EVALUATING THE ROLE OF THE RECEPTOR TYROSINE KINASE AXL IN BLADDER CANCER

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By

James Edward Dyer

Department of Cancer Studies
University of Leicester

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Abstract

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James E. Dyer

Introduction & Objectives

There is mounting evidence that expression of the receptor tyrosine kinase, AXL, correlates with poor prognosis in many human cancers. We aimed to evaluate this association in bladder cancer and to examine the *in vitro* effect of foretinib, an oral inhibitor of VEGF, c-MET and AXL, in bladder cancer cell lines.

Material & Methods

Immunohistochemistry was performed on 65 bladder cancer specimens collected from TURBT prior to radical cystectomy (series 1), in addition to a TMA (n=284) comprising bladder cancers of differing stages and grades encompassing both NMIBC and MIBC (series 2). AXL expression was correlated with pathological stage, grade, subsequent need for radical treatment in NMIBC and survival. We used Western blotting and densitometry to evaluate the effects of foretinib on the phosphorylation of signalling molecules downstream of AXL, namely MEK, AKT and MAPK in J82 bladder cancer cells.

Results

AXL immunopositivity was associated with invasion in both patient series. In patients undergoing cystectomy, AXL positivity was associated with pre-operatively unsuspected lymph node metastasis. Furthermore, in patients with NMIBC in series 2, AXL expression was associated with an eventual need for cystectomy and with reduced disease-specific survival.

Incubation with foretinib (10nM) produced a significant reduction in phosphorylation of MEK and AKT but not MAPK in serum starved J82 cells following stimulation with GAS6, the ligand for AXL.

Conclusions

We provide evidence that AXL expression is aligned with the phenotypes of bladder cancers associated with poor outcomes and that AXL signalling can be abrogated with foretinib. Taken together this supports the continued study of AXL inhibitors in bladder cancer.

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Chapter 1: Introduction

Bladder cancer: Clinical perspective

Epidemiology and pathology of bladder cancer

In the UK, carcinoma of the bladder was the fourth and fourteenth most common new cancer diagnosis amongst men and women respectively in 2014. In England, there were 8514 new diagnosis giving an annual incidence of 23.3 per 100, 000 in men and 8.2 per 100, 000 in women (1). The vast majority of these cancers (90% in the UK) are Urothelial Carcinomas (UC) termed so as they arise from the urothelium. Whilst the majority (90%) of UC have little morphological variation some rare variants do exist. These are important as they confer reduced survival and so can influence prognosis and treatment decisions. Rare morphological variants of UC, include micropapillary UC, UC with squamous differentiation, nested carcinoma, small cell carcinomas and spindle cell carcinomas. Other forms of bladder cancer include squamous cell carcinoma (10%), adenocarcinoma (<1%).

Historically squamous cell carcinoma of the bladder had predominated over UC in regions such as North Africa, owing to endemic schistosomiasis, a known risk factor for the disease. Current evidence, however, suggests that following advances in schistosomiasis treatment and a relative increase in cigarette smoking exposure, UC now predominates worldwide (2). UC will form the subsequent focus of this thesis and where the specific subset of bladder cancer is not given this should be interpreted as referring to UC.

Economics of bladder cancer

In the only published cost analysis of bladder cancer in the UK, Sangar et al. calculated the cost to the NHS of patients diagnosed with bladder cancer in 2001 at £55.39m over five years. This equates to £8349 per patient (3). The majority of this spending was on patients diagnosed with non-muscle-invasive bladder cancer (NMIBC) rather than muscle-invasive bladder cancer (MIBC). In this study the cost of all diagnostic tests, treatment and follow up was combined with estimates of cost owing to loss of earnings and economic productivity. The authors identify that the majority of cost is incurred as a result of the currently mandated frequent cystoscopic follow up for both NMIBC and MIBC. It follows, therefore, that treatments that can reduce recurrence or identify patients who will fail standard therapy in NMIBC earlier will have significant economic benefit.

