothelium identified in original slides.
- Insufficient tumour or urothelium identified on original slides.
- Tissue was of poor quality due to poor fixation or preservation.
In cases where the pathologists (CW and SV) identified insufficient tumour or urothelium, on the new H&E slides, all of the original slides from the time of cystectomy were re-reviewed to confirm tumour status.

### 2.2.5.2.2 TMA construction

Using a Manual Tissue Arrayer, MTA-1 (Beecher Instruments Inc., Sun Prairie, WI, USA) up to 3 cores of normal urothelium, tumour and CIS 1mm diameter x 3mm depth were removed from the donor FFPE tissue blocks, from areas identified and marked on the corresponding H&E slides. These cores were then randomly inserted into recipient paraffin wax cassette mounted blocks. The position of each core was recorded, including the donor patient identifier and the nature of the core (tumour, normal appearing urothelium or CIS). Once all of the cores had been implanted into the recipient blocks they were baked face down at 50°C for 2 hours.

From the TMA blocks 4μm sections were cut, mounted and stained with H&E prior to being evaluated by bright field microscopy to confirm the tissue content of each core, by a pathologist (CW).

### 2.2.6 Automated image analysis

All automated image analysis (AIA) was performed using Definiens® Tissue Studio 3.0 (Definiens AG, München, Germany).

Individual TMA sections, DAB chromogen stained for the marker of interest and counterstained with haematoxylin, were scanned into digital format using a Leica® brightfield scanner. Slide image files were subsequently loaded into Definiens® alongside CSV files containing data on slide core layout and
individual core identification. Each slide was then subjected to an automated core detection step, which outlined the region of interest (ROI) at each core location for further analysis. The accuracy of ROI definition was then subjected to quality control by visual screening with areas of artefact manually excluded, and missed areas defined as ROI’s.

The solution (analysis algorithm) ultimately used for the analysis of expression of each marker was then developed within Definiens®, using ROI from within 10 representative tissue cores. Once solution development was complete, all cores were then subjected to automated analysis, using the appropriate solution, producing a CSV file containing multiple data points for each core. The Definiens® data output files were then linked to the patient outcome database using Microsoft® Access prior to analysis.

2.2.7 Statistical Methods

All statistical analysis and graphing was performed in either GraphPad Prism® (GraphPad Software, CA, USA) or SPSS® (IBM, Portsmouth, U.K.).

Data from all of the cell line experiments is reported in the form of mean values from individual or combined experiments ± the standard error, except where stated.

In all cell culture experiments comparisons between different cell lines and between cell lines under different experimental conditions were conducted using an unpaired T-test, with significance set at p=0.05.

To analyse the baseline tumour demographics of all patients within original the database, and ultimately those included in the TMA, Kaplan-Meir plots were performed combined with a Mantel-Cox Log-Rank test, significance was set at p=0.05. Patients lost to follow up were censored. Multivariate Cox regression analysis was used to determine the independent prognostic power
of differing patient characteristics, tumour characteristics and marker expression.

The accuracy of Definiens®, to correctly classify tumour cells within the tissue cores, was directly compared to the actual classification of each cell within a core, as defined by the human eye, by calculating the sensitivity and specificity of this classification.

The solutions in Definiens® were programmed to output a number of parameters regarding each tissue core, including: total number of cells, histological score (H-score), the percentage of cells scored as either negative, low, medium or high (according to pre-determined thresholds) and the average absolute staining intensity in each cell compartment. The H-score was calculated as the sum of the individual products of the percentage of tumour cells within each core at each intensity threshold and the numerical score for that threshold on the scale 0, 1, 2 and 3. This is summarised in the formula:

\[
H\text{-score} = (1 \times \% \text{ of cells scoring low}) + (2 \times \% \text{ of cells scoring intermediate}) + (3 \times \% \text{ of cells scoring high}).
\]

This results in a range of 0-300. Cores were scored by human eye using the same H-score criteria, by two separate blinded observers on two separate occasions. Intra and inter-observer variability for these H-scores, including between Definiens® and human eye classification, were compared by calculating the intra-class correlation coefficient (ICC) using a single measures two-way mixed model. The same variability was also compared using Bland-Altman plots.
2.3 Identification of putative targets for further evaluation

The online database Oncomine™ (Oncomine 4.4.3, Compendia Bioscience, Ann Arbor, MI, USA, www.oncomine.org) was searched to identify putative targets for further investigation.

The following search filter was applied:

- Cancer Type: Infiltrating Bladder Urothelial Cancer

Only datasets based on human bladder tissue were included, cell line datasets were excluded. Datasets were also excluded if the data within Oncomine™ included only a data sub-set from the original source publication / dataset.

An analysis of included datasets was then conducted to compare and rank putative targets across the included datasets based upon gene over-expression / copy number gain. The top 50 ranked targets were then exported from the database and subjected to an assessment of their potential as treatment targets / biomarkers based upon the following factors:

- Published data on the target.
- Whether the target is likely to have originated from the inflammatory cell infiltrate that contaminates the tumour cell population in such analyses. Such targets were excluded from further investigation.
- Whether the target relates to a ubiquitous protein e.g. collagen. Such targets were excluded from further investigation.
- The “drugability” of the target.

This filtering process generated a list of putative targets for further investigation.
3 Results

3.1 Case selection and patient demographics

3.1.1 Case selection

The original Christie cystectomy database contained data on 743 patients who underwent a cystectomy for the treatment of bladder cancer (429 primary cystectomy and 310 salvage cystectomy following failed radiotherapy and 4 where this data was not available) and 210 who underwent a cystectomy as part of pelvic surgery for non-bladder cancer reasons.

A total of 137 cases were initially excluded on the basis of: 1 duplicate entry in database, 1 insufficient clinical data, 2 cases with previous upper tract TCC and cystectomy performed for other reasons, 6 cases with cystectomy performed for benign inflammatory bladder disorder, 34 cases not identified in pathology department database/logbooks, 64 cases operated on past the 1st September 2006 cut-off, 15 cases where the blocks were damaged and 14 cases where the blocks were not found within the archive; leaving 816 cases available for initial screening of the tissue blocks. This resulted in the retrieval of 12,421 original slides from the time of cystectomy for primary review. There were 54 patients where the original slides were missing. For these cases the individual blocks were retrieved and a further 174 new slides produced for more detailed screening.

Following initial review of all 12,595 original H&E slides a further 110 cases were excluded on the basis of: 81 cases with no bladder tissue within the blocks, 26 cases with insufficient urothelium and 3 with no tumour and insufficient normal urothelium.

Subsequent to review by a histopathologist, a further 139 patients were excluded due to: 53 cases with insufficient tumour or CIS and no normal
urothelium, 50 cases where the tissue was too poorly preserved, 20 cases with no urothelium and 16 cases with urothelial inflammation only.

This resulted in the inclusion of a total of 567 cases in the TMA overall, comprising 70 non-bladder cancer controls and 497 (66.9% of the original cohort of bladder cancer cases) bladder cancer cases (314 primary and 183 salvage).

Within these overall figures, 50 bladder cancer cases (all G3pT3 primary cystectomy) and two non-bladder cancer controls were used to construct a pilot TMA. This pilot TMA process was conducted prior to construction of the final TMA from the entire cohort. This sequential process is summarised in figure 3.1.

3.1.2 Demographics

The median age of the bladder cancer patients included in the TMA was 64.3 years (range 29 to 87), 73.6% were male and 63.2% had undergone a primary cystectomy. Overall 90.3% of tumours were of urothelial histological type (TCC and TCC with squamous differentiation combined), of which 9.6%, 17.4% and 64.8% were of 1973 WHO grades 1, 2 or 3 respectively; the remainder were of unknown grade. The cancers were staged as 7.8% pTis, 7.8% pTa, 18.1% pT1, 16.7% pT2, 30.6% pT3 and 16.9 pT4, with 4.8% pNx, 77.5% pN0 and 15.3% ≥pN1. The patient demographics and tumour characteristics (based upon histopathological assessment of the cystectomy specimen and lymphadenectomy) are summarised in figure 3.2 and tables 3.1 to 3.3.
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<td>30</td>
<td>6.0</td>
</tr>
<tr>
<td>TCC</td>
<td>266</td>
<td>84.7</td>
<td>162</td>
<td>88.5</td>
<td>428</td>
<td>86.1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>314</strong></td>
<td></td>
<td><strong>183</strong></td>
<td></td>
<td><strong>497</strong></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.1 - Histological subtypes of bladder cancer patients included in the TMA

<table>
<thead>
<tr>
<th>Tumour grade TCC only</th>
<th>Primary</th>
<th>% of primary group</th>
<th>Salvage</th>
<th>% of salvage group</th>
<th>Total cohort</th>
<th>% of total cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1 TCC only</td>
<td>25</td>
<td>9.0</td>
<td>18</td>
<td>10.5</td>
<td>43</td>
<td>9.6</td>
</tr>
<tr>
<td>G2 TCC only</td>
<td>54</td>
<td>19.4</td>
<td>24</td>
<td>14.0</td>
<td>78</td>
<td>17.4</td>
</tr>
<tr>
<td>G3 TCC only</td>
<td>183</td>
<td>65.8</td>
<td>108</td>
<td>63.2</td>
<td>291</td>
<td>64.8</td>
</tr>
<tr>
<td>Unknown</td>
<td>16</td>
<td>5.8</td>
<td>21</td>
<td>12.3</td>
<td>37</td>
<td>8.2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>278</strong></td>
<td></td>
<td><strong>171</strong></td>
<td></td>
<td><strong>449</strong></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tumour stage all patients</th>
<th>Primary</th>
<th>% of primary group</th>
<th>Salvage</th>
<th>% of salvage group</th>
<th>Total cohort</th>
<th>% of total cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tx</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>T0</td>
<td>1</td>
<td>0.3</td>
<td>6</td>
<td>3.3</td>
<td>7</td>
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<tr>
<td>CIS</td>
<td>26</td>
<td>8.3</td>
<td>13</td>
<td>4.1</td>
<td>39</td>
<td>7.8</td>
</tr>
<tr>
<td>Ta</td>
<td>26</td>
<td>8.3</td>
<td>13</td>
<td>4.1</td>
<td>39</td>
<td>7.8</td>
</tr>
<tr>
<td>T1</td>
<td>64</td>
<td>20.4</td>
<td>26</td>
<td>8.3</td>
<td>90</td>
<td>18.1</td>
</tr>
<tr>
<td>T2</td>
<td>53</td>
<td>16.9</td>
<td>30</td>
<td>9.6</td>
<td>83</td>
<td>16.7</td>
</tr>
<tr>
<td>T3</td>
<td>90</td>
<td>28.7</td>
<td>62</td>
<td>19.8</td>
<td>152</td>
<td>30.6</td>
</tr>
<tr>
<td>T4</td>
<td>52</td>
<td>16.6</td>
<td>32</td>
<td>10.2</td>
<td>84</td>
<td>16.9</td>
</tr>
<tr>
<td>Unknown</td>
<td>2</td>
<td>0.6</td>
<td>1</td>
<td>0.3</td>
<td>3</td>
<td>0.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nodal stage all patients</th>
<th>Primary</th>
<th>% of primary group</th>
<th>Salvage</th>
<th>% of salvage group</th>
<th>Total cohort</th>
<th>% of total cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nx</td>
<td>11</td>
<td>3.5</td>
<td>13</td>
<td>4.1</td>
<td>24</td>
<td>4.8</td>
</tr>
<tr>
<td>N0</td>
<td>238</td>
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<td>147</td>
<td>46.8</td>
<td>385</td>
<td>77.5</td>
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<tr>
<td>N1</td>
<td>43</td>
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<td>15</td>
<td>4.8</td>
<td>58</td>
<td>11.7</td>
</tr>
<tr>
<td>N2</td>
<td>18</td>
<td>5.7</td>
<td>0</td>
<td>0</td>
<td>18</td>
<td>3.6</td>
</tr>
<tr>
<td>Unknown</td>
<td>4</td>
<td>1.3</td>
<td>8</td>
<td>2.6</td>
<td>12</td>
<td>2.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gender all patients</th>
<th>Primary</th>
<th>% of primary group</th>
<th>Salvage</th>
<th>% of salvage group</th>
<th>Total cohort</th>
<th>% of total cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>224</td>
<td>71.3</td>
<td>142</td>
<td>77.6</td>
<td>366</td>
<td>73.6</td>
</tr>
<tr>
<td>Female</td>
<td>90</td>
<td>28.7</td>
<td>41</td>
<td>22.4</td>
<td>131</td>
<td>26.4</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>314</strong></td>
<td></td>
<td><strong>183</strong></td>
<td></td>
<td><strong>497</strong></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Age all patients</th>
<th>Primary</th>
<th>% of primary group</th>
<th>Salvage</th>
<th>% of salvage group</th>
<th>Total cohort</th>
<th>% of total cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median</td>
<td>63.9</td>
<td></td>
<td>65.3</td>
<td></td>
<td>64.3</td>
<td></td>
</tr>
<tr>
<td>Min</td>
<td>29</td>
<td></td>
<td>33</td>
<td></td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Max</td>
<td>87</td>
<td></td>
<td>85</td>
<td></td>
<td>87</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2 - Demographics and tumour characteristics of patients included in TMA

The individual columns indicate the breakdown of the number of patients in the primary cystectomy and salvage cystectomy groups with the proportions of those groups, expressed as percentages.
Figure 3.1 - Breakdown of selection for inclusion of cases into TMA
Red=excluded, blue=included for further analysis, green=final inclusion
Table 3.3 - Indication for cystectomy in the control cohort

<table>
<thead>
<tr>
<th>Indication for surgery</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anal carcinoma</td>
<td>3</td>
</tr>
<tr>
<td>Cervical carcinoma</td>
<td>29</td>
</tr>
<tr>
<td>Colonic carcinoma</td>
<td>3</td>
</tr>
<tr>
<td>Ovarian carcinoma</td>
<td>2</td>
</tr>
<tr>
<td>Prostatic carcinoma</td>
<td>1</td>
</tr>
<tr>
<td>Rectal carcinoma</td>
<td>26</td>
</tr>
<tr>
<td>Urethral carcinoma</td>
<td>1</td>
</tr>
<tr>
<td>Uterine carcinoma</td>
<td>2</td>
</tr>
<tr>
<td>Vaginal carcinoma</td>
<td>1</td>
</tr>
<tr>
<td>Vulval carcinoma</td>
<td>1</td>
</tr>
<tr>
<td>Other indication</td>
<td>1</td>
</tr>
</tbody>
</table>

Figure 3.2 - Patient age at the time of cystectomy

Absolute number of patients in each age category included in the TMA who underwent a cystectomy for the treatment of bladder cancer.
3.2 Patient Survival Data

3.2.1 Original database

Overall survival at 6 years, within the entire cohort of bladder cancer patients identified in the original Christie database, was 45.7% with a median survival of 48.7 months. There was a significant difference between the primary and salvage groups (p=0.047, Log-rank test), with 6-year survival rates of 48.0% and 42.2%, with a median survival of 57.1 and 37.2 months respectively [figure 3.3]. In a Cox regression model that included: gender, age at time of cystectomy, year of cystectomy, type of cystectomy (primary/salvage), histological type, T stage, N stage (either positive or negative) and WHO grade; only age at cystectomy (HR 1.039, 95%CI 1.026-1.051, p<0.0001), year of cystectomy (HR 0.965, 95%CI 0.955-0.975), T stage (comparator pTa: pT1 HR 1.872, 95%CI 0.744-1.895; pT2 HR 0.8157, 95%CI 0.489-1.361; pT3 HR 2.32, 95%CI 1.4112-3.531; pT4 HR 3.157 95%CI 1.975-5.048)) and N stage (lymph node positive HR 2.56, 95%CI 1.921-3.400, p<=0.0001) were predictive of overall survival (OS). Applying the same model to cancer specific survival (CSS), excluding 70 and 50 patients who had died by 6 years due to non-cancer causes and treatment related complications (death within 30 days of surgery due to complications) respectively, revealed an even greater effect for T-stage and lymph node status [table 3.4]. Death due to complications of surgery were excluded in the CSS model as those deaths are related more to to patient co-morbidity than the biology of the tumour [260].
Figure 3.3- Overall survival following cystectomy stratified by type of surgery.
Overall survival for bladder cancer patients in the original Christie cystectomy database, 1969-2006, stratified: primary cystectomy or salvage cystectomy after failed radiotherapy.

Figure 3.4 - Overall survival following cystectomy stratified by T-stage
Overall survival for bladder cancer patients in the original Christie cystectomy database, 1969-2006: stratified by tumour T-stage
Figure 3.5 - Overall survival following cystectomy stratified by patient age
Overall survival for bladder cancer patients in the original Christie cystectomy database, 1969-2006: stratified by patient age at the time of cystectomy.

Figure 3.6 - Overall survival following cystectomy stratified by year of surgery
Overall survival for bladder cancer patients in the original Christie cystectomy database, 1969-2006: stratified date of cystectomy.
Table 3.4 - Cox regression model for overall and cancer specific survival for all bladder cancer patients in the original Christie database

<table>
<thead>
<tr>
<th>Variable</th>
<th>Overall survival</th>
<th></th>
<th>Cancer specific survival</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P</td>
<td>HR</td>
<td>95% CI of HR Lower</td>
<td>Upper</td>
</tr>
<tr>
<td>Gender*</td>
<td>0.2498</td>
<td>1.1538</td>
<td>0.9043</td>
<td>1.4720</td>
</tr>
<tr>
<td>Cystectomy type§</td>
<td>0.9552</td>
<td>1.0065</td>
<td>0.8025</td>
<td>1.2625</td>
</tr>
<tr>
<td>Age at cystectomy</td>
<td>&lt;0.0001</td>
<td>1.0388</td>
<td>1.0263</td>
<td>1.0514</td>
</tr>
<tr>
<td>Year of surgery</td>
<td>&lt;0.0001</td>
<td>0.9647</td>
<td>0.9547</td>
<td>0.9748</td>
</tr>
<tr>
<td>Histological type</td>
<td>0.7566</td>
<td></td>
<td></td>
<td>0.7068</td>
</tr>
<tr>
<td>Adenocarcinoma♦</td>
<td>0.9479</td>
<td>0.9704</td>
<td>0.3938</td>
<td>2.3912</td>
</tr>
<tr>
<td>Anaplastic♦</td>
<td>0.3201</td>
<td>2.0440</td>
<td>0.4994</td>
<td>8.3656</td>
</tr>
<tr>
<td>Other♦</td>
<td>0.5438</td>
<td>1.3782</td>
<td>0.4893</td>
<td>3.8821</td>
</tr>
<tr>
<td>Sarcoma♦</td>
<td>0.3964</td>
<td>1.4788</td>
<td>0.5987</td>
<td>3.6525</td>
</tr>
<tr>
<td>SCC♦</td>
<td>0.3662</td>
<td>0.7904</td>
<td>0.4745</td>
<td>1.3165</td>
</tr>
<tr>
<td>SCC+SCC diff.♦</td>
<td>0.9997</td>
<td>0.9999</td>
<td>0.6060</td>
<td>1.6500</td>
</tr>
<tr>
<td>Unknown♦</td>
<td>0.4307</td>
<td>0.5250</td>
<td>0.1057</td>
<td>2.6073</td>
</tr>
<tr>
<td>T stage</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Node positive</td>
<td>&lt;0.0001</td>
<td>2.5554</td>
<td>1.9205</td>
<td>3.4003</td>
</tr>
<tr>
<td>Overall WHO grade</td>
<td>0.4102</td>
<td></td>
<td></td>
<td>0.3118</td>
</tr>
<tr>
<td>WHO grade 2#</td>
<td>0.5371</td>
<td>0.8729</td>
<td>0.5668</td>
<td>1.3443</td>
</tr>
<tr>
<td>WHO grade 3#</td>
<td>0.9509</td>
<td>1.0128</td>
<td>0.6747</td>
<td>1.5205</td>
</tr>
</tbody>
</table>

* in comparison to female gender, § in comparison to primary surgery, ♦ in comparison to TCC, # in comparison to WHO grade 1. HR=hazard ratio. CI=confidence interval.
3.2.2 Patients included in the TMA

3.2.2.1 Overall survival

Comparing those patients included and excluded from the TMA construction there was no statistically significant difference in overall survival (p=0.215, Log Rank), although there was trend towards a worse outcome in those included, with 6 year survival rates of 43.9% and 50.2% respectively [figure 3.7]. The breakdown of the pT-stage of these two cohorts is shown table 3.5.