Trends in UK incidence, mortality and survival

Bladder cancer incidence increases with age; the majority of new diagnoses are made in patients above 65 years (figure 1). Bladder cancer incidence has, however, fallen since the mid 1970s (figure 2 and 3). This observed decline is likely to be contributed to by a reduction in the number of cases related to occupational carcinogen exposure and a reduction in cigarette smoking. These

factors also dictate why there has been a more accelerated decline in incidence in males compared to females. Government legislation in the 1950s and 60s banned the use of the aromatic amines 2-napthylamine and benzidine in the manufacture of azo dyes. Manual work in such industries was traditionally male dominated, hence the observed gender-specific impact on the withdrawal of these chemicals. The lag between such legislation and decline in incidence is attributed to the 20 to 40-year delay between exposure and clinically manifest disease. Mortality from bladder cancer mirrors incidence in so far as it increases with age, rising rapidly from 65 years onward. Similarly, there has been a downward trend since the 1970s, which has been most pronounced in males.

Although bladder cancer is more common in males, survival, standardised for age and stage, is better in comparison to females (figure 4). The largest published database interrogation suggests that the stage-controlled survival difference is most significant in women over 70 years old (4). The precise underlying mechanism, or mechanisms, responsible for the observed differential in bladder cancer survival between men and women is yet to be adequately explained. Whilst delayed presentation in women with bladder cancer can be explained by misattribution of presenting symptoms common to other benign causes, this would not explain why several studies have observed a reduced survival, when controlling for stage at presentation, in women (5,6). Potential explanations

include a protective role of the androgen receptor/signalling in bladder cancer, or a preponderance of aggressive pathological subtypes of MIBC (7).

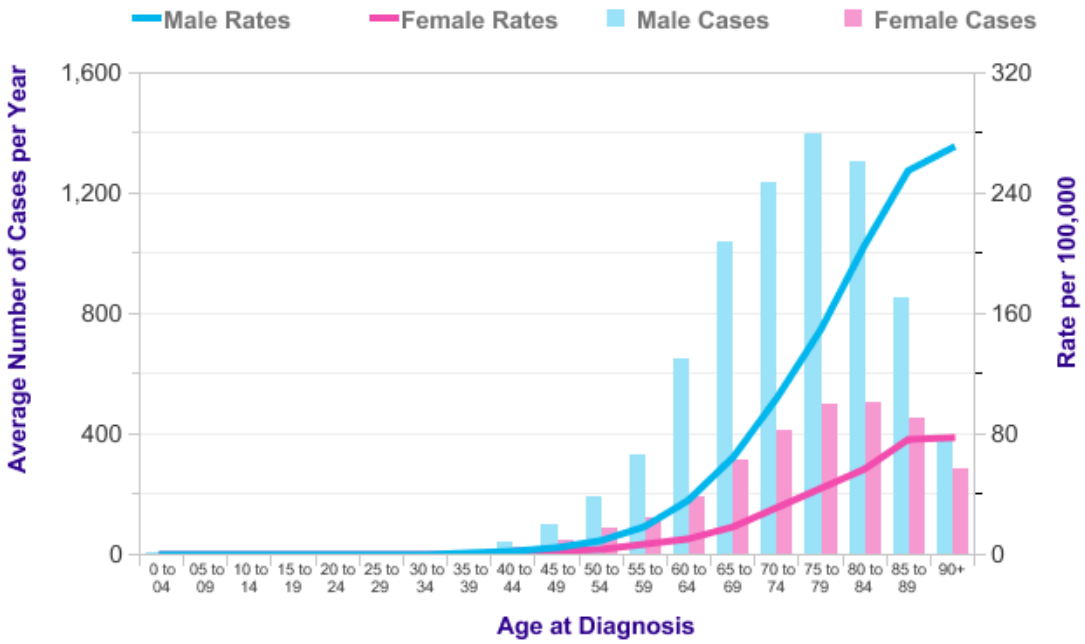

Figure 1. Average number of new cases per year and age specific incidence rates per 100,000 population, UK 2011-2013. http://www.cancerresearchuk.org/cancerinfo/cancerstats/types/bladder/incidence/ last accessed June 2016

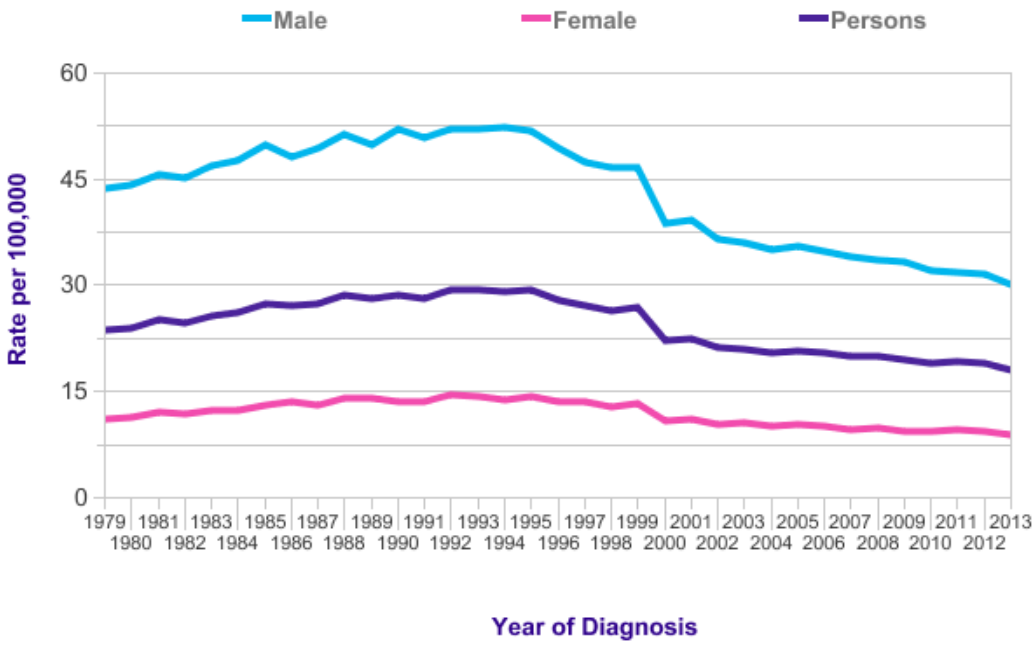

Figure 2. European age-standardised incidence rates per 100,00 population by gender in Great Britain 1979-2013

http://www.cancerresearchuk.org/cancerinfo/cancerstats/types/bladder/incidence/ last accessed June 2016