Figure 3.7 - Comparison of overall survival between those patients included and excluded from the TMA
Comparison of all overall survival for the 203 cystectomy cases excluded from and the 497 cystectomy cases included in the TMA from 1969 to 2006 (bladder cancer cases only).
<table>
<thead>
<tr>
<th>Pathological tumour stage</th>
<th>Included in the TMA (%)</th>
<th>Excluded from TMA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pT0</td>
<td>1.4</td>
<td>13.3</td>
</tr>
<tr>
<td>pTis</td>
<td>7.8</td>
<td>4.4</td>
</tr>
<tr>
<td>pTa</td>
<td>7.8</td>
<td>11.8</td>
</tr>
<tr>
<td>pT1</td>
<td>18.1</td>
<td>19.7</td>
</tr>
<tr>
<td>pT2</td>
<td>16.7</td>
<td>13.3</td>
</tr>
<tr>
<td>pT3</td>
<td>30.6</td>
<td>21.2</td>
</tr>
<tr>
<td>pT4</td>
<td>16.9</td>
<td>9.9</td>
</tr>
</tbody>
</table>

**Table 3.5** – Percentage of patients at each pathological T-stage in the cohorts included and excluded from the TMA construction.

In a Cox regression model of patients included in the TMA, age at the time of cystectomy (HR 1.033, 95% CI 1.018-1.048, p<0.0001), year of cystectomy (HR 0.964, 95% CI 0.951-0.9755, p<0.0001), T-stage (p<0.0001) and lymph node status (HR 2.738, 95% CI 1.9728-3.8006) remained predictive of overall survival at 6 years. Gender, type of surgery (primary vs. salvage), histological subtype and WHO grade were not independent predictors of survival [table 3.6].

<table>
<thead>
<tr>
<th>Variable</th>
<th>Overall survival (n=497)</th>
<th>Cancer specific survival (n=416)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p</td>
<td>HR</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender*</td>
<td>0.2899</td>
<td>1.1663</td>
</tr>
<tr>
<td>Cystectomy type§</td>
<td>0.5143</td>
<td>1.0927</td>
</tr>
<tr>
<td>Age at cystectomy</td>
<td>&lt;0.0001</td>
<td>1.0330</td>
</tr>
<tr>
<td>Year of surgery</td>
<td>&lt;0.0001</td>
<td>0.9634</td>
</tr>
<tr>
<td>Histological type</td>
<td>0.7573</td>
<td>0.7510</td>
</tr>
<tr>
<td>T stage</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Node positive</td>
<td>&lt;0.0001</td>
<td>2.7382</td>
</tr>
<tr>
<td>WHO grade</td>
<td>0.9107</td>
<td>0.8333</td>
</tr>
</tbody>
</table>

**Table 3.6** - Cox regression model for overall and cancer specific mortality for bladder cancer patients included in the TMA.

* in comparison to female gender, § in comparison to primary surgery, HR=hazard ratio. CI=confidence interval.
At 6 years following cystectomy 10 patients (2%) were lost to follow up, 15 (3%) had died due to unknown causes, 36 (7.2%) due to treatment related complications (death within 30 days of surgery), 45 (9.1%) due non-bladder cancer causes, 193 (38.8%) were alive and 198 (39.8%) had died due to bladder cancer [table 3.7].

<table>
<thead>
<tr>
<th>Status of patient at 6 years</th>
<th>n</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alive</td>
<td>193</td>
<td>38.8</td>
</tr>
<tr>
<td>Lost to follow up</td>
<td>10</td>
<td>2.0</td>
</tr>
<tr>
<td>Death due to bladder cancer</td>
<td>198</td>
<td>39.8</td>
</tr>
<tr>
<td>Death due to other causes</td>
<td>45</td>
<td>9.1</td>
</tr>
<tr>
<td>Death within 30 days of surgery</td>
<td>36</td>
<td>7.2</td>
</tr>
<tr>
<td>Death due to unknown cause</td>
<td>15</td>
<td>3.0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>497</td>
<td>100.0</td>
</tr>
</tbody>
</table>

*Table 3.7 - Status of all patients included in the TMA at 6 years following cystectomy.*
3.2.2.2 Cancer specific survival

To calculate cancer specific survival at 6 years the 45 confirmed non-cancer deaths and 36 deaths within 30 days of surgery were excluded, leaving 416 cases for analysis of CSS. Of the 36 patients who died of treatment related complications 21, 9, 3 and 3 were operated on in the 1970’s, 1980’s, 1990’s and 2000’s respectively. This equated to a treatment related death rate of 16.6%, 10.8%, 2.3% and 1.9% respectively [figure 3.8]. The median time to treatment related death was 14 days.

In patients included in the TMA the median time from the end of radiotherapy to undergoing salvage cystectomy was 16.9 months. There were no significant differences in cancer specific survival between the primary and salvage groups (p=0.112, Log Rank) with 6 year survival rates of 54.6% and 48.3% respectively [figure 3.9].

Figure 3.8 - Treatment related mortality rate stratified by date of cystectomy.
Data expressed as the percentage treatment related death rate of all patients undergoing cystectomy in each 5 year period.
An identical Cox regression model to that used for overall survival used was applied to cancer specific survival in the patients included in the TMA. In this model only age at cystectomy (HR 1.099, 95%CI 1.003-1.037, p=0.0184), year of cystectomy (HR 0.970, 95%CI 0.956-0.985, p<0.0001), T-stage (p<0.0001) and lymph node status (HR 3.421, 95%CI 2.373-4.931, p<0.0001) were independent predictors of bladder cancer related death at 6 years following cystectomy [table 3.6 and figures 3.10-3.13]. There were no significant differences in CSS between pTis, pTa, pT1 or pT2 or between pT3 and pT4 in pair wise comparisons (Log Rank test), although there was a clear trend towards a worse outcome with increasing T-stage [figure 3.13 and table 3.8]. There were significant differences between both pT3 and pT4 in pairwise comparison either to pTis, pTa, pT1 or pT2 (p<0.0001, Log Rank). The same pattern was also seen in the Cox regression model, where in comparison to stage pTa as a baseline, pT3 had a HR 3.583 (95%CI 1.453-8.35, p=0.0056) and pT4 had a HR 5.893 (95%CI 2.407-14.426, p<0.0001), [table 3.6].
Figure 3.9 - Cancer specific survival following cystectomy for patients included in the TMA stratified by type of surgery
CSS comparing 269 primary and 147 salvage cystectomy cases from 1969 to 2006

Figure 3.10 - Cancer specific survival following cystectomy for patients included in the TMA stratified by year of surgery
CSS for all 416 bladder cancer patients included in the TMA, stratified by the date of cystectomy.
Figure 3.11 - Cancer specific survival following cystectomy for patients included in the TMA stratified by patient age

Cancer specific survival in 416 bladder cancer patient included in the TMA, stratified by the age of patient at the time of cystectomy.

Figure 3.12 - Cancer specific survival following cystectomy for patients included in the TMA stratified by lymph node status

Cancer specific survival in 416 bladder cancer patient included in the TMA, stratified by pathological lymph node status.
Figure 3.13 - Cancer specific survival following cystectomy for patients included in the TMA stratified by T stage

Cancer specific survival in 416 bladder cancer patient included in the TMA, stratified by the pathological T stage of the bladder tumour.

<table>
<thead>
<tr>
<th></th>
<th>pTa</th>
<th>pT1</th>
<th>pT2</th>
<th>pT3</th>
<th>pT4</th>
<th>pTis</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTa</td>
<td></td>
<td>0.1937</td>
<td>0.2870</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.7367</td>
</tr>
<tr>
<td>pT1</td>
<td>0.1937</td>
<td></td>
<td>0.7620</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.0778</td>
</tr>
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</table>

Table 3.8 - Statistical significance of pairwise comparisons of differences in 6 year cancer specific survival stratified by T stage for patient included in the TMA

Pairwise comparisons represent p values of statistical significance calculated by Log Rank test (Mantel Cox) for the differences in cancer specific survival for figure 3.13.
3.2.3 Patients included in the TMA from 1985 onwards

Due to the marked differences in OS prior to and post 1985 [figure 3.6] and differences in the expression of panCK in cores taken from patients who underwent cystectomy prior to 1985 [figure 3.41] the final analysis was conducted only in patients who underwent surgery from 1985 onwards only, discussed in more detail in section 4.1.1.

Excluding patients from prior to 1985 left 329 bladder cases from the final TMA, of whom 89.4% were of TCC histological sub-type. The median age was 65.4 years (range 29.5 - 85.1), 72.6% male and 43.5% salvage cystectomy [table 3.9].

In Cox regression modelling the year of surgery no longer remained a significant predictor of OS or CSS [table 3.10]. Age at cystectomy remained predictive of OS (HR 1.0280, 95%CI 1.0100-1.0470, p=0.0030) but not CSS (HR 1.0180, 95%CI 0.9970-1.390, p=0.0910). T stage overall was a significant predictor of survival (p<0.0001) and specifically stage pT4 predicted OS (HR 3.0890, 95% CI 1.3650-6.9930, p=0.0070) and CSS (HR 5.0370, 95% CI 1.4400-17.6120, p=0.0110). Lymph node status also remained a significant predictor of OS (HR 2.6140, 95%CI 1.750-3.8970, p<0.0001) and CSS (HR 3.1730, 95%CI 2.0410-4.9340, p<0.0001)
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<td>52</td>
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<tr>
<td>T3</td>
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<td>28.3</td>
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<tr>
<td>Nx</td>
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<td>1.8</td>
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<tr>
<td><strong>Surgery type all patients</strong></td>
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<tr>
<td>Primary</td>
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<td>56.5</td>
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<tr>
<td>Salvage</td>
<td>104</td>
<td>43.5</td>
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<tr>
<td><strong>Gender all patients</strong></td>
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<tr>
<td>Male</td>
<td>239</td>
<td>72.6</td>
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<td>Min</td>
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<tr>
<td>Max</td>
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Table 3.9 - Demographics and tumour characteristics of patients included in TMA from 1985 onwards
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<th>Overall survival (n=329)</th>
<th>Cancer specific survival (n=288)</th>
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<td>p</td>
<td>HR</td>
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<td>Gender*</td>
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<td>0.9750</td>
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<td>Histological type</td>
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</tr>
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<td>Node positive</td>
<td>&lt;0.0001</td>
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<tr>
<td>WHO grade</td>
<td>0.9250</td>
<td></td>
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</table>

Table 3.10 - Cox regression model for overall and cancer specific mortality for bladder cancer patients included in the TMA from 1985 onwards

* in comparison to female gender, § in comparison to primary surgery, ^ in comparison to stage pTa. HR=hazard ratio. CI=confidence interval.
3.3 Identification of a panel of bladder cancer cell lines for in-vitro modelling of bladder cancer

In parallel to the IHC analysis of the TMA a cell line model of bladder cancer was required, to study in-vitro the mechanisms involved with the putative targets identified. A detailed search of the published literature for research involving bladder cancer cell lines and the commercially available bladder cancer cell lines from the European Collection of Cell Cultures (ECACC) and the American Type Culture Collection (ATCC) produced a list of 32 bladder cancer cell lines. Examination of the published literature in relation to the grade and stage of the tumour of origin, invasive potential and immune-phenotype enabled the characterisation of these cell lines [table 3.11]. On the basis of these characteristics and availability, six cell lines: RT112, 5637, UM-UC-3, HT1376, T24 and J82 were chosen. These cell lines were felt to represent the spectrum of bladder cancer as they included both low grade papillary and high grade invasive tumours of origin, and had reportedly differing invasive potentials in-vitro and immuno-phenotypes.

The 5637 cell line was already held by the genito urinary cancer research group at the University of Manchester (UoM). The UM-UC-3, HT1376, T24 and J82 cell lines were kindly donated by Dr. Amanda Williams of the Translational Radiobiology Group at the UoM. The RT112 cell line was purchased from EACC. All six cell lines were successfully authenticated by the Molecular Biology Core Facility at the CRUK Manchester Institute.
<table>
<thead>
<tr>
<th>Cell line</th>
<th>Grade &amp; Stage</th>
<th>Nature of tumour</th>
<th>Invasive potential</th>
<th>p16 gene status</th>
<th>PIK3CA gene status</th>
<th>p53 gene status</th>
<th>Ras gene status</th>
<th>FGFR3 gene status</th>
<th>FGFR3 expression</th>
<th>E-Cad expression</th>
<th>N-Cad expression</th>
<th>Vimentin expression</th>
<th>c-MET expression</th>
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<td>N-Cad expression</td>
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<td>++[267]</td>
<td>+[267]</td>
<td>-[267]</td>
</tr>
</tbody>
</table>

**Table 3.11** - Characteristics of all bladder cancer cell lines commercially available from ECACC and ATCC.

Mut = mutated, WT = wild type, + = low expression, ++ = intermediate expression, +++ = high expression, - = no expression, [number] = reference from which data derived.
3.4 Oncomine™ search results

A search of Oncomine™ using the filter: Cancer Type - Infiltrating Bladder Urothelial Cancer returned a total of 13 datasets. Three were excluded as they were cell line datasets and a further dataset from Modlich et al. was excluded as it only included a subset of data from the original publication [283]. The remaining nine datasets combined contained data from 1075 patients. Of these nine datasets, eight were associated with publications [161, 283-288] with the remaining one comprising data submitted to Oncomine™ by the Cancer Genome Atlas on 58 patients.

Of the nine datasets included eight were based upon mRNA expression data, measured using a number of different platforms, and one (the TCGA dataset) was based upon DNA copy number alteration data. All of the data was normalised and expressed either as log₂ copy number units for DNA data, or log₂ median centred intensity for mRNA expression. Based upon the classification filter applied Oncomine then ranks each gene, based upon copy number gain or overexpression, in each of the individual datasets. Each gene is then ranked across all datasets, based upon the median rank of the gene in the individual datasets.

This process of pooled analysis has potential advantages in identifying target genes, but also a number of weaknesses. The main advantage is that Oncomine allows the investigator to combine datasets originally derived from completely separate studies unrelated to the question asked in the pooled Oncomine analysis being conducted. Systematic bias should therefore be largely eliminated. The use of median centered normalised data also allows the direct comparison of datasets collected at different time points and between different external cohorts. Combined analysis of multiple datasets from multiple studies should reduce the effects of confounding factors, such as age or race etc., which may significantly contribute to the findings in
individual studies examined in isolation. The use of multiple external cohorts, acts as a form of external validation.

However, there are some issues with this type of analysis for identifying target genes. They can be broadly grouped as pathological bias, cohort bias and technical bias. Firstly the tissue samples used to generate the raw data will have contained tissue types and cells other than bladder cancer tissue. This is particularly likely as the filter used in the search was that of ‘infiltrating bladder cancer’. The gene data will therefore in part reflect the biology of the stroma and infiltrating immune cells, and not purely that of the cancer. This tissue contamination is not quantifiable based upon the data available, but the high expression of genes usually associated with the extracellular matrix/stoma (eg. collagen and fibronectin) makes this phenomenon seem likely.

The patient populations in each of the included datasets will also differ, due to variations in parameters including tumour stage, grade, subtype, geographical variations and prior treatment received. There are also technical biases inherent to each of the platforms used for raw data generation. Combining these datasets, as takes place in Oncomine, without accounting for these biases may result in a misleading data output.

The basis for using Oncomine was to identify a starting point in relation to putative targets. As the final conclusions regarding the roles of the targets identified was not based upon the analysis of the Oncomine data is seems an acceptable place to start; accepting the potential weaknesses of the this form of pooled analysis.

The top 50 genes, based upon copy number gain or over-expression are shown in the supplementary data in appendix 1, with the top ten shown in table 3.12. These 50 genes were assessed based upon the criteria described in section 2.3, including whether they were ubiquitous proteins or potentially originating from inflammatory cell infiltration and their potential role in EMT, invasion and metastasis. Connective tissue growth factor (CTGF) and
cysteine rich angiogenic inducer 61 (CYR61) were selected as putative treatment targets for further investigation, based upon this process. There is evidence that there is interaction between the HGF/MET axis and CTGF/CYR61 [289, 290], summarised in figure 3.14. Combined with the increasing interest in MET axis targeting therapies in other cancers, and only sparse data on MET in bladder cancer, MET was also selected as a target for further investigation.
Figure 3.14 - Schematic representation of the proposed interactions between the MET axis and the CCN proteins.

A&β=integrin subunits, FAK=focal adhesion kinase, ILK=integrin linked kinase.
1: Ligand-independent MET activation by hetero-dimerization with integrin activated by CCN class proteins 2: Canonical MET activation by ligand HGF. 3&4: Canonical integrin-agonist interaction with CCN class proteins.
<table>
<thead>
<tr>
<th>Rank in Oncomine™</th>
<th>Gene</th>
<th>Median rank (in original datasets)</th>
<th>Product</th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td>COL1A1</td>
<td>23</td>
<td>Pro-α1 chain of type 1 collagen</td>
</tr>
<tr>
<td>2</td>
<td>PLEKHG2</td>
<td>35</td>
<td>Pleckstrin homology domain containing, family G member 2</td>
</tr>
<tr>
<td>3</td>
<td>FN1</td>
<td>39</td>
<td>Fibronectin 1</td>
</tr>
<tr>
<td>4</td>
<td>PSTN</td>
<td>39</td>
<td>Periostin, osteoblast specific factor</td>
</tr>
<tr>
<td>5</td>
<td>AEBP1</td>
<td>40</td>
<td>AE binding protein 1</td>
</tr>
<tr>
<td>6</td>
<td>GLIPR2</td>
<td>42</td>
<td>Glioma pathogenesis-related protein 2</td>
</tr>
<tr>
<td>7</td>
<td>CTGF</td>
<td>52</td>
<td>Connective tissue growth factor</td>
</tr>
<tr>
<td>8</td>
<td>NNMT</td>
<td>52</td>
<td>Nicotinamide N-methyltransferase</td>
</tr>
<tr>
<td>9</td>
<td>MT2A</td>
<td>53</td>
<td>Metallothionein 2A</td>
</tr>
<tr>
<td>10</td>
<td>CYR61</td>
<td>57</td>
<td>Cysteine-rich, angiogenic inducer, 61</td>
</tr>
</tbody>
</table>

Table 3.12 - Genes of interest identified from and Oncomine™ comparison of 9 bladder cancer datasets

The top 10 genes of interest identified from an Oncomine™ list of the top 50 over-expressed/copy number gain genes when comparing invasive and non-invasive bladder TCC from 9 independent datasets. The Oncomine™ analysis rank indicates the position of the gene in the top 50 list. The median rank across original individual datasets column indicates the median rank of the gene across the individual datasets based upon the same comparative criteria – invasive vs. non-invasive.
3.5 Cell line characteristics

3.5.1 Cell proliferation rates of the six bladder cancer cell lines

SRB proliferation assays were conducted, of the six different cell lines (RT112, 5637, UM-UC-3, HT-1376, T24 and J82), to determine the proliferation rates of the cell lines and optimum seeding density for further cytotoxicity screening assays [figure 3.14]. During the time point 72-96 hours incubation, all six cell lines remained in an exponential phase of growth from an initial seeding number of 4000 cells per well. At seeding numbers of 8000 and 1600 the J82 and all six cell lines respectively had existed the exponential phase of growth and plateaued. The optimum seeding density, based upon these proliferation profiles, to conduct a 72 hour cytotoxicity screen, was therefore estimated at 4000 cells per well. At this initial density and after 72 hours of incubation the cell lines in order of increasing proliferation rate were: HT1376, UMUC3, J82, 5637, T24 and RT112.
Figure 3.15 - 120 hour SRB assays comparing the proliferation rates of 6 bladder cancer cell lines

4,000 (A), 8,000 (B) and 16,000 (C) cells were seeded per well in 96 well plates. After two hours of incubation (5% CO₂ in air at 37°C) and then after each subsequent 24 hours for a total of 120 hours the plates were fixed, stained with SRB and the optical density of each well measured using plate reader at λ490nm. Values were calculated as mean fold change per cell line from two hours post seeding. Error bars = standard error of the mean.
3.5.1 Comparison of invasive potential of the six bladder cancer cell lines

3.5.1.1 Invasion response to foetal calf serum

The invasive characteristics of each cell line was assessed using a Boyden chamber Matrigel™ assay towards either 5% FCS or tissue culture plastic (TCP). Combined data from 3 independent experiments, adding $10^6$ cells per insert followed by 18 hours incubation, revealed marked differences in invasion across the six cell lines investigated [figure 3.15]. There was a statistically significant invasion response ($p<0.05$, unpaired T-test) to 5% FCS compared to TCP for all cell lines other than the UMUC3 and HT1376. The mean number of cells invading towards 5% FCS per 10mm x 10mm graticule at 100x magnification was: 53.3(±21.5), 134.6(±72.8), 150.4(±25), 190.4(±93.1), 873.3(±41.2) and 1245.7(±78.9) for the 5637, HT-1376, RT112, UM-UC-3, J82 and T24 cell lines respectively.

In pairwise comparisons there were significant differences (unpaired T-test, $p<0.05$) in invasion towards 5% FCS between all cell lines, other than between: RT112 and UMUC3, RT112 and HT1376, 5637 and UMUC3, 5637 and HT1376, and between UMUC3 and HT1376 [table 3.13].