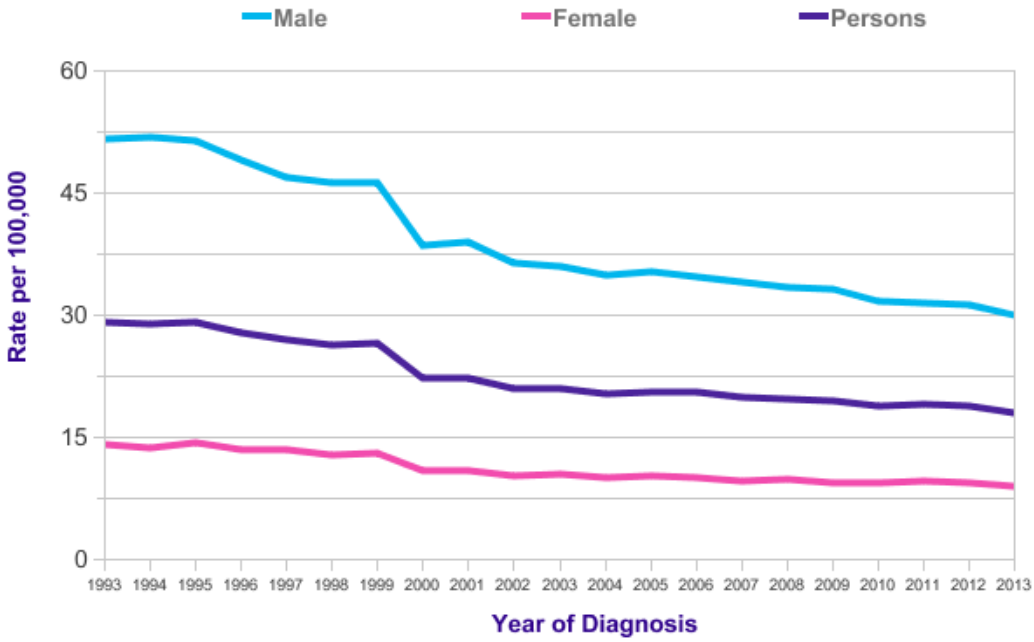

Figure 3. European age-standardised incidence rates per 100,000 population, UK, 1993-2013.

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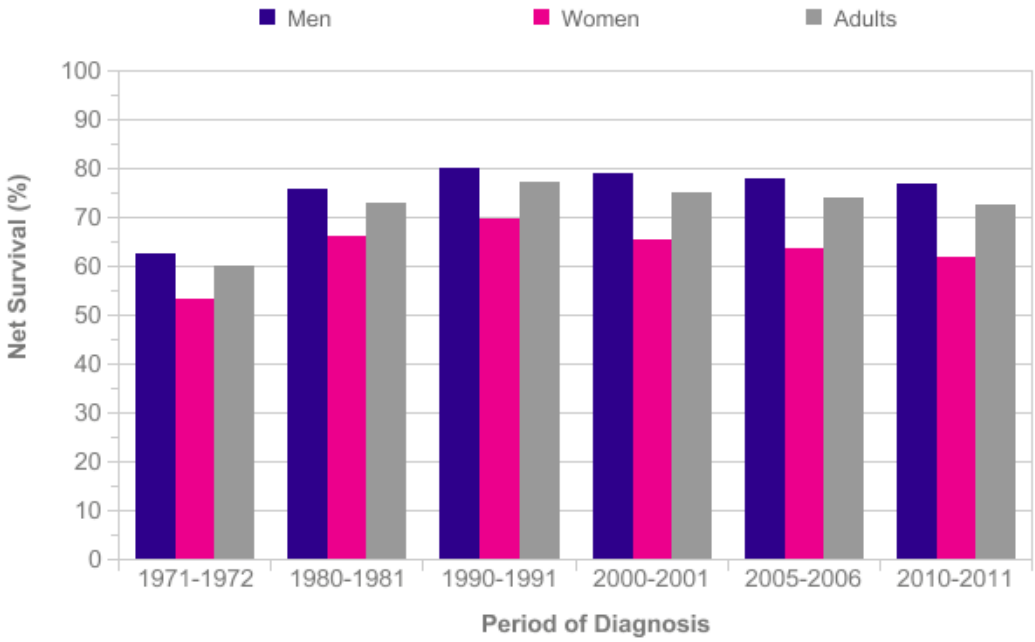


Figure 4. Age-standardised one-year net survival rates, England and Wales 1971-1995, England 1971-2011

http://www.cancerresearchuk.org/cancerinfo/cancerstats/types/bladder/incidence/ last accessed June 2016

The staging and grading of TCC of the bladder

The worldwide convention for bladder cancer staging is to use the TNM classification system (8-10) (table 1).

Table 1. TNM staging of bladder cancer. Taken from Sobin et al (11)

T - Primary Tumour	
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Ta	Non-invasive papillary carcinoma
Tis	Carcinoma *in situ*: "flat tumour"
T1	Tumour invades subepithelial connective tissue
T2	Tumour invades muscle
	T2a Tumour invades superficial muscle (inner half)
	T2b Tumour invades deep muscle (outer half)
T3	Tumour invades perivesical tissue:
	T3a microscopically
	T3b macroscopically (extravesical mass)
T4	Tumour invades any of the following: prostate stroma, seminal vesicles, uterus, vagina, pelvic wall, abdominal wall
	T4a Tumour invades prostate stroma, seminal vesicles, uterus, or vagina
	T4b Tumour invades pelvic wall or abdominal wall
N - Regional Lymph Nodes	
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph-node metastasis
N1	Metastasis in a single lymph node in the true pelvis (hypogastric, obturator, external iliac, or presacral)
N2	Metastasis in multiple lymph nodes in the true pelvis (hypogastric, obturator, external iliac, or presacral)
N3	Metastasis in common iliac lymph node(s)
M - Distant Metastasis	
M0	No distant metastasis
M1	Distant metastasis

At initial diagnosis, most (75%-85%) urothelial cancers are non-muscle-invasive (NMIBC) (12) comprising pTa, pT1 and Carcinoma in situ, (CIS) lesions. The remainder are muscle-invasive (MIBC) that are either still confined to the muscle layer (pT2) or have already advanced, either locally (pT3, pT4), regionally (N1, N2) or with distant metastasis (M1). Traditionally, bladder cancer grade was categorised on a scale of G1-G3, with Grade 3 disease representing the most poorly differentiated disease. The World Health Organisation (2004) suggested an alternative grading system comprising Papillary Urothelial Neoplasia of Low Malignant Potential (PUNLMP), low-grade and high-grade disease (13) (figure 5). Histopathological standards are to report both grading systems for each specimen,

indicating that no widespread consensus has been reached in determining which is more useful. As many specimens used in this thesis predate this reporting method all grading will be reported as grade 1 to 3.

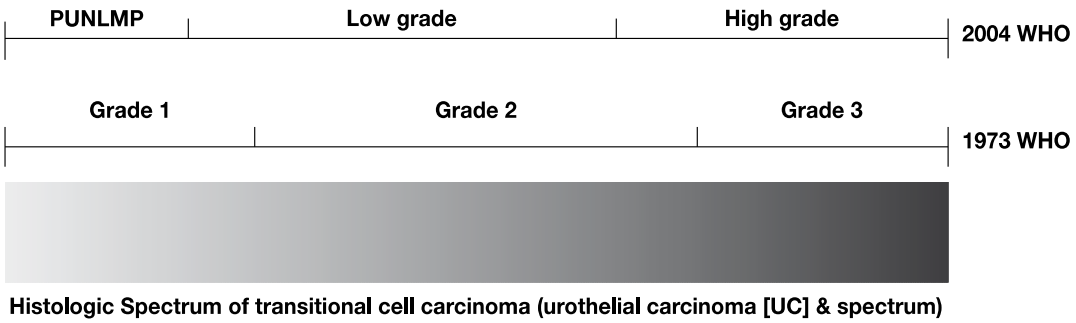


Figure 5. Current and historical grading systems for bladder cancer. Taken from Maclennan et al (13).

Assessment and management of NMIBC

The cornerstone of NMIBC assessment is accurate histological diagnosis, allowing confirmation of the histological subtype in addition to staging and grading the disease. This is most frequently achieved through transurethral resection of bladder tumour (TURBT). Tumours are endoscopically resected from the bladder in piecemeal fashion, the harvested tissue then being fixed in formalin prior to processing.

Broadly speaking, NMIBC has a divergent prognosis and tends to fall into one of three categories, those that do not recur, those that recur as further NMIBC and those that progress to MIBC. Five-year recurrence rates vary between 31-78% and 5-year progression rates varies between 0.8-45%. Risk factors for both recurrence and progression have been elucidated by multivariate analysis. The presence of high-grade (G3) lesions, concomitant CIS and pT1 disease predict progression whereas multiplicity, previous recurrence and size of lesions predict recurrence (table 2 and 3, 14-16).