3.5.1.2 Defining the optimum HGF concentration for invasion assays

The optimum HGF concentration required to stimulate cellular invasion during a Boyden chamber Matrigel™ assay was determined using the RT112 and J82 cell lines using the same methodology as in section 2.4.2.1. Different concentrations of HGF were added to media either with or without 5% FCS in the lower chamber. The highest number of invading cell occurred for both cell lines in the presence of RPMI + 1% L-glutamine + 5%FCS + 10ng/ml HGF in the lower chamber. Increasing the concentration of HGF to 100ng/ml in the presence of 5% FCS resulted in a reduction in invasion compared to an HGF
The optimum concentration for all future HGF invasion assays was therefore set at 10ng/ml.

### 3.5.1.3 Invasion response to hepatocyte growth factor

The invasive characteristics of each cell in response to stimulation with HGF were assessed using a Boyden chamber Matrigel™ assay, as described in section 2.2.1.3, towards either 10ng/ml HGF or TCP. Combined data from 3 independent experiments, revealed marked differences in invasion across the six cells in response to HGF with a mean of 1.8, 30.5, 38.8, 106.3, 369.8 and 528.8 cells invading towards HGF for the RT112, 5637, HT1376, UMUC3, J82 and T24 cell lines respectively [figure 3.17]. The increase in cell invasion towards HGF compared to TCP alone was statistically significant (p<0.05, unpaired T-test) for all cell lines other than the UMUC3 and HT1376 lines. In pairwise comparison of invasion towards HGF (unpaired T-Test) there was a significant difference between the J82 and T24 cell lines and all other cell lines (p<0.05) but not between the J82 and T24 cell line (p=0.1422), [table 3.14].
Figure 3.16 - Comparison of the invasive ability of six bladder cancer cell lines towards TCP or 5% FCS

Combined data from three independent experiments. 1 x 10^5 cells were seeded into the upper insert of a Boyden chamber with a Matrigel™ coated 8μm pore membrane, above either serum free media (TCP) or media containing 5% FCS. The number of cells invading post incubation (18hrs, 5% CO₂ in air at 37°C) per 10mm x 10mm graticule at 100x magnification were counted. Error bars = standard error of the mean, FCS = Foetal calf serum, TCP = tissue culture plastic. Statistical significance shown, unpaired T-test compares each cell line towards TCP or 5%FCS, * p<0.05, ****p<0.0001, ns = not significant.

Table 3.13 - Statistical significance of pairwise comparisons of cell line invasion towards 5% FCS

<table>
<thead>
<tr>
<th></th>
<th>RT112</th>
<th>5637</th>
<th>UMUC3</th>
<th>HT1376</th>
<th>T24</th>
<th>J82</th>
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<td>RT112</td>
<td>0.0122</td>
<td>0.0122</td>
<td>0.6856</td>
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</tr>
<tr>
<td>5637</td>
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<td>0.1769</td>
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<td>0.6450</td>
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<td>UMUC3</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>J82</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Pairwise comparisons represent p values of statistical significance calculated by an unpaired T-test for the differences between invasion towards 5% FCS in the six bladder cancer cell lines shown in figure 3.15.
Figure 3.17 - Comparison of the invasion of the J82 (A) and RT112 (B) cell lines towards varying concentrations of HGF in either plain media or media with 5% FCS

$1 \times 10^5$ cells were seeded into the upper insert of a Boyden chamber with a Matrigel™ coated 8μm pore membrane, above increasing concentrations of HGF in either serum free media or media containing 5% FCS. The number of cells invading post incubation (18hrs, 5% CO$_2$ in air at 37°C) per 10mm x 10mm graticule at 100x magnification were counted. Error bars = standard error of the mean, FCS = Foetal calf serum. Bar appearance indicated composition of media in lower chamber: plain bars = RPMI +1% L-Glutamine, hatched bars = RPMI +1% L-Glutamine + HGF, diagonal bars = RPMI +1% L-Glutamine + 5%FCS, chequered bars = RPMI +1% L-Glutamine + 5%FCS + HGF.
Figure 3.18 - Comparison of the invasion of six bladder cancer cell lines towards TCP and HGF 10ng/ml

N=3. 1 x 10^5 cells were seeded into the upper insert of a Boyden chamber with a Matrigel™ coated 8μm pore membrane, above either serum free media (plain bars) or serum free media containing 10ng/ml HGF (hatched bars). The number of cells invading post incubation (18hrs, 5% CO₂ in air at 37°C) per 10mm x 10mm graticule at 100x magnification were counted. Error bars = standard error of the mean, FCS = Foetal calf serum, TCP = tissue culture plastic. Statistical significance shown, unpaired T-test, compares each cell line towards serum free media or 10ng/ml HGF, * p<0.05, **p<0.01, ns = not significant.

<table>
<thead>
<tr>
<th></th>
<th>RT112</th>
<th>5637</th>
<th>UMUC3</th>
<th>HT1376</th>
<th>T24</th>
<th>J82</th>
</tr>
</thead>
<tbody>
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<td>0.0024</td>
<td>0.1627</td>
<td>0.0002</td>
<td>0.0019</td>
</tr>
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<tr>
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<td>0.0112</td>
<td>0.0041</td>
<td>0.1466</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.14 - Statistical significance of pairwise comparisons of cell line invasion towards 10ng/ml HGF

Pairwise comparisons represent p values of statistical significance calculated by an unpaired T-test for the differences between invasion towards HGF 10ng/ml in the six bladder cancer cell lines shown in figure 3.17.
3.5.2 Screening for cisplatinum sensitivity of the bladder cancer cell lines

Cytotoxicity screening was undertaken to determine the sensitivity of the six cell lines to cisplatinum, a chemotherapy drug commonly used in the treatment of bladder cancer. The lowest IC₅₀ value for cisplatinum across the six cell lines was estimated, based upon published data, to be approximately 1.0μg/ml. Cytotoxicity screens were therefore performed on all six cell lines across the drug dose ranges 0.001-50μg/ml for cisplatin. The drug vehicle used for dilution was 0.9% saline. Cells were seeded at 4000 per well and incubated in the presence of the drug for 72 hours. The mean fold changes in cell proliferation for each drug concentration were normalised against the mean values for each cell line cultured in the presence of the drug vehicle alone and used to plot a dose inhibitory response curve [figure 3.18]. Plots and IC₅₀ values were determined using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA).

The calculated IC₅₀ values for cisplatinum were 0.046, 0.453, 0.729, 0.846, 0.708, 0.917 and 0.974 (μg/ml) for the T24, J82, 5637, HT1376, UMUC3 and RT112 cell lines respectively.
Figure 3.19 - Dose inhibitory response curve for cell proliferation for six bladder cancer cell lines across a range of concentrations of cisplatinum

4,000 cells were seeded per well in 96 well plates and cultured for 72 hours (5% CO₂ in air at 37°C) in the presence of the drug vehicle (0.9% saline) and increasing concentrations of cisplatinum. Cell proliferation was assessed using an SRB assay. Using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA), the mean absolute values were normalised to cells grown in the presence of the drug vehicle. Error bars = standard error of mean normalised values.
3.5.3 Bladder cancer cell line migration

3.5.3.1 Migratory potential of the six bladder cell lines

The migratory potential of each cell line was assessed using an overnight scratch ‘wound’ assay of an 85% confluent monolayer of each cell line [section 2.2.1.5]. In the initial experiment the cells were incubated for 24 hours in the presence of 5% FCS following ‘wounding’ and the plates re-imaged. The ‘wound’ was completely bridged by three cell lines (5637, J82 and T24) at 24 hours. On this basis the experiment was repeated and the plates were re-imaged 14 hours after ‘wounding’. The migration indices of the cell lines were 56(±13), 197(±49), 218(±35), 398(±29), 605(±29) and 861(±40) for the HT1376, RT112, UM-UC-3, 5637, J82 and T24 lines respectively [figure 3.19]. There were statistically significant differences between the migration indices of all cell lines, in pairwise comparisons (p<0.01, unpaired T-test) except when comparing the RT112 and UM-UC-3 lines (p=0.729, unpaired T-test).

Figure 3.20 - Mean migration indices of the six bladder cancer cell lines

Cells at 85% confluence were scratch ‘wounded’ using a p1000 pipette tip. The ‘wounds’ were imaged immediately and then after 14 hours incubation with 5% FCS (5% CO₂ in air at 37°C). The mean difference in width of the ‘wounds’ at these time points was calculated as the migration index. Error bars = standard error of mean.
3.5.3.2 Migration of the bladder cell lines in response to HGF

Cells were cultured and wounded as in section 2.2.1.5, except that the experiment was conducted under two different conditions. After wounding, the media was changed to either RPMI + 1% L-glutamine or RPMI + 1% L-glutamine + 10ng/ml HGF and the cells re-imaged after 14 hours. In combined data from three independent experiments there was a 1.37, 1.43, 2.32, 2.74, 3.22 and 3.50 fold change in the migration indices of the HT1376, UMUC3, 5637, J82, T24 and RT112 and cell lines in response to 10ng/ml HGF [figure 3.20]. This change was statistically significant for the RT112, 5637, T24 and J82 cell lines (p<0.0001, unpaired T-test), but not for the UMUC3 (p=0.0644) or HT1376 cell lines (p=0.0911). In order of increasing migration the overall mean migration indices in response to HGF were 140.0 (±18.4), 301.3 (±35.6), 329.5 (±26.6), 494.0 (±27.9), 506.4 (±24.3) and 585.3 (±24.0) for the HT1376, UMUC3, RT112, J82, 5637 and T24 cell lines respectively.

Figure 3.21 - Mean migration indices of the six bladder cancer

n= 3 independent experiments. Cells at 85% confluence were scratch 'wounded' and imaged immediately and after 14 hours incubation either with or without 10ng/ml HGF (5% CO₂ in air at 37°C). The mean difference in 'wound' width over 14hrs was calculated as the migration index (MI). Error bars = SEM, plain bar = RMPMI + 1% L-glutamine, hatched bar = RPMI + 1% L-glutamine + 10 ng/ml HGF. Statistical significance - unpaired T-test. ****p<0.0001.
3.5.4 Cell line expression of putative targets and EMT markers

The expression of MET, CTGF and CYR61 was assessed by western blotting, as described in section 2.2.3, in the six bladder cancer cell lines. The expression of the epithelial marker E-cadherin and the mesenchymal markers vimentin and N-cadherin were also assessed in the same manner.

The initial blots were performed using lysates from all six bladder cell lines and lysates from the renal cancer cell lines 786-0 and A498 as positive controls. Abcam polyclonal rabbit anti-CTGF and CYR61 and Millipore anti-MET antibodies did not produce results as expected. The Abcam antibodies produced no clear bands [figure 3.21], and the Millipore anti-MET antibody produced a band at approximately 110 KDa, whereas the predicted band for MET should occur at 170KDa [figure 3.22]. Repeats of the western blots were therefore performed and incubated with a Santa Cruz goat anti-CTGF and rabbit anti-CYR61 and with an Invitrogen mouse anti-MET antibody. This produced bands as expected at 38, 40 and 170 KDa for CTGF, CYR61 and MET respectively [figures 3.21 and 3.22].

The six cell lines had differing expressions of the EMT markers and the putative targets, as shown in figure 3.33. The UMUC3 line had the highest relative expression of vimentin with the 5637 and J82 expressing low levels and the T24, HT1376 and RT112 all being negative. N-cadherin was expressed in all cell lines except the RT112. E-cadherin was expressed at a relative high level in the RT112 cell lines and at a low level in the 5637, whereas all of the other lines were negative. MET was expressed in all six cell lines, although at a relative low level in the RT112 line. CTGF expression was highest in the HT1376 and T24 lines with relative low levels in the UMUC3 and J82, the RT112 and 5637 were negative for expression. CYR61 was expressed at the highest level in the J82 line with relative low levels in the T24, UMUC3 and HT1376, both the 5637 and RT112 were negative for expression.
Figure 3.22 - Western blots of the six bladder cancer cell lines shown for CYR61 and CTGF
Left column CYR61 - predicted molecular weight 40 KDa (red arrows)
Right column CTGF - predicted molecular weight 38 KDa (green arrows)
Abcam primary antibodies (top row) and Santa-Cruz primary antibodies (bottom row)
Western blots were performed using lysates, of the cell lines shown, from cells grown in standard media with 10% FCS to 80% confluence. Experiments conducted to confirm the specificity of the antibodies. 786-0 and A498 – renal cancer cell lines as positive controls.
Figure 3.23 - Expression of MET following incubation with siRNA directed against MET and RNAiMAX assessed using a Millipore (top) and Invitrogen (bottom) anti-MET antibody

T24 and J82 cells were cultured for 48 hours in standard media + 10% FCS alone or with the addition of 30nM siRNA directed against MET and RNAiMAX or RNAiMAX alone. Western blots were then produced from cell lysates to evaluate the resulting knockdown of MET. The 768-0 renal cancer cell line was used as a control. Detection of MET was undertaken using the primary antibodies shown. Predicted molecular weight of MET 170 KDa = red arrow.
Figure 3.24 - Western blot of the expression of EMT markers and putative targets in six bladder cancer cell lines

Lysates, produced from sub-confluent monolayers of six different bladder cancer cell lines grown in standard media +10% FCS, were run in a 10% acrylamide with 15µg of protein added per well. The membrane was incubated with primary antibodies for the EMT markers vimentin, N-cadherin and E-cadherin in addition to the putative targets MET, CTGF and CYR61. GAPDH was used as a loading control antibody.
3.5.5 Time lapse HGF stimulation

Cells in culture at 80% confluence were serum starved for 24 hours prior to stimulation with 10ng/ml HGF in a serum free media. Lysates were produced at time 0 and then at 15, 30, 60, 120, 240, 360, 480 and 1440 minutes after stimulation with HGF. Western blotting was undertaken to determine the expression of MET, CTGF and CYR61 following stimulation with HGF at these time points [figures 3.24 and 3.25]. In response to stimulation with HGF there was an increase in the expression of CTGF in all cell lines, except the HT1376 which had basal high expression, between 120 and 360 minutes following which expression returned to basal or below basal levels by 1440 minutes. There was a small increase in the expression of CYR61 in the UMUC3 and RT112 cell lines following HGF stimulation, with peaks at 360 and 240 minutes respectively. No changes in CYR61 expression were observed in the other four cell lines. The level of MET declined and remained low in all cell lines following stimulation. This decline occurred over a range of times, between 60 and 360 minutes.

The HGF stimulation experiment was conducted on at least two further occasions for the T24 and J82 cell line, including experiments following siRNA knockdown of MET. No change in the expression of CYR61 was observed in any subsequent experiment for both cell lines following HGF stimulation. There was a statistically significant increase in mean CTGF expression from baseline at 60 minutes (p<0.05, unpaired T-test), 120 minutes and 240 minutes (p<0.01, unpaired T-test) for the T24 cell line (combined data from five independent experiments) and at 240 minutes (p<0.0001, unpaired T-test) for the J82 cell line (combined data from three independent experiments). The mean fold changes in expression at 30, 60, 120, 240 and 480 minutes were 1.49(±0.24), 1.70(±0.28), 2.53(±0.4), 2.06(±0.31) and 1.39(±0.21) for the T24 and 1.09(±0.18), 1.31(±0.14), 2.88(±0.41), 2.17(±0.03) and 1.37(±SE 0.4) for the J82 line respectively. Following siRNA knockdown of MET, these increases in CTGF expression were reduced [figure 3.26].
Monolayers of cells at 80% confluence were serum starved for 24 hours and then stimulated with 10ng/ml HGF. Lysates were produced at the times shown and then used to undertake western blotting to detect CTGF, CYR61 and MET. Lysate from the 768-0 renal cancer cell line was used as a control.
Monolayers of cells at 80% confluence were serum starved for 24 hours and then stimulated with 10ng/ml HGF. Lysates were produced at the times shown and then used to undertake western blotting to detect CTGF, CYR61 and MET. Lysate from the 768-0 renal cancer cell line was used as a control.
Figure 3.27 - Expression of CTGF in the T24 and J82 cell lines following stimulation with HGF and siRNA knockdown of MET

Cells at 80% confluence were serum starved for 24 hours, following 24 hours incubation with either non-targeting (NT) siRNA or siRNA directed against MET, and then stimulated with 10ng/ml HGF. Lysates were then produced at the time points indicated and western blotting conducted to evaluate the expression of CTGF at each time point. Using combined data from five and three independent experiments for the T24 and J82 cell line respectively the mean fold change in expression from baseline at time 0 was calculated as shown in the histograms, by densiometric analysis in Image-J. Error bars = standard error of the mean. Statistical significance, unpaired T-test, in comparison to baseline, *p<0.05, **p<0.01 and ****p<0.0001.
3.5.6 siRNA knockdown of MET, CTGF and CYR61

3.5.6.1 Confirmation of target knockdown

Knockdown of the targets of interest was performed using the siRNA’s listed in section 2.1.4 against either MET, CTGF or CYR61 and using a non-targeting siRNA as a negative control. To validate the siRNA transfection methodology described in section 2.2.1.2, T24 and J82 cells were cultured for 48 hours in standard media + 10% FCS, standard media +10% FCS + RNAiMAX or standard media + 10% FCS + RNAiMAX + 30nM siRNA directed against MET. Only the latter combination resulted loss of expression of MET, confirming effective knockdown at 48 hours [figure 3.22]. In subsequent experiments, efficient knock down was demonstrated to occur for MET, CTGF and CYR61 in both the J82 and T24 cell lines following 48 hours incubation [figure 3.27], using an initial 30nM siRNA concentration in the media.

3.5.6.2 Effects of knockdown on expression of mesenchymal markers

Knockdown of CTGF had no effect upon the expression of either N-cadherin or vimentin in the T24 and J82 cell lines. The expression of vimentin was significantly reduced (p<0.01, unpaired T-test) in the T24 cell line following knockdown of CYR61, with an 86.4% reduction in expression compared to cells cultured with NTsiRNA [figures 3.27 and 3.28]. There was no change in the expression of vimentin in the J82 cell line or in the expression of N-cadherin in both cell lines following knockdown of CYR61.
Sub-confluent cells were cultured in the presence of RNAiMAX and a 30nM concentration siRNA directed against either MET, CYR61 or CTGF or with a non-targeting (NT) siRNA for 24 hours. The media as then removed and replaced with serum free media for a further 24 hours. Lysates were then produced for use in western blotting to confirm levels of expression. GAPDH was used a loading control.

Figure 3.28 - Western blots confirming efficient knockdown of CTGF, CYR61 and MET following incubation with specific siRNA’s and the subsequent expression of the mesenchymal markers vimentin and N-cadherin in the T24 and J82 lines

Figure 3.29 - Mean change in the expression of vimentin following incubation with siRNA’s against MET, CTGF and CYR61 (in comparison to siNT) for the T24 cell line

N=3. The western blots described in figure 3.27 were analysed by densiometric analysis using Image-J and the mean fold change, in comparison to incubation with NTsiRNA were calculated. Statistical comparison performed using an unpaired T-test. Error bars = standard error of the mean. **p<0.01.
3.5.6.3 The effects of target knockdown on cellular proliferation and chemosensitivity.

Cells were cultured, as described in section 2.2.1.4.1, in standard media + 10% FCS for 24 hours, followed by 48 hours incubation with the addition of the required siRNA at 30nM + RNAiMAX. The cells were then trypsinised and seeded, 4000 cells per well, in 96 well plates and cultured for 72 hours. Proliferation was evaluated using an SRB assay, in comparison to cells exposed to 48 hours culture with non-targeting siRNA as a control. Knockdown of MET, CTGF and CYR61 caused a reduction in cellular proliferation at 72 hours in both the J82 and T24 lines, although this reduction did not reach statistical significance for knockdown of MET and CYR61 in the J82 line (unpaired T-test, in comparison to NT siRNA control). The mean fold change, data from four independent experiments, from baseline following incubation with NT-control, MET, CTGF and CYR61 siRNA was 4.98 (±0.48), 3.987 (±0.35) p=0.099, 2.45 (±0.23) p<0.0001, and 3.88 (±0.35) p=0.072 for the J82 line and 15.75 (±0.99), 12.22 (±0.74) p=0.007, 2.18 (±0.23) p<0.0001 and 10.96 (±0.68) p=0.0003 for the T24 cell line respectively [figure 3.29].

To test the effects of target knockdown on sensitivity to cisplatinum identical siRNA knockdown and proliferation experiments were conducted with the addition of cisplatinum to the culture media 2 hours after cell seeding in the 96 well plates. Initially cisplatinum was added to a concentration of 0.05µg/ml and 0.5µg/ml for the T24 and J82 cell lines respectively, as was defined in the initial chemosensitivity experiments in section 3.5.2. Cisplatinum at a 0.05µg/ml concentration had no effect upon the proliferation of T24 cells in two independent experiments. The concentration was therefore increased incrementally in sequential experiments until a statistically significant 50% reduction in proliferation was observed. This occurred at a concentration of 0.5µg/ml. At the same 0.5µg/ml a statistically significant 50% reduction in proliferation in the J82 cell line occurred during experiments conducted in parallel with the T24 experiments, therefore no change was made to dosing for the J82 line.
In both cell lines, knockdown of MET increased cell proliferation in the presence of cisplatinum 0.5µg/ml, in comparison to the NT control cells, although this was only significant for the T24 cells, p=0.008 and not for the J82 line, p=0.093. Knockdown of CTGF and CYR61 produced a statistically significant reduction in cell proliferation in the presence of cisplatinum 0.5µg/ml for both cell lines (T24 – 3 independent experiments, J82 – 4 independent experiments) in comparison to NT control cells (p=0.039 for knockdown of CYR61 in the J82 line and p<0.0001 for CTGF in both cell lines and for CYR61 in the T24 cell line). The mean fold change in proliferation from day one, in the presence of 0.5µg/ml cisplatinum, for NT control cells and following knockdown MET, CTGF and CYR61 was 2.26 (±0.18), 2.75 (±0.22), 1.20 (±0.06) and 1.77 (±0.14) for the J82 line and 6.57 (±0.34), 8.55 (±0.62), 1.04 (±0.05) and 1.40 (±0.06) for the T24 line respectively. The reduction in proliferation following knockdown of CTGF and CYR61 was also statistically significant for both cell lines (p<0.0001) when comparing proliferation following knockdown with and without cisplatinum at 0.5µg/ml figures 3.30 and 3.31.
Figure 3.30 - Proliferation of the J82 (top) and T24 (bottom) cell lines following siRNA knockdown of either MET, CTGF or CYR6.

n=4. Following 48 hours incubation with a specific siRNA (as shown on x-axis, control cells (grey bars) were cultured without siRNA), 4,000 cells were seeded per well in 96 well plates. Cell proliferation was assessed using an SRB assay. Values were calculated as mean fold for each cell line from two hours post seeding. NT = non-targeting. Error bars = standard error of the mean. Statistical significance shown, unpaired T-test, compares the mean fold change following knockdown of each target to the fold change following incubation with non-targeting siRNA in the presence of cisplatinum. Statistical significance shown, unpaired T-test **p<0.01, ***p<0.001, ****p<0.0001.
Figure 3.31 - Proliferation of the J82 cell lines following siRNA target knockdown and cisplatinum treatment

N=4. Following 48 hours incubation with a specific siRNA (as shown on x-axis, control cells (grey bars) were cultured without siRNA), 4,000 cells were seeded per well in 96 well plates. Cisplatinum was added, where indicated, to a final concentration of 0.5 µg/ml after 2 hours. Proliferation was assessed using an SRB assay. Proliferation was calculated as mean fold change from two hours post seeding. NT = non-targeting. Error bars = SEM. Statistical significance shown, unpaired T-test **p<0.01, ****p<0.0001.
Figure 3.32 - Proliferation of the T24 cell lines following siRNA target knockdown and cisplatinum treatment

N=4. Following 48 hours incubation with a specific siRNA (as shown on x-axis, control cells (grey bars) were cultured without siRNA), 4,000 cells were seeded per well in 96 well plates. Cisplatinum was added, where indicated, to a final concentration of 0.5 µg/ml after 2 hours. Proliferation was assessed using an SRB assay. Proliferation was calculated as mean fold change from two hours post seeding. NT = non-targeting. Error bars = SEM. Statistical significance shown, unpaired T-test **p<0.01, ****p<0.0001.
3.5.6.4 The effects of target knockdown on cellular invasion

Cells were cultured, as described in section 2.2.1.3, in standard media + 10% FCS for 24 hours, followed by 24 hours incubation with the addition of the required siRNA at 30nM + RNAiMAX. The cells were then serum starved in RPMI + 1% L-glutamine (v/v) for 24 hours prior to the 18 hour Boyden chamber Matrigel™ invasion assay, in response to HGF and FCS.

There was a statistically significant (p<0.0001, unpaired T-test) reduction in invasion towards 10ng/ml HGF, following knockdown of MET, CTGF and CYR61 in comparison to NT siRNA control cells, for both the J82 and T24 cell line (five independent experiments combined). The mean number of cells invading towards 10ng/ml HGF, per 10x10mm graticule at 100x magnification, for NT control and following knockdown of MET, CTGF and CYR61 was 112.4 (±4.5), 7.4 (±2.8), 15.5 (±5.4) and 20.6 (±6.1) for the J82 line and 223.3 (±28.8), 44.2 (±8.3), 48.8 (±19.3) and 29.2 (±17.0) for the T24 line respectively [figure 3.32].

There was a statistically significant reduction in invasion towards 5% FCS following knockdown of CYR61 for both the J82 (p<0.0001, unpaired T-test) and T24 (p=0.0027) cell lines, in comparison to NT control cells (combined data from seven independent experiments). There was no significant reduction in the invasion response to 5% FCS following knockdown of MET or CTGF in either cell line. The mean number of cells invading towards 5% FCS, per 10x10mm graticule at 100x magnification, for NT control and knockdown of MET, CTGF and CYR61 was 529.6 (±49.5), 497.6 (±36.0), 396.2 (±50.7) and 115.0 (±13.3) for the J82 line and 567.0 (±52.5), 598.5 (±38.4), 515.9 (±32.7) and 293.9 (±63.3) for the T24 line respectively [figure 3.33].
Figure 3.33 - Comparison of invasion, in response to 10ng/ml HGF, of the J82 (A) and T24 (B) cell lines following siRNA knockdown

Combined data from T24 (N=5) and J82 (N=4) independent experiments. Following incubation with specific siRNA's (x-axis, control cells (grey bars) were cultured without siRNA), 1 x 10^5 cells were seeded into the upper insert of a Boyden chamber with a Matrigel™ coated 8μm pore membrane, above either serum free media (plain bar) or serum free media containing 10ng/ml HGF (hatched bars). The number of cells invading post incubation (18hrs, 5% CO₂ in air at 37°C) per 10x10mm graticule at 100x magnification. Error bars = standard error of the mean. NT = non-targeting. Statistical significance shown, unpaired T-test ****p<0.0001.
Figure 3.34 - Comparison of invasion, in response to 5% FCS, of the J82 (A) and T24 (B) cell lines following siRNA knockdown

N=7. Following incubation with specific siRNA’s (as shown on x-axis, control cells (grey bars) were cultured without siRNA), 1 x 10^5 cells were seeded into the upper insert of a Boyden chamber with a Matrigel™ coated 8μm pore membrane, above either serum free media (plain bar) or media containing 5%FCS (diagonal bars). The number of cells invading post incubation (18hrs, 5% CO₂ in air at 37°C) per 10x10mm graticule at 100x magnification. Error bars = standard error of the mean. NT = non-targeting. Statistical significance shown, unpaired T-test. ** p<0.01,****p<0.0001.
3.5.6.5 The effects of target knockdown on cellular migration

Cells were cultured, as described in sections 2.2.1.2 and 2.2.1.5, in standard media + 10% FCS for 24 hours, followed by 24 hours incubation with the addition of the required siRNA at 30nM + RNAiMAX. The cells were then serum starved in RPMI + 1% L-glutamine (v/v) for 24 hours prior to a 12 hour scratch ‘wound’ migration assay in response to HGF or FCS.

Knockdown of MET in the J82 cell line caused a large and significant (p<0.0001, unpaired T-test) reduction in cell migration in response to 10ng/ml HGF, in comparison to NT control cells (three independent experiments combined). There were small, although significant, reductions following knockdown of CTGF (p=0.008) and CYR61 (p=0.009). In the T24 cell line knockdown of MET had a similarly large and significant effect (p<0.0001), knockdown of CTGF had no effect (p=0.24) and knockdown of CYR61 caused a small, but again significant (p=0.026), reduction in migration. The mean migration indices after stimulation with 10ng/ml HGF, for NT control and knockdown of MET, CTGF and CYR61 were 396.8 (±18.1), 160 (±12.5), 325.3 (±18.1) and 319.0 (±21.70) for the J82 line and 509.1 (±26.7), 259.9 (±24.6), 545.1 (±14.4) and 421.8 (±27.0) for the T24 line respectively [figure 3.34].

In response to stimulation with 5% FCS there was a moderate and significant reduction in migration following knockdown of MET (p<0.0001, unpaired T-test) and CYR61 (p<0.0001), but no effect of knockdown of CTGF (p=0.884) in the J82 cell line. In the T24 cell line there was no effect on migration from knockdown of MET (p=0.341) or CYR61 (p=0.439) and only a small, although significant (p=0.016), reduction following knockdown of CTGF. The mean migration indices after stimulation with 5% FCS, for NT control and knockdown of MET, CTGF and CYR61 were 386.4 (±11.5), 251.9 (±18.1), 390.9 (±28.8) and 258.5 (±19.4) for the J82 line and 486.4 (±30.0), 451.5 (±20.5), 401.6 (±15.8) and 446.7 (±41.0) for the T24 cell line respectively [figure 3.35].
Figure 3.35 - Comparison of migration, in response to 10 ng/ml HGF, of the J82 (A) and T24 (B) cell lines following siRNA knockdown

N=3. Following incubation with specific siRNA’s (as shown on x-axis), cells at 85% confluence in a 6 well plate were scratch ‘wounded’. The ‘wounds’ were imaged immediately and after 14 hours incubation either with or without 10ng/ml HGF (5% CO₂ in air at 37°C). The mean difference in width of the ‘wounds’ at these time points was calculated as the migration index. Error bars = standard error of mean. Statistical significance shown, unpaired T-test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
Figure 3.36 - Comparison of migration, in response to 5% FCS, of the J82 (A) and T24 (B) cell lines following siRNA knockdown

N=3. Following incubation with specific siRNA’s (as shown on x-axis), cells at 85% confluence in a 6 well plate were scratch ‘wounded’. The ‘wounds’ were imaged immediately and after 14 hours incubation either with or without 5% FCS (5% CO₂ in air at 37°C). The mean difference in width of the ‘wounds’ at these time points was calculated as the migration index. Error bars = standard error of mean. Statistical significance shown, unpaired T-test. *p<0.05, ****p<0.0001.
3.6 IHC antibody optimisation

3.6.1 Bench top vs. robotic IHC

Comparative staining was undertaken between the bench top protocol and the Leica BOND-MAX™ robot. Sequential 4\(\mu\)m sections of FFPE high grade bladder cancer tissue were DAB stained for MET and pan-cytokeratin, using Invitrogen mouse monoclonal anti-MET and Sigma mouse monoclonal anti-pan-cytokeratin antibodies. Additional sections were stained with the primary antibody substituted for a mouse IgG1 isotype control as a negative control. Sections of human prostate cancer were stained as positive controls for MET.

The Leica BOND-MAX™ robot produced highly specific pan-cytokeratin staining, with almost no background staining and no staining of muscle [figure 3.37]. In contrast the bench top protocol produced widespread background staining and strong staining of muscle. The bench top protocol also produced inferior MET staining with widespread background staining and a lower intensity of tumour staining [figure 3.36]. All subsequent IHC was therefore undertaken with the Leica BOND-MAX™ robot.
Sequential 4μm sections of grade 3 human bladder TCC were stained using monoclonal antibodies to MET using both a bench top protocol and the Leica BOND-MAX™ in conjunction with the Bond ™ Polymer Refine Detection System. Epitope retrieval was performed at pH 6.0.

Sequential 4μm sections of grade 3 human bladder TCC were stained using a monoclonal antibody to pan-cytokeratin using a bench top protocol (A) and the Leica BOND-MAX™ in conjunction with the Bond ™ Polymer Refine Detection System with epitope retrieval pH 6.0 (B) and pH 9.0 (C). m = muscle within image A.
3.6.2 Individual antibody optimisation

Initial staining for MET was conducted using an R&D Systems mouse monoclonal anti-MET antibody, which produced widespread non-specific background staining. Adjusting different parameters of the protocol, as described in section 2.2.4.5, did not improve staining quality. Subsequently an Invitrogen mouse monoclonal anti-MET antibody was used which resulted reduced background staining. Initial staining for both CTGF and CYR61 using Abcam rabbit polyclonal antibodies produced similarly poor results with profound background staining. Subsequent use of a Santa Cruz rabbit polyclonal anti-CYR61 antibody produced markedly reduced background staining. For CTGF a Santa Cruz goat polyclonal anti-CTGF was used, which as a non-mouse/rabbit primary antibody required the use of the Bond™ Open Secondary Detection System. Significant non-specific staining occurred, which persisted despite the use of either a goat IgG1 isotype control or TBS in place of the primary antibody, despite the inclusion of either a serum block or Candor block, avidin-biotin block and the use of an alternative secondary in the form of a donkey anti-goat polyclonal antibody. The use of this antibody in combination with the Bond™ Open Secondary Detection System was therefore abandoned. A third anti-CTGF antibody was therefore obtained, Sigma rabbit polyclonal anti-CTGF, which produced far superior results and was used for all subsequent IHC. The optimised antibody protocols are listed in table 3.15.
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Table 3.15 - Immuno-histochemistry protocols for DAB based staining of human bladder FFPE tissue sections
3.7 Validation and staining results for pilot TMA

A pilot TMA was constructed using cores of tissue from 50 patients with G3pT3 TCC treated by primary cystectomy and two non-bladder cancer controls. This produced three individual TMA blocks containing a total of 200 individual tissue cores. Of these three blocks the third TMA block (block 10_NOCL_01D 8-11-11 3A [figures 3.38 and 3.39] was constructed using tissue cores from patients whose tissue was felt to be less well preserved. This assessment of poor preservation was made by a histopathologist on the basis of the tissue appearances using bright field microscopy of H&E stained slides cut and stained during the TMA selection process. Sections of this TMA were stained for pan-cytokeratin, a protein that was expected to be well preserved, and phospho-tyrosine, which was expected to be less stable. The TMA sections were stained according to the optimised protocols in table 3.15. Although the tissue architecture was distorted in some of the less well preserved cores the pan-cytokeratin staining intensity remained high [figure 3.40]. The sections of this pilot TMA were then scored, based upon bright field microscopy appearances to the human eye, using the following criteria:

- 3 = Strong staining in $\geq$5% of cancer or urothelial cells.
- 2 = Intermediate staining in $\geq$5% of cancer or urothelial cells.
- 1 = Weak staining in $\geq$5% of cancer or urothelial cells.
- 0 = No staining or staining equal to background.

Of the 193 cores that contained cancer or urothelial cells, 189 (98%) stained positive for pan-cytokeratin, with: 7 (3.7%), 8 (4.2%) and 179 (92.1%) of the 189 cores staining at intensity levels 1, 2 and 3 respectively [figure 3.39. Staining for phospho-tyrosine was more variable than the staining observed for pan-cytokeratin with: 38 (20.1%), 85 (45%), 38 (20.1%) and 28 (14.8%) of the 189 cores staining at intensity levels 0, 1, 2 and 3 respectively [figure 3.38].

Based upon these staining results heat maps, indicating the staining intensity of each core, were constructed [figures 3.38 and 3.39]. This revealed a distribution
of staining throughout the TMA blocks that was consistent with true intra and inter tumour variability, rather than artefact due to a staining error. The block constructed from tissue deemed to be poorly preserved based upon H&E evaluation had the highest proportion of cores that were grade 3 for phospho-tyrosine staining at 37.9%; and 86.2% of cores grade 3 for pan-cytokeratin staining.
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Figure 3.39 - Heat map of pilot TMA phospho-tyrosine staining for the three pilot TMA blocks and their corresponding photomicrographs

Paired images represent the heat maps and corresponding photomicrographs for three TMA blocks of grade 3 pT3 TCC bladder DAB stained for phospho-tyrosine. Blocks 10_NOCL_01D 8-11-11-1A and 10_NOCL_01D 8-11-11-2A were constructed from tissue assessed on H&E sections of the donor tumour block as of good fixation / preservation, block 10_NOCL_01D 8-11-11-3A was assessed as of poor quality by the same criteria.

White = IHC score 0 (No staining or staining equal to background); Light blue = IHC score 1 (Weak staining intensity ≥5% of cancer or urothelial cells in core); Medium blue =IHC score 2 (Intermediate staining intensity ≥5% of cancer or urothelial cells); Dark blue = IHC score 3 (Strong staining in ≥5% of cancer or urothelial cells); Yellow = no tumour cells or urothelium in core; N = normal urothelium; T= tumour; Number indicates patient identifier.
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Figure 3.40 - Heat map of pilot TMA pan-cytokeratin staining for the three pilot TMA blocks and their corresponding photomicrographs

Paired images represent the heat maps and corresponding photomicrographs for three TMA blocks of grade 3 pT3 TCC bladder DAB stained for pan-cytokeratin. Blocks 10_NOCL_01D 8-11-11-1A and 10_NOCL_01D 8-11-11-2A were constructed from tissue assessed on H&E sections of the donor tumour block as of good fixation / preservation, block 10_NOCL_01D 8-11-11-3A was assessed as of poor quality by the same criteria.

White = IHC score 0 (No staining or staining equal to background); Light blue = IHC score 1 (Weak staining intensity ≥5% of cancer or urothelial cells in core); Medium blue =IHC score 2 (Intermediate staining intensity ≥5% of cancer or urothelial cells); Dark blue = IHC score 3 (Strong staining in ≥5% of cancer or urothelial cells); Yellow = no tumour cells or urothelium in core; N = normal urothelium; T= tumour; Number indicates patient identifier.
Figure 3.41 - Images of normal bladder and high grade TCC bladder sections stained for H&E and DAB stained for pan-cytokeratin

Paired sections of the same area stained with H&E (left column) and DAB stained for pan-cytokeratin (right column). Images A&B (high grade TCC) poor fixation / preservation as determined by pathologist on H&E stained sections. Images C&D (normal urothelium) and E&F (high grade TCC) good fixation / preservation as determined by pathologist on H&E stained sections.
3.8 Validation of final TMA

The complete TMA, including the three block pilot TMA, included 2031 cores of tissue embedded into 35 paraffin blocks (in triplicate). Evaluation of these blocks by H&E staining of 4\(\mu\)m sections confirmed the presence of bladder cancer or urothelial cells in 1788 (88.0%) cores, from 557 patients of whom 491 were bladder cancer cases. Sections from each block were stained for pan-cytokeratin and phospho-tyrosine using the protocols in table 3.15, and analysed using Definiens®. The analysis solutions in Definiens® were programmed to assign a score to each urothelial or cancer cell in each core according to the scale: 0=negative staining, 1=low intensity staining, 2=intermediate intensity staining and 3=high intensity staining and from this calculate an H-score. Definiens® also measured the absolute staining intensity (on a linear scale) in each cell and then calculated mean staining intensity parameters per core.

A total of 1.2% of the cores were negative for pan-cytokeratin staining with 12.1%, 15.8% and 70.9% of cores having H-score of 1-99, 100-199 and 200-300 respectively. The percentage of cores staining for phospho-tyrosine in each H-score category were 0.9%, 49.2%, 30.5% and 19.6% respectively. The median IHC intensity per patient across the TMA was 0.733 (range 0.177 – 1.263) for pan-CK expression and 0.146 (range 0.030 – 1.095) for phospho-tyrosine.

There was a positive and highly statistically significant, correlation between the year of surgery and both mean H-score and mean IHC core intensity per patient for pan-CK staining, with Pearson’s r values of 0.4445 (p<0.0001) and 0.4216 (p<0.0001). The opposite correlation was observed for phospho-tyrosine staining with Pearson’s r values of -0.4577 (p<0.0001) and -0.3412 (p<0.0001) for mean H-score and mean IHC intensity respectively, [figure 3.41 and 3.42]. There was a notable difference between the expression of both pan-CK and phospho-tyrosine pre and post 1980. When patients from prior to 1980 were excluded the correlation between year of surgery and staining intensity
disappeared [figures 3.41 and 2.42] with Pearson’s r values of $2.3 \times 10^{-6}$ ($p>0.999$) and -0.0465 ($p=0.339$) for pan-CK and phospho-tyrosine mean IHC intensity respectively.