Table 2. Scores assigned to risk factors for recurrence and progression. Taken from Sylvester et al (14)

Factor	Recurrence	Progression
Number of tumours		
Single	0	3
2 to 7	3	3
≥8	6	3
Tumour size		
<3cm	0	0
≥3cm	3	3
Prior recurrence rate		
Primary	0	0
≤1 recurrence/year	2	2
>1 recurrence/year	4	2
T category		
Ta	0	0
T1	1	4
CIS		
No	0	0
Yes	1	6
Grade		
G1	0	0
G2	1	0
G3	2	5
Total Score	0-17	0-23

Table 3. Probability of recurrence and progression of NMIBC according to score. Taken from Sylvester et al (14,10)

Recurrence	Probability of recurrence at 1 years	Probability of recurrence at 5 years
0	15% (10%, 19%)	31% (24%, 37%)
1-4	24% (21%, 26%)	46% (42%, 49%)
5-9	38% (35%, 41%)	62% (58%, 65%)
10-17	61% (55%, 67%)	78% (73%, 84%)
Progression	Probability of progression 1 year	Probability of progression 5 years
0	0.2% (0%, 0.7%)	0.8% (0%, 1.7%)
2-6	1.0% (0.4%, 1.6%)	6% (5%, 8%)
7-13	5% (4%, 7%)	17% (14%, 20%)
14-23	17% (10%, 24%)	45% (35%, 55%)

It is now standard practice for all patients undergoing TURBT, for a suspected new diagnosis of bladder cancer, to have a single dose of intravesical chemotherapy immediately after TURBT (17). This is thought to eradicate tumour cells shed during resection and prevent subsequent re-implantation in addition to treating any tumours missed intraoperatively (18-20). In several studies, and indeed in a meta-analysis, intravesical chemotherapy results in a significant reduction of 38% at one year in recurrence of NMIBC compared to no treatment (21,22).

Following a diagnosis of NMIBC, and using the variables listed above patients are allocated to low, intermediate or high-risk groups (table 4).

Table 4. Categorisation of low, intermediate and high risk NMIBC. Taken from Nice guidelines NG2: Bladder cancer: diagnosis and management (17)

Low risk NMIBC	**Intermediate risk NMIBC**	**High risk NMIBC**
Solitary pTaG1<3cm	Solitary pTaG1>3cm	pTaG3
Solitary pTaG2 (low grade) <3cm	Multifocal pTaG1	pT1G2
Any PUNLMP	Solitary pTaG2 (low grade) >3cm	pT1G3
	Multifocal pTaG2 (low grade)	CIS
	pTaG2 (high grade)	Any morphological variants
	Any low risk recurring within 12 months	

Those with low risk of progression and recurrence often undergo a period of cystoscopic surveillance with no further treatment. Those in the intermediate-risk and high-risk groups often undergo further intravesical therapy depending on the predominant risk, be it progression or recurrence. If recurrence is the greater concern then further instillations of chemotherapy, over a one-year period, have been shown to improve recurrence rate per year by 62% (23). If, however,

progression is the principal concern, then immunotherapy, with intravesical Bacillus Calmette Guerin (BCG) is the preferred option. Although the precise mechanism of action remains unknown, BCG appears to induce an inflammatory response within the bladder, which culminates in an anti-cancer effect. In meta-analysis of clinical trials, BCG therapy reduced the rate of progression to MIBC from 13.8% to 9.8% in intermediate-risk and high-risk NMIBC patients over a median follow-up period of 2.5 years (23,24). Furthermore, BCG also has greater efficacy in reducing the risk of recurrence when compared with intravesical chemotherapy. A meta-analysis of nine randomised controlled trials reported a 32% reduction in risk of recurrence when compared with mitomycin (25). However, the side effect profile of BCG is much less favourable than chemotherapy, therefore, BCG is reserved for patients in whom the risk of progression predominates or recurrence has occurred despite chemotherapy.