Figure 3.42 - Correlation between pan-cytokeratin staining and the year of surgery
TMA sections were DAB stained for pan-cytokeratin and then analysed using Definiens® automated image analysis software. Data points represent the mean staining intensity per patient, on a linear scale. Red line represents the Pearson’s correlation between mean intensity and year of surgery.
Figure 3.43 - Correlation between phospho-tyrosine staining and the year of surgery

TMA sections were DAB stained for phospho-tyrosine and then analysed using Definiens® automated image analysis software. Data points represent the mean staining intensity per patient, on a linear scale. Red line represents the Pearson’s correlation between mean intensity and year of surgery.
3.9 Optimisation and validation of Definiens® image analysis

DAB based chromogenic and fluorescence IHC staining of tissue sections were both undertaken to determine which provided the most robust approach for AIA. It was initially hoped that the use of fluorescence IHC would allow the application of a pan-cytokeratin antibody, to identify the cells of interest for AIA, with synchronous application of at least two further markers for analysis. Sequential sections of FFPE human bladder tissue were dewaxed and rehydrated, as described in section 2.2.4.2.1. The first section was simply coverslipped with ProLong® Gold Antifade Reagent with DAPI. The subsequent sections incubated with a mouse monoclonal anti-pan-cytokeratin antibody (Sigma) at 1:200 dilution, followed by either an Alexa Fluor® 488, 555 or 647 conjugated rabbit anti-mouse secondary antibody (Life Technologies). The slides were coverslipped as for the first slide, prior to scanning in a Mirax fluorescence slide scanner, as described in section 2.2.4.2.2. Autofluorescence of the tissue occurred at excitation wavelengths of 488nm and 555nm. No discernible auto-fluorescence occurred at an excitation wavelength of 647nm figure 3.43. The intensity of the auto-fluorescence seen was very similar to the intensity observed from cells labelled for pan-cytokeratin with the Alexa Fluor® 488 and 555, as shown in figure 3.44. The Mirax scanner does not possess a filter capable of delivering light of higher wavelengths, making it impossible to investigate the use of fluorophores with excitation wavelengths greater than 647nm. It was therefore felt that fluorescence IHC did not offer any advantages over chromogenic staining as with the scanning resources it was not possible to reliably use more than one fluorophore in addition to DAPI.
Figure 3.44 - Immunofluorescence micrographs of a section of normal bladder urothelium stained with only DAPI, imaged at varying excitation wavelengths

A FFPE 4µm section of normal bladder urothelium was de-waxed, rehydrated and cover-slipped with ProLong® Gold Antifade Reagent with DAPI. The section was then imaged using a Mirax slide scanner at an excitation wavelength of 358nm for DAPI (image A) and then at was combined with increasing wavelengths to produce images B-D. The compound images shown represent: DAPI & 488nm (image B), DAPI & 555nm (image C) and DAPI & 647nm (image D).
Figure 3.45 - Immunofluorescence micrographs of a section of normal bladder urothelium stained for pan-cytokeratin with varying fluorophores and DAPI

Three FFPE 4µm section of normal bladder urothelium were de-waxed, rehydrated and incubated with an anti-pan-cytokeratin antibody, followed by either an Alexa Fluor® 488 (image A&B), 555 (image C&D) or 647 (image E&F) conjugated secondary antibody and cover-slipped with ProLong® Gold Antifade Reagent with DAPI. The sections were then imaged using a Mirax slide scanner at an excitation wavelength of 358nm for DAPI, and then either 488nm, 555nm or 647nm as appropriate. Left hand column = compound images including DAPI and excitation at wavelength shown. Right hand column = excitation at wavelength shown only. Image F demonstrates no auto-fluorescence and correctly labels only the normal urothelial cells.
The analysis algorithms available in Definiens® allow for segmentation of the tissue in a core into different categories, such as stroma, muscle, tumour etc. (termed region of interest classification) and then a cellular analysis step. Unfortunately, despite considerable effort, it was not possible to develop an algorithm that could correctly classify the differing core regions of interest. The most troublesome misclassifications were that of labeling muscle as tumour and classifying tumour cells that were negative for the marker/target of interest as non-tumour [figure 3.45]. Differing counterstains were used, including Van Gieson’s and Masson’s in an attempt to correctly classify muscle. Unfortunately the counter stains were taken up by other tissue compartments and as such did not help with segregation. On this basis the region of interest classification step was removed from the AIA algorithm. The final algorithm developed relied upon the classification at a cellular level based upon nuclear and cell morphology and size. To validate this classification algorithm a single TMA section, containing 79 cores, was DAB stained for CYR61 according to the protocol in table 3.15. The AIA cell classification of each cell in each of the 79 cores was then reviewed by human eye. The sensitivity and specificity of the AIA algorithm to correctly identify tumour/urothelial cells was 82.5% (95%CI 79.1-85.8) and 99.7% (95%CI 99.6-99.9) respectively.

To further validate the AIA process the same TMA section, stained for CYR61, was subjected to AIA to generate H-scores and also scored, by human eye, on two separate occasions by two blinded observers (A and B). The scores were then compared, as described in section 2.2.7. The intra-class correlation coefficient values for the intra-observer variability of scorers A and B were 0.907 (95%CI 0.857-0.940) and 0.797 (95%CI 0.697-0.867) respectively; compared to 0.701 (95%CI 0.564-0.800) for the inter-observer variability between the scorers A and B. The correlation coefficient for AIA was 1.0, for intra-observer variability, as AIA returned the same results on repeated analyses. The correlation coefficient values comparing scorer A and AIA were 0.810 (95%CI 0.714-0.875), scorer B and AIA were 0.758 (95%CI 0.642-0.840) and between the average of all four scores by scorers A and B with AIA were 0.857 (95%CI 0.873-0.907). These relationships are summarised in the Bland-Altman plots in figure 3.46 and 3.47.
Figure 3.46 - Examples of region of interest classification by Definiens®


Good classification occurs when clear distinction occurs between tumour and non-tumour - A&B. Poor classification occurs when tumour cells are negative for the marker of interest - C&D, or when there is background staining of muscle - E&F. m = muscle in image E.
Figure 3.47 - Intra and inter-rater variability in H-score analysis of IHC quantified expression of CYR61

A single TMA section containing 79 cores was DAB stained for CYR61. H-scores for the expression of CYR61 were calculated by 2 raters (A and B) and by Definiens® (AIA) on 2 different occasions. For the inter-rater comparison the mean scores for each core for each rater were compared. Each dot represents an individual tissue core. Dotted horizontal line=95% limits of agreement.
Figure 3.48 - Inter-rater variability in H-score analysis of IHC quantified expression of CYR61.

A single TMA section containing 79 cores was DAB stained for CYR61. H-scores for the expression of CYR61 were calculated by 2 raters (A and B) and by Definiens® (AIA) on 2 different occasions. Each dot represents an individual tissue core. Dotted horizontal line=95% limits of agreement.
3.10 Bladder tissue expression of MET, CTGF and CYR61

For each of the three putative targets 4μm TMA sections were cut from each of the 35 blocks and stained according to the protocols in table 3.15. In addition to the TMA sections, separate sections of prostate and renal cancer tissue were stained as positive controls for MET and CYR61/CTGF respectively. As negative controls, sections of bladder tissue were stained with substitution of the primary antibody for an IgG1 isotype control. The expression was analysed using AIA with Definiens®. For each target the thresholds for classification of expression were adjusted, but remained consistent throughout analysis of each target. The staining intensity threshold between 0 (negative) and 1 (low) expression was set at the level of background staining for that antibody. The threshold between 2 (intermediate expression) and 3 (high expression) was set at a level determined by reviewing a large number of cores. The threshold between 1 and 2 was derived as the numerical mid point of IHC staining intensity between the thresholds for 1 and 3. The individual thresholds for each marker are shown in table 3.16.

In addition to H-scores the mean IHC staining intensity (MIHCI) per cell was calculated, in addition to the calculation of various staining intensity parameters. Examples of the staining intensity thresholds for each of the three targets and the cell classification generated by AIA are shown in figure 3.48 to 3.50.

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Table 3.16 - IHC intensity thresholds used in each analysis algorithms in Definiens® for each target of interest
Figure 3.49 - Representative MET expression in 1mm TMA cores
Left column = original photomicrograph. Right column = final analysis by Definiens® following cell identification and classification.
IHC score: white=0, yellow=1, orange=2 and brown=3. Grey=cell excluded from classification.
Figure 3.50 - Representative CYR61 expression in 1 mm cores
Left column = original photomicrograph. Right column = final analysis by Definiens® following cell identification and classification.
IHC score: white=0, yellow=1, orange=2 and brown=3. Grey=cell excluded from classification.
Figure 3.51 - Representative CTGF expression in 1mm TMA cores

Left column = original photomicrograph. Right column = final analysis by Definiens® following cell identification and classification.

IHC score: white=0, yellow=1, orange=2 and brown=3. Grey=cell excluded from classification.
3.10.1 Target expression by tumour grade and stage

There was a high degree of correlation between the mean H-scores and mean IHC intensity score of the tumour cores of each patient for the expression of MET, CYR61 and CTGF with Pearson’s r values of 0.969, 0.952 and 0.955 respectively (p<0.0001 for all correlations). The expression of each of the three proteins was evaluated in the entire cohort used to construct the TMA and separately in the cohort who underwent cystectomy from 1985 onwards.

Overall the expression of MET was inversely associated with increasing grade of TCC [figure 3.51] with mean IHC intensities of 0.285, 0.211 and 0.170 for grades 1, 2 and 3 respectively (p<0.0001, unpaired T-test for all pairwise comparisons). This correlation disappeared when examining only the ≥ 1985 cohort (p>0.05, all comparisons) although only 1.4% and 14.3% of the cohort were TCC grade 1 and 2 respectively. The expression was also inversely associated with increasing stage of tumour in both the overall and ≥ 1985 cohort (p<0.0001, ANOVA analysis including control, normal adjacent and adjacent CIS in conjunction with pTis-pT4 tumours); although the range of expression was less variable across the different stages in the ≥ 1985 cohort [figure 3.52].

The MIHCI values in the ≥ 1985 cohort for control, normal adjacent, adjacent CIS and stages pTis, pTa, pT1, pT2, pT3 and pT4 were 0.1866, 0.1711, 0.1814, 0.1881, 0.1685, 0.1637, 0.1735, 0.1339 and 0.1300 respectively; with significant differences between control cases and pT1 (p=0.0491, unpaired T-test) and pT3 (p<0.0001) and pT4 (p<0.0001) but not for other stages of tumour.

There was no difference in the expression of CYR61 between the different grades of TCC in all pairwise comparisons (p>0.05, unpaired T-test) in the overall or ≥ 1985 cohort [figures 3.53 and 3.54]. In the post 1985 cohort the two lowest MIHCI values were for normal controls and normal adjacent urothelium at 0.1866 and 0.1862 respectively, compared to 0.2265, 0.2129, 0.2172, 0.2152, 0.2167, 0.1963 and 0.2104 for adjacent CIS, pTis, pTa, pT1, pT2, pT3 and pT4 respectively. There were statistically significant differences between
CYR61 expression in the control cases and for each T stage of tumour (p<0.05, unpaired T-test) for all stages other than pT3 (p=0.191).

Overall there was a significantly lower level of expression of CTGF in grade 3 TCC tumours compared to grade 2 (p=0.027, unpaired T-test) but no difference in other pairwise comparisons between grades [figure 3.55]. As for MET and CYR61 this difference was not seen in the ≥1985 cohort [figure 3.56]. The expression of CTGF was inversely associated with increasing stage of tumour (p<0.0001, ANOVA) in both cohorts. The MIHCl values in the ≥1985 cohort for control, normal adjacent, adjacent CIS and stages pTis, pTa, pT1, pT2, pT3 and pT4 were 0.2901, 0.2756, 0.2621, 0.2852, 0.2490, 0.2386, 0.2252, 0.2003 and 0.2170 respectively; with significant differences between control cases and each T stage of tumour (p<0.05, unpaired T-test) other than for stage pTis (p=0.808).

Figure 3.52 - Expression of MET by stage and grade of tumour all cases 1969-2006
Box and whisker plots of mean IHC staining intensity for all patients (top) and for those with a TCC histological subtype of bladder cancer (bottom). 4µm sections of the TMA were stained for MET and then analysed by AIA. The mean IHC staining intensity per patient was then calculated for the tumour (pTis-pT4), adjacent CIS (aCIS), normal appearing urothelium for patients with bladder cancer (N) and normal bladder urothelium from the control patients (C). Horizontal line = median. Box = interquartile range. Whiskers = 10th – 90th centile. Dots = outlying patients. Tumour grade = WHO 1973 classification. Cases by grade compared using an unpaired T-test in pairwise comparisons.
Figure 3.53 - Expression of MET by stage and grade of tumour in cases post 1985

Box and whisker plots of mean IHC staining intensity for all patients (top) and for those with a TCC histological subtype of bladder cancer (bottom). 4µm sections of the TMA were stained for MET and then analysed by AIA. The mean IHC staining intensity per patient was then calculated for the tumour (pTis-pT4), adjacent CIS (aCIS), normal appearing urothelium from patients with bladder cancer (N) and normal bladder urothelium from the control patients (C). Horizontal line = median. Box = interquartile range. Whiskers = 10th – 90th centile. Dots = outlying patients. Tumour grade 1-3 based upon WHO 1973 classification. Cases by stage compared with one-way ANOVA test. Cases by grade compared using an unpaired T-test in pairwise comparisons.
Figure 3.54 - Expression of CYR61 by stage and grade of tumour all cases 1969-2006

Box and whisker plots of mean IHC staining intensity for all patients (top) and for those with a TCC histological subtype of bladder cancer (bottom). 4µm sections of the TMA were stained for CYR61 and then analysed by AIA. The mean IHC staining intensity per patient was then calculated for the tumour (pTis-pT4), adjacent CIS (aCIS), normal appearing urothelium from patients with bladder cancer (N) and normal bladder urothelium from the control patients (C). Horizontal line = median. Box = interquartile range. Whiskers = 10th – 90th centile. Dots = outlying patients. Tumour grade 1-3 based upon WHO 1973 classification. Cases by stage compared with one-way ANOVA test. Cases by grade compared using an unpaired T-test in pairwise comparisons.
Figure 3.55 - Expression of CYR61 by stage and grade of tumour in cases post 1985

Box and whisker plots of mean IHC staining intensity for all patients (top) and for those with a TCC histological subtype of bladder cancer (bottom). 4µm sections of the TMA were stained for CYR61 and then analysed by AIA. The mean IHC staining intensity per patient was then calculated for the tumour (pTis-pT4), adjacent CIS (aCIS), normal appearing urothelium from patients with bladder cancer (N) and normal bladder urothelium from the control patients (C). Horizontal line = median. Box = interquartile range. Whiskers = 10th – 90th centile. Dots = outlying patients. Tumour grade 1-3 based upon WHO 1973 classification. Cases by stage compared with one-way ANOVA test. Cases by grade compared using an unpaired T-test in pairwise comparisons.
Figure 3.56 - Expression of CTGF by stage and grade of tumour all cases 1969-2006

Box and whisker plots of mean IHC staining intensity for all patients (top) and for those with a TCC histological subtype of bladder cancer (bottom). 4μm sections of the TMA were stained for CTGF and then analysed by AIA. The mean IHC staining intensity per patient was then calculated for the tumour (pTis-pT4), adjacent CIS (aCIS), normal appearing urothelium from patients with bladder cancer (N) and normal bladder urothelium from the control patients (C). Horizontal line = median. Box = interquartile range. Whiskers = 10th – 90th centile. Dots = outlying patients. Tumour grade 1-3 based upon WHO 1973 classification. Cases by stage compared with one-way ANOVA test. Cases by grade compared using an unpaired T-test in pairwise comparisons.
Figure 3.57 - Expression of CTGF by stage and grade of tumour in cases post 1985

Box and whisker plots of mean IHC staining intensity for all patients (top) and for those with a TCC histological subtype of bladder cancer (bottom). 4µm sections of the TMA were stained for CTGF and then analysed by AIA. The mean IHC staining intensity per patient was then calculated for the tumour (pTis-pT4), adjacent CIS (aCIS), normal appearing urothelium from patients with bladder cancer (N) and normal bladder urothelium from the control patients (C). Horizontal line = median. Box = interquartile range. Whiskers = 10th – 90th centile. Dots = outlying patients. Tumour grade 1-3 based upon WHO 1973 classification. Cases by stage compared with one-way ANOVA test. Cases by grade compared using an unpaired T-test in pairwise comparisons.
3.10.2 *Classified target expression by tissue type*

In the further analysis of target expression in relation to tissue type (control case, normal urothelium from bladder cancer cases and tumour) patients were additionally classified using a categorical system defined as negative (0) = background staining or <5% of cells at any higher intensity level, low (1) = ≥5% of cells at intensity level 1, intermediate (2) = ≥5% of cells at intensity level 2 and high (3) = ≥5% of cells at intensity level 3 for MET and CYR61. For CTGF the percentage threshold was increased to 10%, as discussed in section 3.10.4. Analysis based upon tissue type was also conducted using the MIHCI, and by dichotomising patients as either negative/low or intermediate/high, based upon the categorical system above. Analyses using these classifiers of expression were only undertaken in the ≥1985 cohort.

There was no significant difference in the expression of MET between control and normal adjacent or adjacent CIS, but there was a difference between control and tumour expression with MIHCI values of 1.817 and 1.534 respectively (p=0.001, unpaired T-test). A similar significant difference between control and tumour cases (p=0.001, Chi square) was observed with categorical classification. This equated to 56.0%, 44.2%, 49.1% and 31.7% of control, normal adjacent, adjacent CIS and tumour cases being classified as intermediate/high respectively [figure 3.57].

There was a significant difference in the expression of CYR61 between control and all other tissue types, with MIHCI values of 0.1856, 0.2051, 0.2265 and 0.2099 respectively for control, normal adjacent, adjacent CIS and tumour (p=<0.05, unpaired T-test). The same significant differences (p=0.005, Chi square) were observed with categorical classification, equating to 62.7%, 72.8%, 75.0% and 83.0% being classified as intermediate/high respectively [figure 3.58].

There was a significant difference in the expression of CTGF between control and both adjacent CIS and tumour (p<0.01, unpaired T-test) but not in
comparison to normal adjacent, with MIHCl values of 0.2901, 0.2632, 0.2279 and 0.2778 respectively. The same significant differences were observed with categorical classification (p<0.0001, Chi square), equating to 78.3%, 74.7%, 58.9% and 31.5% of control, normal adjacent, adjacent CIS and tumour cases being classified as intermediate/high respectively [figure 3.59].
4µm sections of the TMA (≥ 1985 cohort in both figures) were stained for MET and then analysed by AIA.

**Top** - Box and whisker plots of mean IHC staining intensity for all patients ≥1985. The mean IHC staining intensity per patient was then calculated for control cases, normal appearing urothelium from the patients with bladder cancer, adjacent CIS and tumour. Horizontal line = median. Box = interquartile range. Whiskers = 10th – 90th centile. Dots = outlying patients. Statistical comparison to the control cases using an unpaired T-test. ns=not significant, ***p<0.001.

**Bottom** - Patients were categorised based upon the mean percentage of cells per core staining at each pre-determined staining intensity for control cases, normal appearing urothelium from the patients with bladder cancer, adjacent CIS and tumour. Negative = background staining or <5% of cells at any higher intensity level. Low = ≥5% of cells at intensity level 1. Intermediate = ≥5% of cells at intensity level 2. High = ≥5% of cells at intensity level 3.

**Figure 3.58** - Mean IHC staining intensity of MET (top) and percentage of cases expressing MET at each level according to percentage thresholds (bottom)
Figure 3.59 - Mean IHC staining intensity of CYR61 (top) and percentage of cases expressing CYR61 at each level according to percentage thresholds (bottom)

4µm sections of the TMA (≥ 1985 cohort in both figures) were stained for CYR61 and then analysed by AIA.

**Top** - Box and whisker plots of mean IHC staining intensity for all patients ≥1985. The mean IHC staining intensity per patient was then calculated for control cases, normal appearing urothelium from the patients with bladder cancer, adjacent CIS and tumour. Horizontal line = median. Box = interquartile range. Whiskers = 10th – 90th centile. Dots = outlying patients. Statistical comparison to the control cases using an unpaired T-test. ns=not significant, *p<0.05, ***p<0.001, ****p<0.0001.

**Bottom** - Patients were categorised based upon the mean percentage of cells per core staining at each pre-determined staining intensity for control cases, normal appearing urothelium from the patients with bladder cancer, adjacent CIS and tumour. Negative = background staining or <5% of cells at any higher intensity level. Low = ≥5% of cells at intensity level 1. Intermediate = ≥5% of cells at intensity level 2. High = ≥6% of cells at intensity level 3.
Figure 3.60 - Mean IHC staining intensity of CTGF (top) and percentage of cases expressing CTGF at each level according to percentage thresholds (bottom)

4µm sections of the TMA (≥ 1985 cohort in both figures) were stained for CTGF and then analysed by AIA.

**Top** - Box and whisker plots of mean IHC staining intensity for all patients ≥1985. The mean IHC staining intensity per patient was then calculated for control cases, normal appearing urothelium from the patients with bladder cancer, adjacent CIS and tumour. Horizontal line = median. Box = interquartile range. Whiskers = 10th – 90th centile. Dots = outlying patients. Statistical comparison to the control cases using an unpaired T-test. ns=not significant, ***p<0.001.

**Bottom** - Patients were categorised based upon the mean percentage of cells per core staining at each pre-determined staining intensity for control cases, normal appearing urothelium from the patients with bladder cancer, adjacent CIS and tumour. Negative = background staining or <10% of cells at any higher intensity level. Low = ≥10% of cells at intensity level 1. Intermediate = ≥10% of cells at intensity level 2. High = ≥10% of cells at intensity level 3.
3.10.3 **Survival stratified by target expression**

Patients were stratified dichotomously by expression of the targets of interest according to the mean percentage of cells in each core at classified as either IHC intensity level 0,1,2 or 3, as described in section 3.10.2. For MET and CYR61 this percentage threshold was set at 5% and for CTGF at 10% (based upon the Cox-regression analysis in tables 3.16 and 3.17). The survival of patients was then examined by Kaplan-Meir analysis with statistical significance calculated by Mantel-Cox Log-Rank test.