Where NMIBC progresses to MIBC despite BCG therapy, and major surgery is possible, radical cystectomy, surgical removal of the bladder, should be offered. It has been shown in a systematic review of 19 studies, that patients who fail BCG treatment, defined as either recurrence within 9 months of BCG commencement or muscle-invasive recurrence, which is followed by cystectomy have a cancer-specific survival of 32%, which is inferior to those with *de novo* MIBC who undergo radical treatment (26,27). This group of patients therefore represent an important challenge in developing therapies that can prevent or defer both recurrence and progression.

Occasionally, and most often in younger patients, the lifelong risk of progression may be so high that radical cystectomy is mandated as a primary treatment.

Future perspectives in NMIBC treatment

Treatment options for patients who fail BCG and are unwilling or unfit for cystectomy are limited. In this scenario, newer treatments aim to deliver standard therapeutic agents more effectively. Two techniques to achieve this are electromotive drug administration (EMDA) and chemohyperthermia. EMDA enhances mitomycin absorption by creating an electrical gradient across the urothelium and abdominal wall by means of electrodes both in a urethral catheter and placed externally on the patient. Chemohyperthermia describes the combination of intravesical chemotherapy and intravesical hyperthermia. Hyperthermia renders the urothelium more permeable to mitomycin and enhances its effect. The HYMN trial is a phase 3 randomised controlled trial comparing hyperthermia and mitomycin with further BCG treatment in patients with BCG failure (28). The results however, are awaited.

Assessment and management of MIBC

If a diagnosis of muscle invasive bladder cancer is established following TURBT, then this alone or in conjunction with other intravesical therapies is not considered sufficient to induce cure. Further radiological tests (MRI/CT) are required to determine the suitability for further radical treatment. Specific consideration is given to concurrent upper tract tumours, local stage, nodal status and distant metastasis.

In the setting of localised disease, standard radical treatment takes the form of either cystectomy or radiotherapy. Cystectomy can then be further categorised, dependent upon the urinary diversion strategy, into ileal conduit, orthotopic or heterotopic neobladder. Radical cystectomy is the standard treatment for localised MIBC (T2-T4a, N0, M0) worldwide (29, 30) with 5-year recurrence free and cancer-specific survival of 58% and 66% respectively (31, 32). During radical cystectomy, in addition to removal of the bladder, pelvic lymph nodes are removed, to allow removal of unsuspected metastasis and also to facilitate accurate staging. Unfortunately, despite no prior radiological evidence of nodal metastasis, in up to 25% of patients undergoing radical cystectomy, harvested lymph nodes contain metastatic bladder cancer (N1). This is an important prognostic finding and has been shown to be associated with reduced survival, regardless of the bladder pathological stage, in comparison to node-negative

patients (32). In one series of 1054 patients, the 5-year recurrence free survival of patients with node-positive disease was 35% in comparison to 50-91% (across different stages of bladder primary tumour) of node-negative cancers.
External beam radiotherapy (EBRT) has been reported to yield similar 5-year overall survival of 21-68% and cancer-specific survival of 48-75% (33-42).
Several randomised control trials have failed to demonstrate a survival advantage with surgery over radiation therapy for MIBC. In five studies included in the most recent guidance issued by the National Institute for Health and Care Excellence (NICE) no significant difference was found in disease specific survival following either surgery or radiotherapy (17). NICE recognises that the overall quality of evidence is low and one potential confounding factor is discrepancy between pathological and clinical disease stage. In a large multicentre study, clinical staging, via a combination of TURBT histology and radiological findings underestimated the local stage bladder cancers, when compared with cystectomy specimens in up to 50% of patients (43). It follows, therefore, that those patients selected for radiotherapy may have under-staged disease which will clearly skew subsequent clinical correlation and interpretation of radiotherapy efficacy. On-the-other-hand, cystectomy allows accurate pathological staging of MIBC and, therefore, accurate subsequent clinical correlation. Radiotherapy is usually delivered in one of two regimes, these include 52.5-55 Gy in 20 fractions over 4 weeks and 64Gy in 32 fractions over 6.5 weeks. There have been recent advances in strategies to enhance the effect of radiotherapy with radiosensitisors or concurrent chemoradiotherapy. Radiosensitisation with carbogen and