3.10.3.1 **Entire cohort included in the TMA**

There was no significant difference in OS or CSS between patients with negative/low and intermediate/high MET expression, p=0.189 and p=0.748 respectively [figure 3.60].

There was a large difference in median OS of 49 months versus 26 months and in CSS of >72 months versus 37 months for negative/low versus intermediate/high CYR61 expression respectively. These differences did not reach statistical significance, p=0.226 and p=0.243 respectively [figure 3.61].

Median OS was longer at 43 months versus 25 months for intermediate/high versus negative/low expression of CTGF, but this was not statistically significant, p=0.189. There was, however, a significant difference (p=0.021) in CSS between those with intermediate/high versus negative/low expression (using the 10% threshold) with a median survival of >72 versus 28 months respectively [figure 3.62].
Figure 3.61 - Overall survival (top) and cancer specific survival (bottom) stratified by MET expression

4µm sections of the TMA were stained for MET and then analysed by AIA. Survival stratified by mean tumour core expression. Negative expression (background staining or <5% of cells at any higher intensity level) and low expression (≥5% of cells at intensity level 1) were combined, as were intermediate expression (≥5% of cells at intensity level 2) and high (≥5% of cells at intensity level 3). Statistical significance calculated by Mantel-Cox Log Rank test.
Figure 3.62 - Overall survival (top) and cancer specific survival (bottom) stratified by CYR61 expression

4µm sections of the TMA were stained for CYR61 and then analysed by AI/A.
Survival stratified by mean tumour core expression. Negative expression (background staining or <5% of cells at any higher intensity level) and low expression (≥5% of cells at intensity level 1) were combined, as were intermediate expression (≥5% of cells at intensity level 2) and high (≥5% of cells at intensity level 3).
Statistical significance calculated by Mantel-Cox Log Rank test.
Figure 3.63 - Overall survival (top) and cancer specific survival (bottom) stratified by CTGF expression

4µm sections of the TMA were stained for CTGF and then analysed by AIA. Survival stratified by mean tumour core expression. Negative expression (background staining or <10% of cells at any higher intensity level) and low expression (≥10% of cells at intensity level 1) were combined, as were intermediate expression (≥10% of cells at intensity level 2) and high (≥10% of cells at intensity level 3). Statistical significance calculated by Mantel-Cox Log Rank test.
3.10.3.2 Post 1985 cohort included in the TMA

There was no significant difference in OS or CSS between patients with negative/low and intermediate/high MET expression with a median survival of 39.0 vs. 50.0 months p=0.689 and 46.0 vs. 58.0 p=0.490 respectively [figure 3.63].

There was a large difference in median OS of >72.0 vs. 34 months and in CSS of >72 months vs. 43 months for negative/low versus intermediate/ high CYR61 expression respectively. These differences did however not reach statistical significance, p=0.081 and p=0.131 respectively [figure 3.64].

Median OS and CSS were significantly longer for intermediate/high versus negative/low expression of CTGF at >72.0 vs 28.0 months, p=0.010 and >72.0 vs 28.0 months, p=0.021 respectively (again using the 10% threshold) [figure 3.65].

3.10.3.3 Post 1985 TCC only cohort included in the TMA

Further subgroup analysis was conducted, for the TCC cases only from 1985 onwards, of target expression in relation to OS and CSS. There was no significant difference in OS (p=0.547) or CSS (p=0.388) based upon dichotomised expression of MET. Within this cohort there was a large and significant difference in median survival between negative/low and intermediate/high expression of CYR1 for both OS >72.0 vs 33.0months (p=0.026) and CSS >72.0 vs 39.0 (p=0.046) respectively [figure 3.66]. The difference based upon CTG expression remained relatively unchanged with a median OS of >72.0 vs 28.0 months (p=0.009) and CSS of >72.0 vs 29.0 months (p=0.001) for intermediate/high and low/negative respectively [figure 3.67].
Figure 3.64 - Overall survival (top) and cancer specific survival (bottom) stratified by MET expression in the 1985 onwards cohort

4µm sections of the TMA were stained for MET and then analysed by AIA. Survival stratified by mean tumour core expression. Negative expression (background staining or <5% of cells at any higher intensity level) and low expression (≥5% of cells at intensity level 1) were combined, as were intermediate expression (≥5% of cells at intensity level 2) and high (≥5% of cells at intensity level 3).
Statistical significance calculated by Mantel-Cox Log Rank test.
4µm sections of the TMA were stained for CYR61 and then analysed by AIA. Survival stratified by mean tumour core expression. Negative expression (background staining or <5% of cells at any higher intensity level) and low expression (≥5% of cells at intensity level 1) were combined, as were intermediate expression (≥5% of cells at intensity level 2) and high (≥5% of cells at intensity level 3). Statistical significance calculated by Mantel-Cox Log Rank test.
Figure 3.66 - Overall survival (top) and cancer specific survival (bottom) stratified by CTGF expression in the 1985 onwards cohort

4 µm sections of the TMA were stained for CTGF and then analysed by AIA. Survival stratified by mean tumour core expression. Negative expression (background staining or <10% of cells at any higher intensity level) and low expression (≥10% of cells at intensity level 1) were combined, as were intermediate expression (≥10% of cells at intensity level 2) and high (≥10% of cells at intensity level 3). Statistical significance calculated by Mantel-Cox Log Rank test.
Figure 3.67 - Overall survival (top) and cancer specific survival (bottom) stratified by CYR61 expression in the 1985 onwards cohort, TCC histological subtype only

4µm sections of the TMA were stained for CYR61 and then analysed by AIA. Survival stratified by mean tumour core expression. Negative expression (background staining or <5% of cells at any higher intensity level) and low expression (≥5% of cells at intensity level 1) were combined, as were intermediate expression (≥5% of cells at intensity level 2) and high (≥5% of cells at intensity level 3). Statistical significance calculated by Mantel-Cox Log Rank test.
Figure 3.68 - Overall survival (top) and cancer specific survival (bottom) stratified by CTGF expression in the 1985 onwards cohort, TCC histological subtype only

4μm sections of the TMA were stained for CTGF and then analysed by AIA. Survival stratified by mean tumour core expression. Negative expression (background staining or <10% of cells at any higher intensity level) and low expression (≥10% of cells at intensity level 1) were combined, as were intermediate expression (≥10% of cells at intensity level 2) and high (≥10% of cells at intensity level 3).

Statistical significance calculated by Mantel-Cox Log Rank test.
### 3.10.4 Cox regression – outcome and target expression

Cox regression analysis was undertaken using a model that included gender, patient age at cystectomy, year of surgery, type of surgery (primary or salvage), T stage, N stage and the expression of each of the targets in turn, to determine their independent prognostic potential. The expression of MET and CYR61 was included in this Cox regression model with expression in the tumour bearing cores defined by the following methods: the mean IHC intensity per patient, the mean IHC intensity of the highest scoring core per patient, the mean percentage of cells scoring at IHC score 3 per tumour core, a score on the scale 0-3 based upon a percentage threshold classification (as described in section 3.10.2), the mean H-score per patient and the highest H-score of all tumour cores per patient. CTGF expression was included in the model using the expression classification methods described for MET and CYR61. In addition, as loss of CTGF was seen to be associated with worse survival in Kaplan-Meir analysis, the mean percentage of cells scoring at IHC score 0 per tumour core, the minimum H-score of all tumour cores per patient and the mean IHC intensity of the lowest scoring tumour core per patient were also included in the Cox regression analysis.

### 3.10.4.1 Cox regression – entire cohort in the TMA

The expression of MET and CYR61 were not found to independent predictors of survival in this model, despite including target expression by the varying methods described [table 3.17]. For CTGF, only the mean percentage of cells per core at IHC score 0 (negative) was an independent predictor of OS, with a hazard ratio (HR) of 1.006 (p=0.033, 95%CI 1.000-1.011). The expression of CTGF was found to be an independent predictor of cancer specific survival when included by multiple methods including: mean H-score (HR 0.996, p=0.013), maximum H-score (HR 0.997, p=0.044), minimum H-score (HR 0.996, 0.032), mean percentage of cells at IHC score 3 (HR 0.982, p=0.044), mean percentage of cells at IHC score 0 (HR 1.009, p=0.008), the percentage threshold classification score at a 10% cut off (HR 0.860, p=0.038), mean IHC
intensity (HR 0.023, p=0.035) and minimum IHC intensity (HR 0.037, p=0.043). The expression of CTGF using a percentage classification score at cut off levels of 2.5% or 5% and the maximum IHC intensity were not predictive of CSS.

3.10.4.2 Cox regression – 1985 onwards TCC subtype only

As was the case in the overall cohort, the expression of MET was not related to OS or CSS in the subgroup of patients with TCC histology from 1985 onwards. Increased expression of CYR61 was associated, in the same subgroup, with worse OS when expression was dichotomised as negative/low vs. intermediate/high at a 2.5% threshold (HR 1.493, 95%CI 1.040-2.143, p=0.030) and 5% threshold (HR 1.40, 95%CI 1.071-1.830, p=0.014). CYR61 expression was not prognostic of CSS. Loss of CTGF expression was significantly prognostic of OS when measured by nine out of the 11 methods described and of CSS in all but one method [table 3.18]. Negative/low vs. intermediate/high expression was predictive of CSS, associated with a HR of 0.783 (95%CI 0.629-0.977, p=0.030) at the 2.5% threshold and a HR of 0.744 (95%CI 0.566-0.979, p=0.035) at the 10% threshold. The lowest HR, was for the MIHCI in the lowest scoring core pre patient with a HR of 0.002 (95%CI <0.0001-0.222, p=0.009). Although this may give the impression that MIHCI of CTGF expression is therefore most prognostic marker it must ne noted that the range of MIHCI for CTGF was <0.0001-0.5100.
<table>
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<th>Overall survival</th>
<th>Cancer specific survival</th>
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**Table 3.17 - Cox-regression of survival following cystectomy based upon expression of the putative targets (entire TMA cohort)**

A Cox-regression model was constructed including gender, age of patient, year of surgery, surgery type (primary or salvage), T-stage and lymph node status (positive or negative). The expression of the target (in the tumour bearing cores) were then added into the model, according to various classification methods. The mean H-score (range 0-300), mean % cells high or negative (0-100) and mean IHC intensity (0-0.5) were analysed on a linear scale. Mean, min. or max. IHC intensity refers to the mean IHC intensity of all tumour cores or the lowest or highest scoring tumour core for each patient. The threshold values were included as 0=background staining or <stated% of cells at any higher intensity level), 1=≥stated% of cells at intensity level 1, 2=≥stated% of cells at intensity level 2 and 3=≥stated% of cells at intensity level 3. HR=hazard ratio. CI=confidence interval.
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Table 3.18 - Cox-regression of survival following cystectomy based upon expression of the putative targets (1985 onwards, TCC only)

A Cox-regression model was constructed including gender, age of patient, year of surgery, surgery type (primary or salvage), T-stage and lymph node status (positive or negative). The expression of the target (in the tumour bearing cores) were then added into the model, according to various classification methods. The mean H-score (range 0-300), mean % cells high or negative (0-100) and mean IHC intensity (0-0.5) were analysed on a linear scale. Mean, min. or max. IHC intensity refers to the mean IHC intensity of all tumour cores or the lowest or highest scoring tumour core for each patient. The threshold values were included as 0=background staining or <stated% of cells at any higher intensity level), 1=≥stated% of cells at intensity level 1, 2=≥stated% of cells at intensity level 2 and 3=≥stated% of cells at intensity level 3. HR=hazard ratio. CI=confidence interval.
3.11 Summary of key findings

Three target proteins were selected for evaluation of their therapeutic and prognostic potential in bladder cancer. CTGF and CYR61 were identified using the Oncomine™ database. MET was the third inclusion as there is published evidence of CTGF and CYR61 involvement in the MET axis, which has itself been targeted therapeutically in a number of cancers.

Stimulation of six bladder cell lines with HGF, the agonist of MET, caused an increase in the expression of CTGF in all but one of the cell lines. However, an increase in CYR61 was only observed in one cell line, the RT112 line. The effects of target knockdown were studied exclusively in the high-grade invasive J82 and T24 cell lines. Knockdown of CYR61 caused a significant reduction in the expression of vimentin in the T24 cell line, although the basal expression of vimentin in this cell line was low in comparison to the other cell lines studied. No other changes in the expression of the EMT markers E-cadherin, N-cadherin or vimentin were noted following knockdown of the three targets.

Knockdown of CTGF caused a significant reduction in cell proliferation in both cell lines, whereas CYR61 knockdown only reduced proliferation in the T24 cell line. Knockdown of CTGF and CYR61 significantly increased sensitivity to cisplatinum in both cell lines. MET knockdown reduced proliferation in the T24 cell line but did not increase sensitivity to cisplatinum in either cell line.

During Matrigel™ invasion assay experiments knockdown of all three targets abrogated the invasive response to HGF, whereas only knockdown of CYR61 significantly reduced the response to FCS. In the scratch ‘wound’ migration assay experiments the results were very mixed, with no single target demonstrating a consistent effect following knockdown during stimulation with either HGF or FCS.

A bladder cancer TMA was successfully constructed from the bladder tissue of 497 bladder cancer patients and 70 control cases. Age at cystectomy, T stage
and nodal status were initially found to be independent predictors of survival. Year of cystectomy was also found to be predictive of outcome, which when combined with the differences in cytokeratin expression observed in relation to sample age, led to the exclusion of all cases prior to 1985. This resulted in the inclusion of 329 bladder cancer cases for the final IHC analysis. In this cohort T stage and nodal status remained independent predictors of outcome. Year of cystectomy was no longer predictive of survival and age at cystectomy was predictive of OS but not CSS.

Automated image analysis with Definiens™ was validated as a robust method for evaluating IHC staining across the bladder TMA, and subsequently used to evaluate the expression of the three putative targets.

MET expression did not correlate with grade of tumour but was inversely related to increasing stage and significantly lower in tumour than in normal control tissue. MET was not a predictor of survival following cystectomy.

The expression of CYR61 was not related to grade of tumour and although there was a significantly higher level of expression in normal adjacent urothelium, adjacent CIS and tumour when compared to normal controls there was no clear relationship between expression and stage of tumour. CYR61 expression was an independent predictor of OS but not CSS.

CTGF expression was not related to tumour grade but was inversely related to increasing stage and was significantly lower in adjacent CIS and tumour than in control tissue. Loss of expression of CTGF was an independent predictor of both OS and CSS following cystectomy.
4 Discussion

4.1 Patient population and TMA construction

4.1.1 Patient population

In the study of putative biomarkers or treatment targets the optimal starting point would be the prospective enrolment of patients with prospective collection of patient samples including tissue, blood and urine. In this ideal situation uniformity of methodological process and data collection can be applied to reduce confounding factors within the data generated [259]. In this current project it would simply not have been practical to prospectively enrol such patients, as given the number of patients who undergo cystectomy in the South Manchester area and the need for follow up of at least 5 years to obtain meaningful outcomes, it would have taken over a decade to enrol and follow up sufficient patients.

There existed a database, retrospectively generated for patients undergoing cystectomy from 1969 to 1990 and prospectively from 1990 onwards. The pragmatic approach taken was therefore to obtain archival tissue from the Christie pathology archives based upon patients included in this database. It is important to consider, however, that as a tertiary referral centre for the management of complex cancer the population of patients undergoing surgery at the Christie may not be a true reflection of the general population in the U.K. undergoing cystectomy for the treatment of bladder cancer. This is exemplified by the fact that 43.5% of the post 1985 cohort underwent cystectomy as a salvage procedure, whereas this figure nationally is <10% [291].

Appropriate steps were undertaken in an attempt to control for confounding factors with respect to both patient data and tissue quality for inclusion in the TMA. The recording of prior treatments for bladder cancer, such as intra-vesical chemotherapy or BCG, and co-morbidities; which could affect cancer biology
and outcome were inconsistently recorded and it would not have been possible to accurately collect this data retrospectively. The decision was made, therefore, to include in the subsequent database generated for this project only data that was consistently recorded in all patients. This meant that only patient age, year of surgery, type of surgery (either primary or salvage following failed radiotherapy), tumour type, T stage of tumour, nodal status, WHO grade and date and cause of death were included. Despite this narrow spectrum of included data there were still variations within the recording of these parameters and missing data, particularly with respect to outcome. The use of the North West Cancer Intelligence Service (NWCIS) was a valuable resource in providing death certification data for patients with unrecorded outcomes in the database. This meant that the cause of death was known in 95.0% of patients who died during follow up; very high in relation contemporary series [188, 189, 191]. The WHO grading system changed during the study period, being first introduced in 1973 and subsequently changed in 2004. Fortunately very few patients were included prior to 1973 and the Christie pathologists continue to use the 1973 system to this date in preference to the 2004 system. In the final analysis patients prior to 1985 were excluded. This removed any potential confounding effects that may have resulted from variances in histological grading following publication of the 1973 WHO guidelines.

Lymph node status presented a slightly more complicated issue. When examining the original Christie database it was apparent that lymphadenectomy was only performed very occasionally prior to 1978, and after that sporadically until 1994 when was more consistently recorded. This change correlated with trends in U.K. practice and is in line with the current recommendations regarding lymphadenectomy when performing a cystectomy [181]. It is likely that prior to 1994 only in cases where the lymph nodes looked or were palpably involved would they have been removed. There are therefore likely to have been a significant number of patients prior to 1994 in whom there would have been occult lymph node involvement who did not undergo lymphadenectomy. This makes the inclusion of lymph node status into multivariate analysis models open to criticism. Rejecting or accepting the null hypothesis in relation to the prognostic power of a novel marker, based upon to its ability to predict nodal...
status or for it’s correlation with lymph node status has been avoided due to these issues. A similar situation existed for the staging of patients with cross sectional imaging by CT or MRI, which started to be recorded sporadically from around 1990 and became more consistent practice from the mid 1990’s onwards. Due to this situation it is likely that there would have been a greater number of patients with occult metastatic disease at the time of cystectomy prior to the mid-1990’s than after this date. When relating marker expression to events it is important, particularly with retrospective data that the end points chosen have minimal potential for bias. On this basis only OS and CSS were chosen as endpoints. The use of recurrence free survival (RFS) in a retrospective series is fraught with problems. In a retrospective series the detection of recurrence is dependent upon whether a programme of screening for recurrence existed, and if so how that changed over time. Individual clinician choice regarding the use of cross sectional imaging and access to such imaging are also important factors. With no standardised approach applied during follow up of the cohort in question the potential for inconsistency and bias was felt to be too high and RFS was not investigated.

The associated reduction in peri-operative deaths (death within 30 days of surgery) that was notably seen, reducing from 16.6% in the 1970’s to 1.9% in the 2000’s, is probably explained by improvements in surgical technique, peri-operative care and better patient selection for surgery. These values are consistent with current figures from the USA where a study of >50 000 cystectomy patients reported a stable in-hospital mortality rate of 2.5% between 2001-2008 [292]. The potential inclusion of increased numbers of patients with metastatic disease at the time of cystectomy, prior to the mid-1990’s, and improved peri-operative mortality go some way towards explaining the increase in survival following cystectomy over the study period 1969-2006, where year of surgery was associated with increasing OS (HR 0.9647, p<0.0001), and are in keeping with trends seen in other developed nations [293]. Examining the Kaplan-Meir plots for OS [figure 3.6] there was clustering of the date of surgery cohorts pre-1985, with markedly differing survival compared to those patients undergoing surgery after 1985. When only patients from 1985 onwards were examined in Cox multivariate analysis, neither WHO grade or year of surgery
remained as independent predictors of OS, indicating that the post 1985 cohort was far more homogenous in nature, adding weight to the decision to exclude pre-1985 patients.

Although there was an apparent difference in survival between those who underwent primary and salvage cystectomy (in the original Christie Database) at 48.0% and 42.2% at 6 years respectively (p=0.047) by Kaplan Meir analysis, when multivariate analysis was performed this difference in both OS (HR 1.001, p=0.9552) and CSS (HR 0.913, p=0.5253) was not significantly affected by this treatment difference. This is different to the findings of a recent large study containing data on >12 000 primary and 903 salvage cystectomy patients, where the HR was 1.47 for OS and 1.43 for CSS associated with salvage cystectomy [291]. However, the data in that study is open to significant question as the T stage was missing in 25% of cases (compared to 0.6% in the current data) and the whole study was based upon national cancer registry data. Patients undergoing salvage cystectomy represent a self selecting population of patients, who are fit enough to undergo salvage surgery with no identifiable metastatic disease at the time of cystectomy. In those patients included in the TMA construction salvage cystectomy occurred at a median of 16.9 months after radiotherapy, a point by which approaching 25% of patients in the primary cystectomy group had died. This does raise the question as to whether salvage and primary cystectomy patients are truly comparable with respect to tumour behavior. Despite these concerns the absence of a significant difference of OS or CSS in multivariate analysis allows a reasonably confident comparison of the two groups in relation to cancer outcome, and that they do not necessarily need to be considered as two separate groups in the analysis.

4.1.2 TMA construction

Screening of the tissue for inclusion in the TMA by consultant histopathologists resulted in the exclusion of 30.5% of patients within the original database where the tissue blocks were available (36% due to perceived poor tissue fixation and 46% due to insufficient tissue being present within the blocks for construction).
This was in part responsible for the overall inclusion of 63.8% of all eligible patients in the Christie database (those who underwent surgery prior to 2006). This inclusion figure is relatively high and although there are concerns regarding possible selection bias [294], the absence of a significant difference in OS between those included and excluded from the TMA construction indicates a low likelihood of significant bias [figure 3.7]. Although it is noted that the 6-year survival rates were 43.9% and 50.2% respectively for those included and excluded, most likely explained by the lower stages of tumour seen in those excluded [table 3.5].

Despite evaluation of the tissue by bright field microscopy of H&E sections there were clear significant differences in the tissue expression of panCK and phospho-tyrosine, assessed by DAB based IHC, in relation to sample age [figures 3.41 and 3.42], which disappeared when the pre-1980 cohort were excluded. This, in conjunction with the differences in OS by year of surgery [figure 3.6 ], ultimately led to the exclusion of all patients prior to 1985 from the final data analysis. Differences in the preservation of archival FFPE tissue could be explained by variation in tissue fixation and storage [295], in addition to the reduction in IHC staining intensity that has been described to occur tissue as FFPE samples age [296]. The expression profiles of differing cytokeratins in bladder cancer vary between histological sub-types, stage and grade and the expression of cytokeratins 5, 13, 14, 17 and 20 are known to be associated with cancer outcome [154, 297] indicating an association with tumour phenotype. Whilst this differential expression profile may in part explain the differences seen between individual patients [figure 3.41] the effect should have been reduced by the use of a pan-CK antibody, which recognised cytokeratins 4, 5, 6, 8, 10, 13 and 18. The differential cytokeratin profile does, however, not explain the notable dichotomization in expression pre and post 1980. It is notable that there was a flood in the pathology archives in the past, that had clearly affected some of the blocks from the late 1970’s and early 1980’s, in that some of the storage boxes had corroded and a number of the paraffin blocks were disintegrated. The blocks prior to the 1980’s were also not mounted within modern pathology cassettes and as such had to be melted down and re-embedded, which may have had some effect upon the tissue.
Phospho-tyrosine expression was lower overall in comparison to pan-CK, with mean IHC intensities across the TMA of 0.146 and 0.733 respectively [figure 3.42] and 50.1% of all cores categorised as showing negative or low expression by H-score. There are very few publications describing the effects of sample fixation and sample age on protein phosphorylation. It is, however, recognised that delay in sample freezing, when using fresh frozen tissue samples for proteomic studies, can have a significant effect upon phospho-protein levels [298, 299]. FFPE samples are increasingly utilised in tissue proteomics using mass spectrometry [300, 301] with high rates of retrieval of intact proteomics yielding meaningful and reproducible data that is almost comparable to that obtained from fresh frozen tissue. The rate of formalin tissue penetration, reported at between 0.1 and 1mm per hour, means that for a large number of samples there will be a core of tissue that remains unfixed for a number of hours. This rate of fixation is affected by a number of parameters in the fixation process [302]. During this time the tissue will undergo biological changes due to a number of processes including the release of cytokines, changes in temperature and glucose levels that will alter the phosphorylation and presence of certain proteins [299]. This may explain the negative correlation between sample age and phospho-tyrosine expression that was observed. Delay in fixation can lead to increases in the phosphorylation of certain proteins. The improvements in tissue fixation processes that have occurred over time, resulting in faster fixation, may explain why expression was higher in the older, possibly slower fixed samples. It may well be that the evaluation of phosphorylation within archival FFPE samples is possible, but with no process to control for the variables that affect fixation and preservation in an archival cohort, and in particular given the low level of expression of phospho-tyrosine seen, no attempt was made to study the phosphorylation status of proteins across the TMA.

It is interesting that when the pilot TMA was constructed, three blocks were produced, with one of the blocks (10_NOCL-01D 8-11-11 3A, [figure 3.40]) being made exclusively from tissue that was felt by the histopathologist (CW) to be sub-optimally preserved. When stained for pan-cytokeratin and phospho-
tyrosine there were no differences in staining intensity between the three blocks produced. This suggests that assessment of tissue quality for inclusion by brightfield microscopy of H&E sections is insufficient. In retrospect a process of tissue selection that includes assessment of the expression of proteins such as cytokeratin by IHC may have been more robust. The published literature contains many studies utilising IHC and archival tissue samples, yet almost none describe any process of tissue quality control. In relation to the REMARK guidelines (section 1.8) this project adheres to all 20 points of reference [259], although within these guidelines there are no statements regarding the quality control steps that should be employed when using archival tissue for IHC biomarker studies. In 2013 the NCRI Biomarker and Imaging Clinical Studies Group published guidelines for research utilising TMA’s [225], to which all principles were adhered. However, even those guidelines do not describe quality control steps for tissue selection beyond review of the tissue by an experienced histopathologist.

During TMA construction cores were selected that represented the tumour, adjacent CIS, adjacent normal urothelium (as assessed by bright field H&E microscopy) and cores of normal bladder urothelium from patients with non-bladder malignancies. The inclusion of tissue from non-bladder cancer cases is rare in TMA construction and in bladder cancer tissue research in general. It is of critical importance to include these true normal cores due the theory that bladder cancer develops within a ‘field change’ of ‘unstable’ urothelium [303]. As such apparently normal appearing urothelium in patients with bladder cancer may harbor molecular changes on the pathway of progression from normal to malignant urothelium. Indeed it has has previously been described, in a study that included bladder tissue from non-bladder cancer cases, that histologically normal urothelium from patients with CIS harboured a CIS gene expression signature that was not found in the urothelium of non-bladder cancer patients [286]. The findings of the current project are consistent with this theory, in that a statistically significant increase in CYR61 expression was observed in tumour tissue and histologically normal urothelium from bladder cancer patients when compared to the urothelium from non-bladder cancer controls, [figure 3.58].
4.2 TMA and automated image analysis validation

The use of automated image analysis (AIA) software, in the interpretation of FFPE IHC staining, is increasingly being used in a research setting [251] and it’s role in a clinical setting, as an adjunct to reporting by a pathologist is increasingly being explored, with web based applications for IHC analysis increasingly available [255]. It has been shown that AIA can generate reliably interpret FFPE sections stained using IHC with results that are comparable to interpretation by an histopathologist [256, 304]. A number of studies [305] inappropriately apply the use of simple of correlation statistics, such as the use of Spearman’s correlation [306] rather than calculating κ values for categorical data, Cronbachs α or performing Bland-Altman plots. Despite some of these methodological problems there is evidence that Definiens® is a robust platform for analysing FFPE IHC staining and it is increasing cited in the literature [252, 307].

There are a wide variety of scoring systems that can be applied to the analysis of IHC staining which include categorical classifications such as HER2 staining in breast cancer [247], semi-quantitative systems including Allred and H-scores or the generation of quantitative continuous data such as and average threshold measure (referred to as mean IHC staining intensity (MIHCI) by Definiens®) [246]. It could be argued that the use of an IHC classification system that averages out the expression within a TMA core or tissue section, as is the case with Allred, H-score or MIHCI risks a type two statistical error. Comparison to standard H&E section analysis in the grading of bladder TCC in clinical practice exemplifies this. If a tumour contains any area of grade 3 tumour (WHO 1973) then the tumour is designated as a grade 3 tumour as this is the most ‘dangerous’ focus of cancer in the specimen. It is worth noting, however, that is not the case for all cancers and in particular prostate cancer, where the Gleason grading system is based upon the 2 most prevalent grades rather than highest grades of cancer within a tumour. A core that contains a small number of cells staining very strongly for a marker with the remainder of the core staining negative generates an Allred, H-score or MIHCI that is low and similar to a core containing a large number of weakly staining cells. It may be,
however, that the presence of even a small number of cells that are staining strongly positive is important, as is the case for breast cancer where ≥1% of tumour nuclei positive for ER staining is considered positive [308]. As such the use of an MIHCI, H-score or Allred method may therefore overlook the significance of such staining in biomarker discovery. However, the H-score did provide a good method for validating the use of Definiens® in analysing the bladder TMA, as a semi-quantitative method that can be applied to both human scoring and AIA, whereas MIHCI is applicable only to AIA.

In the analysis of the bladder TMA, Definiens® was unable to accurately differentiate between different tissue types using the region of interest classification algorithm and the use of counter stains was unsuccessful [section 3.9]. This meant that the cell classification algorithm alone was utilised to identify cancer and urothelial cells within the tissue cores. This proved to be very reliable when validated against cell classification by the human eye with a high degree of specificity at 99.7% and a sensitivity of 82.5%. The disadvantage of not successfully utilising the region of interest classification algorithm was that it was not possible to examine the expression of proteins within other tissue compartments, such as the stroma, which may have provided valuable data given the matricellular nature of the CCN proteins ultimately studied. Whilst a sensitivity of 82.5% for the identification of urothelial and tumour cells is high it was hoped that the use of immuno-fluorescence (IF) rather than DAB based IHC may provide higher levels of sensitivity and provide a platform to stain for multiple markers on the same TMA section simultaneously. Unfortunately a high level of auto-fluorescence occurred at excitation wavelengths of 555nm and below [figures 3.43 and 3.44], a well recognised phenomenon when using FFPE tissues [309, 310], particularly in relation to erythrocytes [311]. If it had been possible to reduce the impact of tissue auto-fluorescence using tissue quenching or post image capture data manipulation using one of many described methods [312, 313] this may have allowed for multiple markers to be studied. The aim at the outset of using IF was to stain sections using a fluorphore conjugated anti-panCK antibody and then to stain for other proteins of interest using additional fluorophores. The panCK signal would then have been used to identify the urothelial and tumours cells of interest in the cores.
using AIA. With the wide variation in panCK staining observed with DAB based IHC, and the very high levels of auto-fluorescence, particularly from erythrocytes, the application of this methodology to AIA would have most likely markedly reduced the specificity with only marginal increase in sensitivity. Adequate refinement of a fluorescence methodology, which may not be possible, was beyond the scope of the current project and as such DAB based IHC was felt to be a more robust methodology.

The primary differences between human scoring and AIA are that the human eye is only able to generate qualitative or semi-quantitative data, which lends itself to the generation of the canonical classifications (eg. 0, 1+, 2+ and 3+) frequently used in the interpretation of IHC staining. Human scoring is also subject to significant inter and intra-observer variation [314, 315], due to a number of factors including visual bias. In contrast to human scoring, AIA can generate truly quantitative data, with minimal variation in scoring if standardised algorithms are applied [253, 304]. There remains, however, a requirement for validation in comparison to human scoring [112] which remains at present the gold standard.

It is notable, when examining the Bland-Altman plots [figures 3.46-47] that the greatest levels of disagreement were in the H-score range between around 50 and 200, which would be considered as an intermediate range. Above 200 there was a very high level of agreement. AIA is reported to be problematic with the categorisation of intermediate cases, in contrast to when scoring strongly positive or negative expression, where it can demonstrate near perfect agreement with human scoring [253]. However, the categorisation of cases with intermediate expression is not a problem confined to AIA. With human scoring of HER2 expression in breast cancer, there can be high levels of inter-observer variation, particularly in 2+ cases [316]. Accordingly HER 2+ status determined by IHC requires further confirmation, with for example in-situ hybridisation, prior to clinical decision making on treatment [247, 317]. It may well be therefore that the observed failings of AIA in intermediate cases, in published series, are in part explained by variability in the human validation 'control' rather than an inherent weakness of AIA.
The highest rate of intra-observer agreement when generating validation H-scores was with AIA ($\alpha = 1.000$). This was expected, as application of the same algorithm to analysis should produce the same results each time with AIA. There was a varying degree of human intra-rater variability and low level of inter-rater agreement, as predicted from the known weaknesses inherent in human scoring. The highest levels of agreement were the intra-observer agreement for rater A, the author ($\alpha = 0.907$ and Bland Altman 95% limits of agreement 71.7 – 83.3, [figure 3.46]) who also generated the AIA algorithm, and between the mean of all 4 human scores and AIA ($\alpha = 0.857$ and Bland Altman 95% limits of agreement -100.3 – 70.9 [figure 3.47]).

In this project AIA was demonstrated to have a high sensitivity and specificity for cell classification, with an inter-rater agreement (between AIA and human eye) greater than that observed between human raters; and approaching that seen in multi-centre studies of IHC analysis [315]. These factors, combined with the complete removal of intra-observer variability from AIA, validated Definiens® as a platform for the analysis of DAB based IHC staining of the bladder cancer TMA. The AIA process, once the algorithms are developed, is also highly efficient. Definiens® was capable of independently processing 35 slides containing over 2500 cores in <24 hours, whereas the same process by human scoring would take in excess of 40 hours. Definiens® also outputs a large volume of raw data that can be analysed to categorise cores and patients based upon numerous described methods.

4.3 Marker / target expression across the TMA

When defining if expression of the targets studied (CYR61, CTGF and MET) had prognostic value, the level of expression was examined for each of the targets as continuous, categorical and dichotomised values and as part of a Cox multivariate analysis that included known prognostic factors. This use of multivariate analysis to define clinically relevant cut-off values or thresholds was undertaken with the aim of reducing the risk of a type one error that is known to
occur by defining cut-off values without considering the effect of known prognostic factors simultaneously [258].

4.3.1 MET expression

CYR61 and CTGF were identified through Oncomine® as putative novel targets in MIBC. Whilst there was almost nothing known of their role in bladder cancer, CYR61 has been identified to be an HGF responsive gene [318] and to be responsible for promoting migratory and proliferative cell responses to HGF [289]. There is also evidence that CTGF is regulated by HGF [290]. These reports linking CYR61 and CTGF to the HGF/MET axis combined with the well described role of MET in a number of cancers [119] led to the inclusion of MET in the panel of targets studied.

Evaluation of the expression of MET across the TMA failed to demonstrate a prognostic value for MET expression in relation to either OS or CSS in any cohort studied [figures 3.60 & 3.63]. However, the expression of MET was found to be significantly inversely related to increasing stage of tumour [figure 3.52] and when examined as a continuous variable by MIHCI, or when dichotomised, expression was found to be significantly lower in tumour than in control tissue. The levels in normal adjacent urothelium and in adjacent CIS were very similar to that seen in the control tissue, also being higher than in tumour [figure 3.57]. This pattern of expression with maintained levels of MET in adjacent CIS, believed to be the precursor lesion in the pathway to MIBC [31, 144], and the declining expression observed with increasing grade indicate that the loss of MET expression may be a late event in the pathway of developing MIBC. However, the counter to this argument is that if loss of MET expression were a late event then expression might be expected to predict survival in Kaplan-Meir analysis, irrespective of it’s predictive power in multivariate analysis, by stratifying patients based upon the stage of their disease. It is clear from the Kaplan-Meir analysis performed that this is not the case [figure 3.60 & 3.63].
The publications to date on the prognostic significance of MET are contradictory. Two studies, by Yeh et al. [129] and Cheng et al. [130], have described a significant relationship between high MET expression and poor survival in multivariate analysis. A further study by Sanchez-Carbayo et al. [131] demonstrated the same relationship in univariate analysis, although the number of patients with outcome data was small at only 69 and follow up short at 36 months. In the study by Cheng et al. [130] the magnitude of the effect was not presented and the study contained a wide spectrum of disease in 142 patients, of whom only 38 underwent radical treatment, and high expression was described in only 7. The validity of the findings of the studies by Sanchez-Carbayo and Yeh are therefore questionable. Indeed, Cheung et al. subsequently published a further manuscript in 2005 examining the expression of both MET and RON in an increased cohort, that included 77 patients who underwent radical treatment, where MET failed to yield prognostic power in relation to survival in multivariate analysis, citing that their initial study was underpowered [132]. This later manuscript correlates with the findings of three further studies [133-135], which have failed to demonstrate a significant association between survival and MET expression. Although, the study by Miyata et al. [133] did demonstrate that phosphorylation of MET at Y1349, known to lead to the activation of multiple downstream pathways including PI3K and MAPK, was highly prognostic of survival (HR 3.47). Had there been greater confidence in the study of phosphorylation on the TMA it may have been possible to reproduce this finding. A very recent publication by Kluth et al. from 2014, and possibly the most methodologically sound, described IHC analysis of MET expression and FISH analysis of MET amplification in 560 and 504 cases of bladder cancer respectively [134]. The authors identified moderate or high MET expression in 57.5% of histologically normal urothelium from bladder cancer patients. High expression was found in 45.4% and 17.3% of G1pTa and G3pT2-4 tumours respectively with loss of expression being significantly associated with increasing stage of tumour. They found no association between MET expression and survival and a very low rate of MET amplification in tumours of only 0.8%.
The data presented by Kluth et al. correlates very well with the findings of this current project regarding MET expression across the TMA, validating the TMA and MET expression findings. The counter to this statement is that the current findings are contradicted by the of Yeh et al., although their work only included 65 patients of whom all were stage $\geq$ pT2 and 35.4% were lymph node positive. There are no markers that exist in MIBC where there are not publications contradicting the findings of others, exemplified by history of p53 as a biomarker [46]. In respect of the publications described regarding MET expression in bladder cancer, the observed differences could be explained by differences in the patient populations, length of follow up, antibody used, staining protocols and the scoring systems used to quantify expression. These contradictions will remain very difficult, if not impossible, to resolve without collaboration and standardisation of methodology.

**4.3.2 CYR61 and CTGF expression**

The CCN group contains 6 proteins (CCN1-6), which include CCN1/CYR61 (cysteine rich angiogenic inducer 61) and CCN2/CTGF (connective tissue growth factor). The nomenclature CYR61 and CTGF, was used during this thesis to aid clarity. The proteins within this group have divergent actions, with the induction of CYR61 and CTGF occurring in cells that have been stimulated to proliferate with concurrent down regulation of CCN3. The CCN proteins have multiple cellular effects, acting upon multiple pathways, and rather than only acting through agonist-receptor interactions the proteins regulate multiple pathways as part of a ‘centralised communication network’ [319]; and in particular regulate the interaction between cells and the extracellular matrix [320]. It has been recognised for a number of years that the proteins have a role in multiple human cancers [321, 322], although, the roles of the proteins differs between different cancers with both oncogene and tumour suppressor properties when studied in different tumour types [323]. It is clear however that CYR61 and CTGF have important roles in EMT, proliferation and anti-apoptosis, migration, invasion, metastasis formation, tumour angiogenesis, regulation of gene expression and cell senescence [324, 325]. Almost nothing
was known of the role of the CCN proteins in bladder cancer prior to this project. There is only one published study to date, which detailed the expression of CTGF in a single cell line model of bladder cancer [326] whereby cell stimulation with anti-proliferative factor increased the expression of CTGF, reducing cell proliferation.

4.3.2.1 CTGF (CCN2) expression and tumour phenotype

The expression of CTGF across the TMA was in contradiction to that predicted by the Oncomine® data, where CTGF was a top 10 ranked overexpressed gene in invasive compared to non-invasive bladder tumours. It was also contrary to the findings of the in vitro cell line modelling, which demonstrated a significant reduction in cell proliferation and HGF mediated Matrigel® invasion following knockdown. Contrasting this the TMA data revealed that a loss of CTGF expression was significantly related to OS and CSS (by a number of classification methodologies) and highly prognostic of survival.

The relationship between mRNA and protein expression is complex and it is widely accepted that there can be a very poor correlation between mRNA and protein expression [327, 328]. Vogel et al. in a review of the regulation of protein expression cites an overall low correlation between mRNA and protein expression within the literature, with a Pearson’s r value of around 0.4 [329] and at times there can be an inverse correlation [330]; with protein expression being affected to a far greater extent by post transcriptional mRNA modification, degradation and post translational protein modification than by mRNA transcript levels [329]. It is therefore reasonable to hypothesise that the contradiction observed between the TMA data on CTGF protein expression and the Oncomine® data on mRNA transcript levels is due to post-transcriptional phenomena. There may also be differences between the Oncomine® and current project patient populations. The current project contained a population of patients who were undergoing primary or salvage radical treatment, whereas the patient cohorts from which the Oncomine® meta-analysis data was
obtained were mainly of non-muscle invasive bladder cancer (NMIBC), with the invasive group (≥T2) comprising only 38.6%.

The cell line models predicted an association between increased CTGF levels and a more aggressive tumour phenotype, which correlates with the Oncomine® data. This observed difference between the TMA data and the cell line model could be explained by the nature of the environment in which the cells were cultured, on untreated plastic and devoid of interaction with stromal cells or an extra cellular matrix. It is recognised that in-vitro conditions significantly alter the behaviour and gene expression pattern of tumour cells, with mouse mammary tumour initiating cells showing different invasive and differentiation phenotypes when cultured on either collagen, fibronectin or laminin coated substrates [331]. Specifically to CTGF, expression by human corneal fibroblasts stimulated with TGF-β1, known to induce CTGF expression, is altered five fold by culture on collagen compared to fibronectin [332]. This exemplifies the importance of the extracellular matrix interaction when examining CCN protein biology and may explain why the observations in cells grown on untreated plastic are different to that seen in the human samples. The association of reduced CTGF expression and worse survival observed across the TMA is well described in other cancer types. In nasopharyngeal carcinoma reduced expression is associated with worse outcome, although in contrast to the bladder cell line findings in vitro studies of nasopharyngeal cell lines have demonstrated increased proliferation and tumour xenograft growth in nude mice following CTGF knockdown [333], with CTGF knockdown promoting EMT in vitro [334]. Reduced expression in colorectal cancer is associated with a worse prognosis and with reduced Matrigel® Boyden chamber invasion, but no effect upon proliferation following knockdown has been observed [335]. The same trend is also been described in ovarian and lung cancer [336]. In contrast CTGF appears to be oncogenic in melanoma [337] gastric [338], pancreatic [339] and prostate cancer [340]; although there is conflicting evidence in breast cancer with reports that both reduced [341] and increased expression [342] are associated with a worse outcome. In a series of gall bladder cases the findings were more complex. At an mRNA level CTGF was increased in tumour compared to normal epithelium but reduced in metastatic
sites, with loss of CTGF expression at a protein level in the primary tumours being associated with a worse outcome [343]. It was suggested by the authors that this difference in expression between primary and metastatic sites may reflect differences in the requirements of the tumour and phases of tumour progression. This finding in gall bladder cancer is in keeping with the findings to date for bladder cancer from Oncomine® and the TMA, although metastatic site data for bladder cancer is not available.

4.3.2.2 CYR61 (CCN1) expression and tumour phenotype

The expression of CYR61 correlated with bladder cancer development with a significant difference in expression between normal adjacent, adjacent CIS and tumour cores in comparison to normal controls [figure 3.58]. Expression was also found to be prognostic of OS in the TCC histological subtype in patients from 1985 onwards [table 3.16]. Unlike the expression of CTGF this correlated with the prediction from Oncomine® and the cell line modelling. This association of increased expression and worse outcome is described in glioma [344], osteosarcoma [345], breast [341], pancreatic [346] and colorectal cancer [347]. The progressive increase in CYR61 expression seen in the bladder cancer TMA progressing from control through to the CIS and tumour [figure 3.58] is in keeping with findings in pancreatic cancer [346], where increased CYR61 expression was identified in precursor lesions. This was the case in the TMA with a highly significant (p<0.0001) increase in CYR61 in CIS, the precursor of invasive tumours [31, 144], compared to normal control. In contrast to the current findings in bladder cancer, loss of CYR61 expression is seen in endometrial cancer [348], in lung cancer is associated with a worse cancer outcome [336] and is associated with an adverse tumour phenotype in melanoma [349]. In prostate cancer the evidence is conflicting, with loss of expression associated with adverse phenotypic features and outcomes [350], but it has also been reported that increased expression is seen in prostate cancer compared to BPH [351] and that serum levels are associated with more advanced stage disease [352].
CYR61 has been shown to regulate EMT [324] in both pancreatic cancer cells [346, 353] and in non-malignant gastric cancer cells [354]. The cytoskeletal intermediate filament protein vimentin is considered a marker of EMT in cancer, with a positive correlation of its expression and increased invasiveness and metastatic activity [355-357]. Vimentin expression is associated with motility and bone metastases in prostate cancer and is a prognostic biomarker in renal cancer [358-360]. CYR61 appears to have an effect upon EMT in the T24 bladder cancer cell line, where knockdown led to loss of vimentin expression [figure 3.27], and a 48% reduction in invasion towards 5%FCS during invasion assays [figure 3.33]. Although it must be noted that the basal expression of vimentin by the T24 cell line was low in comparison to some of the other five cell lines studied [figure 3.23]. Furthermore, the absence of an effect of knockdown on the expression of N-cadherin in the T24 cells, or of either of these markers in the J82 cell line, indicates that the effect of CYR61 in bladder cancer EMT may be minimal. Further work examining the effects of increasing CYR61 expression or direct stimulation with recombinant CYR61, in the more epithelial bladder cancer cell lines (5637 and RT112) is required to confirm the role of CYR61 in the EMT process in bladder cancer.

4.4 Proliferation and chemotherapy resistance

Knockdown of CYR61 had no significant effect on the proliferation of J82 cells and only a modest but significant 30.4% (p=0.0003) reduction in T24 cell proliferation. In combination with 0.5 µg/ml cisplatinum, knockdown had a highly significant effect on cell proliferation in comparison to knockdown alone or with cisplatinum alone. The exact mechanism by which this was occurring has not been determined by the experiments conducted however there is increasing evidence from a number of cancer types of a role for CYR61 in chemotherapy resistance.

It has been shown that vinblastine resistant renal cell carcinoma (RCC) cell lines express higher levels of CYR61 than vinblastine sensitive lines. Inhibition with an anti-CYR61 monoclonal antibody reduced the vinblastine IC_{50} and
resistance could be induced by the expression of CYR61 in vinblastine sensitive RCC cell lines [361]. This study also demonstrated vinblastine resistance following induced expression of CYR61 may be occurring through MDR1 mechanisms, following signalling through αvβ3 integrin and PI3K/Akt. A finding also observed in glioma cells where CYR61 transfection of U343 cells led to increased expression of αvβ1 and αvβ3 integrins and activation of the PI3K/Akt pathway via ILK. This has relevance as MDR1 is increased in chemotherapy resistant bladder tumors [362], associated with a worse outcome following adjuvant chemotherapy for advanced bladder cancer [219] and increased in chemotherapy resistant bladder cancer cell lines [362]. Integrin β1 signalling has been shown to confer resistance to chemotherapy in T24 bladder cancer cells with inhibition reducing the activation of PI3K/Akt mediated anti-apoptotic signalling [363, 364]. Furthermore ILK is known to play an important role in EMT in bladder cancer [365]. This mechanism of action is contrasted by evidence in MCF7 breast cancer cells where αvβ3 integrin inhibition, following forced expression of CYR61, had no effect upon Akt activation but did reduce the increased activity seen in the ERK1/2 MAPK pathway. Irrespective of the absolute pathway involved, forced expression of CYR61 in MCF7 cells significantly increased resistance to paclitaxel [366] with reduced accumulation of wild type p53. CYR61 also promotes resistance to chemotherapy induced apoptosis by up regulation of NF-κB via αvβ3 and αvβ5 integrin signalling in MCF7 breast cancer cells[367].

In OVCAR-3 ovarian cancer cells CYR61 reverses carboplatin induced de-phosphorylation of the PI3K/Akt pathway, with overexpression and knockdown inhibiting and enhancing carboplatin-induced apoptosis respectively. In the same study, transfection with CYR61 increased the expression of and promoter activity of Bcl-2 and NF-κB and reduced the promoter activity of p53 [368]. Transfection of CYR61 also reduces caspase-3 mediated cisplatin induced apoptosis in HeLa cells [369].

Although the exact mechanism by which CYR61 and CTGF promote resistance has not been elucidated, it is postulated that this occurs through intergrin
signaling. CYR61 was also identified as a candidate gene involved in vinblastine resistance in cervical cancer cells by comparative genomic hybridization analysis of multi-drug resistant KB-v1 and their parental KB-3-1 cells. Whilst not mechanistically informative it adds weight to the growing evidence of the importance of CYR61 in chemotherapy resistance [370].

In both the J82 and T24 cell lines knockdown of CTGF had very similar effects upon sensitivity to cisplatin as knockdown of CYR61, although the effect was more profound following CTGF knockdown. Published evidence in previous work suggest that there are similarities between the mechanisms of action of CTGF and CYR61 in relation to chemotherapy resistance. CTGF expression is associated with chemotherapy resistance in osteosarcoma [371], glioblastoma [372] and pancreatic cancer [373]. In osteosarcoma cells it has been shown that resistance to cisplatin induced apoptosis is increased by CTGF, through Bcl-xL and survivin [371]. Over expression in U343 glioblastoma cells also leads to increased expression of Bcl-2, Bcl-xL, survivin and Flip, associated with increased resistance to chemotherapy [372]. In breast cancer [342] a reduction in paclitaxel and doxurubicin induced apoptosis is mediated via increased Bcl-xL and cIAP1 following activation of the integrin αβ3/FAK /ERK1/2 pathway by CTGF [342]. Increased levels of CTGF have also been demonstrated in residual breast tumours following neo-adjuvant chemotherapy when compared to the pre-chemotherapy paired samples [374].

These are plausible mechanisms of chemotherapy resistance in bladder cancer as the Bcl-2 family of anti-apoptotic proteins are known to be deregulated in bladder cancer [375-377]. Bcl-2 expression is associated with shorter time to recurrence of pT1 bladder tumours following TURBT [378] and the expression is higher in lymph node metastases than in primary tumours following cystectomy after neo-adjuvant chemotherapy [379]. Expression of Bcl-2 is also associated with a worse prognosis following neo-adjuvant chemotherapy and radiotherapy [380] for pT2-4 TCC and in patients receiving synchronous chemo-radiotherapy [375]. Knockdown of Bcl-2 and Bcl-xL in a panel of bladder cancer cells including RT112 and T24 significantly increased sensitivity to cisplatinum, mitomycin-c, gemcitabine and vinblastine [381].
It is reasonable to hypothesise based upon the TMA and cell line modelling data on bladder cancer that CYR61 is a promising target for increasing chemosensitivity via the mechanisms described in other cancers. The conclusions regarding CTGF are less clear, given the TMA data.

4.5 Cell motility and invasion

The majority of bladder cancer mortality arises as a consequence of tumour invasion of local structures and metastases formation. The ability of a cancer cell to form metastases is a complex multi-step process, the first stages of which involve cells acquiring motility and invasive properties [382]. The comparison of invasive tumours and non-invasive tumours made during the Oncomine® search for novel targets in bladder cancer was based upon this key step in the pathway to metastasis formation. The acquisition of an invasive phenotype also correlates well with the separate pathways of bladder cancer development that have been proposed based upon observed molecular events[31, 32]. To study motility and invasion in bladder cancer cell was therefore a logical step in the process of evaluating the role of CYR61 and CTGF. The HGF/MET axis is also widely recognised as playing a key role in cancer cell motility and invasion [118]. This is exemplified by the former name of scatter factor that was given to HGF, due to it's observed ability to cause scattering of human breast epithelial cells and Madin Darby Canine Kidney (MDCK) cells [383]. It has also been demonstrated that CYR61 and CTGF are up regulated in response to HGF stimulation. In human primary renal tubular epithelial cells HGF stimulation transiently increases CTGF levels to a peak at 6 hours [290] and in glioma cell lines a similar increase in CYR61 is seen [289]. The possible relationship between CYR61 and CTGF with the HGF/MET axis in bladder cancer cell motility and invasion was therefore studied.
4.5.1 CCN proteins and cell motility / invasion

Following knockdown of CYR61 there was a significant and notable 78.3% and 48.2% reduction in Matrigel® invasion towards 5%FCS in the J82 and T24 cell lines respectively. CTGF knockdown had no significant effect. The effects of knockdown on cell migration during scratch wound assays were less pronounced and inconsistent between the two lines studied [figure3.35].

In glioblastoma cells, increased expression of CTGF increases cell motility [372], although the exact mechanism has not been identified. In the H460 and TE-7 oesophageal cancer cell lines αvβ5 integrin is an important component of the CYR61 mediated cell migratory pathway [384] and in monocytes and lymphocytes rapid actin polymerisation has been demonstrated after stimulation with CYR61 via integrin linked PI3K/Akt and MAPK dependant pathways [385]. The literature suggests that integrin mediated signaling may be critical for CYR61 induced migration. The cytoskeletal reorganisation and cell migration that occur via Rac1 and ERK1/2 pathways after stimulation of MCF7 cells with CYR61 are abrogated by inhibition of αv integrins, although this is not the case for α6 integrins [386]. Oral SCC cell migration and MMP-3 expression, which are increased following CYR61, is mediated via both αvβ3 and α6β1 integrin in a FAK/MAPK dependant pathway [387]. T24 cells are known to highly express α2, α3 and β1 integrins, show moderate expression of αv integrin and are negative for α4 integrin subunit expression [388]. The same study also demonstrated that inhibition of β1 integrin significantly reduced the migration rate of T24 cells in a 3D collagen matrix. It must be noted however that the pattern of expression of integrins in human bladder cancer tissue is very different from the expression patterns seen in cell lines derived from invasive cancers [389], presumed to be due to adaptive changes following in vitro culture. The α2, α3 and α6 integrins are associated with NMIBC tumour recurrence and β4 integrin is an independent predictor of recurrence in multivariate analysis [390]. β4 integrin is also highly expressed in invasive tumours [389] and α6β4 has been shown to be associated with bladder cancer survival [391].
The literature described outlines the potential role of integrins in mediating the response to CYR61 in MIBC. Further work will be required to examine these putative links and to define the specific integrins that are involved.

**4.5.2 HGF/MET interactions with the CCN proteins**

In the cell line experiments, an increase in CTGF expression following HGF stimulation was seen in all of the cell lines except the HT1376 cell line, which had a high basal level of CTGF expression. Increases in CYR61 expression were seen following HGF stimulation in two of the cell lines, but not in either J82 or T24 cells. CYR61 and CTGF knockdown abrogated Matrigel® invasion in response to 10ng/ml HGF in both cell lines [figure 3.32]. However, knockdown of MET did not have a significant effect upon the invasive response of either the J82 or T24 cell lines in response to FCS.

The role of CYR61 in HGF mediated cellular migration and invasion has previously been demonstrated to occur via modulation of PI3K/Akt signalling. In the U373 glioma cell line, sustained phosphorylation of Akt beyond 2 hours post HGF stimulation is abolished by knockdown of CYR61, although the early peak in phosphorylation at 30 minutes remains unchanged, leading to abrogation of cellular migration [289]. Changes in CYR61 expression following HGF stimulation in the bladder cancer cell lines were lacking, with a small but definite increase in CYR61 expression observed only in two bladder cell lines, the UMUC3 and the non-invasive RT112 cell line [figure 3.24]. Further experimentation on these two cell lines was not conducted, as the basal level of invasion in response to both HGF and FCS was low [figure 3.19]. It is therefore very difficult to directly translate the observations seen in glioma to bladder cancer based upon the data obtained. However, CYR61 knockdown did abrogate HGF induced Matrigel® invasion and cause a 20% and 17% reduction in J82 and T24 cell migration respectively in the scratch wound assays following HGF stimulation. These findings in the bladder cancer cell lines indicate an important role for CYR61 in modulating the migratory and invasive response to
HGF, although whether this is exerted through modulation of PI3K/Akt signalling is an area for further investigation.

The interaction between HGF and integrins may explain how CTGF and CYR61 modulate HGF/MET induced bladder cancer cell invasion; based upon observations of the interactions between integrin and MET signalling. Cross talk between integrins and HGF/MET has been described in three processes, reviewed by Chan et al.,[392]. Firstly direct interaction between integrins and MET occurs at a membrane level, in which integrins containing a β4 subunit provide an increased number of Src binding sites, which are required to promote the invasive cell response seen following MET activation. The second mechanism is based upon the convergence of both pathways via common proteins, most notably FAK, which as previously described [342, 387] can be activated by CYR61 and CTGF. This is of particular importance as FAK/MET interactions play an important role in mediating HGF/MET induced migration and Matrigel® invasion in human lung cancer and MDCK cells [393]. The third proposed mechanism is the increased expression of integrins that occurs following sustained activation of MAPK in response to MET activation.

The cell line and TMA data produced in this project indicates a putative role for CYR61 targeting therapy, although how this relates to the HGF/MET axis in the clinical setting remains uncertain. Whilst no in vivo data was generated from this project it is possible to infer from the MET expression data across the TMA, and the work of Kluth et al. [134] (the only other large series on MET expression in bladder cancer), that targeting MET in bladder cancer may not yield significant benefit. The use of MET targeting therapy is currently being extensively investigated in non-small cell lung cancer (NSCLC) [394], where it has been established that both gene copy number alteration and protein expression are significantly associated with poorer survival [395]. In gastric cancer a recent meta-analysis has demonstrated a significant correlation between reduced survival and both MET gene amplification and protein expression [396]. These findings add further weight to the assertion that targeting MET in bladder cancer may not be of appreciable clinical benefit, as the findings of Kluth et al. also
indicate a low rate of MET gene copy number alterations at 0.8% in bladder cancer.

4.6 Future direction

The therapeutic direction in the CCN group of proteins has been towards the development of monoclonal antibody based therapies. A novel therapeutic monoclonal antibody produced by a Chinese research laboratory has shown efficacy in a mouse model of breast cancer in reducing tumour xenograft proliferation and metastases formation [397]. This is the only publication describing the in-vivo use of a CYR61 targeting therapy, despite over a decade of research indicating the potential role of CYR61 in cancer therapeutics. The situation with CTGF is far more advanced with a number of phase 1 and phase 2 clinical trials underway, or complete, in a number of inflammatory diseases. [325]. A commercially available monoclonal anti-body targeting CTGF (FG-3019) has been shown to significantly reduce tumour growth and metastases formation in mouse xenograft models of CTGF expressing pancreatic cancer [398, 399] with no observed toxicity. The first human phase 1 trial of FG-3019, in combination with gemcitabine and erlotinib, in locally advanced or metastatic pancreatic cancer has completed recruitment and is due to report in early 2015 (www.clinicaltrials.gov NCT01181245). However, some have cautioned regarding the development of therapies targeting the CCN group of proteins [400], citing that CYR61 has been shown to play a critical role in both prostate cancer proliferation and apoptosis [401], indicating that under certain conditions targeting CYR61 may produce an effect opposite the desired clinical response.

The in vitro findings of this current project indicate that targeting both CYR61 and CTGF may yield a therapeutic response in the treatment of bladder cancer, based upon their putative role in cell proliferation, cellular invasive potential and in chemo-sensitisation. The TMA data paints a slightly different picture, with the expression patterns of CYR61 and CTGF diverging. Whilst this data requires validation in an external human tissue cohort, these initial results indicate that
targeting CYR61, alone and combination with chemotherapeutic agents, may be of therapeutic benefit. The role for CTGF appears less convincing.

Irrespective of the therapeutic targeting of CCN proteins in cancer, they have the potential to be used as urine, serum or tissue based biomarkers in cancer prognostication, prediction of treatment response and follow up. There are numerous studies reporting serum and urine CTGF levels in inflammatory diseases including liver and pulmonary fibrosis [402, 403] with notable differences in serum CTGF levels between patients with liver fibrosis and hepatocellular carcinoma due to hepatitis-B [402]. A much smaller number of reports describe the use of CYR61 as a serum biomarkers, although serum levels of CYR61 are predictive of metastasis and recurrence in gastric cancer [404] and of non-organ confined disease in prostate cancer [352].

In bladder cancer there are a number of biomarkers currently undergoing evaluation for the prediction of chemotherapy [405-408] and radiotherapy [198, 409, 410] response, with promising results. The role for CYR61 as a biomarker of chemotherapy response could be validated in a cohort of patients receiving chemotherapy. The group of patients in whom this analysis seems most logical are those receiving neo-adjuvant chemotherapy prior to radical treatment. Tissue for analysis could be obtained at their initial TURBT with response to chemotherapy assessed both radiologically, on pre and post chemotherapy cross-sectional imaging, and pathologically post cystectomy in those undergoing this radical modality. This cohort would also provide an excellent opportunity to study the role of CYR61 as a urine or serum based marker of treatment response.
4.7 Conclusions

This project has successfully produced a bladder cancer tissue microarray from cystectomy patients and validated the use of automated image analysis in conjunction with immuno-histochemistry to quantify protein expression. In conjunction with robust cell line modelling of bladder cancer the TMA has evaluated the role of the novel putative targets CYR61 and CTGF. This has defined CYR61 as a promising target and potential biomarker in the treatment of bladder cancer, most promisingly in conjunction with cisplatinum based chemotherapy. It has also indicated that MET and CTGF may be of limited value as treatment targets.

The IHC expression data upon which these conclusions are drawn require validation in an independent cohort and the cell line modelling expanding to gain a deeper understanding of the mechanisms by which CYR61 is exerting its effects upon bladder cancer behaviour. It is only possible to infer putative mechanisms based upon observations in other cell types. It seems likely, based upon published evidence in other cancers, that CYR61 and CTGF exert their effects though the modulation of other signalling pathways mediated by integrins, in response to cell-cell and cell-matrix interactions.

Further work both in vitro and in vivo is required to define the role of CYR61 as a biomarker and treatment target in bladder cancer.
5 References


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## Appendix 1

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<td>49</td>
<td>CTSK</td>
<td>Coding</td>
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<td>NEK6</td>
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<td>NIMA related kinsae 6</td>
</tr>
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Table 6.1 – Top 50 ranked overexpressed genes comparing invasive to non-invasive bladder cancer from Oncomine™