nicotinamide has been shown to improve absolute 3-year survival by 13% (from 46% to 59%) when compared to radiotherapy alone (44). Concurrent chemoradiotherapy with 5-fluorouracil (5-FU) and mitomicin-C has been shown in a randomised control trial to improve loco regional disease free survival (45). No statistically significant difference in overall 5-year survival was observed. However, this is likely to have been contributed to by different salvage cystectomy rates between the radiotherapy alone and chemoradiotherapy groups. Gemcitabine has also been shown, in phase 2 trials, to improve loco regional recurrence rates when compared to radiotherapy alone (46).

At present, NICE recommends that, following a diagnosis of MIBC, all patients should be considered for both radical cystectomy and radiation therapy (17). In practice there are often patient related factors which determine the most appropriate treatment strategy.

Historically, systemic chemotherapy had been reserved for patients with metastasis at diagnosis or those who develop recurrence after radical treatment. Since the 1980s the use of neoadjuvant chemotherapy has been explored. This is offers advantages in terms of delivering chemotherapy when micrometastatic burden is lower and allows an assessment of in vivo cancer sensitivity. Furthermore, patient fitness is better prior to radical therapy than after it. Although there is no definite consensus on the most effective chemotherapeutic regime, meta-analysis of clinical trials have shown that cisplatin in combination

with other agents results in meaningful benefit. Such strategies result in absolute values of 5-year survival advantages of 5-11% (47-49). Regimes included in the above analysis include methotrexate, vinblastine, adriamycin (doxorubicin) and cisplatin (MVAC) each two weeks apart, cisplatin/5-FU (5 Fluorouracil), CMV, CM (cyclophosphamaide and methotrexate) and carboMV (carboplatin, methotrexate and vinblastine). In the UK, the standard neoadjuvant chemotherapy agents are gemcitabine and cisplatin (GC). The class and mechanism of actions of these classical chemotherapy agents is listed in table 5.

Table 5. Commonly used chemotherapeutic agents used in the treatment of bladder cancer and their mode of action.

Agent	Class	Mechanism of Action
Methotrexate	Antimetabolite	Dihydrofolate reductase inhibition leading to reduced biosynthesis of thymidylic acid, methionine, serine, adenine and guanine. This results in reduced DNA, RNA and protein synthesis and cell death.
Vincristine	Microtubule inhibitor	Block mitosis in metaphase resulting in cell death
Adriamycin	Anthracycline antibiotic	Three major actions: 1. Non-specific binding between base pairs and sugar-phosphate backbone causing DNA uncoiling and preventing DNA, RNA and protein synthesis. 2. Binding to cell membranes and interrupting transport processes. 3. Generation of oxygen radicals.
Cisplatin **Carboplatin**	Platinum coordination complex	Forms inter-DNA and intra-DNA strand cross resulting in inhibition of DNA and RNA synthesis.
5-FU	Antimetabolite	Pyrimidine analogue which prevents conversion of deoxyuridylic acid to thymidylic acid, an essential precursor for DNA synthesis.
Gemcytabine	Nucleoside analogue	Inhibition of ribonucleotide reductase leading to inhibited DNA synthesis.

Management of metastatic MIBC

Approximately 10-15% of patients presenting with MIBC will have metastasis at the time of diagnosis. Furthermore, of those with pre-operatively unsuspected metastasis, 25% will be found to have nodal metastasis on pathological staging following cystectomy (30).

Up to 30% of patients relapse with distant metastasis after radical cystectomy. The use of chemotherapeutic regimes listed above, MVAC or Gemcitabine/Cisplatin (GC) can improve median survival from 3-6 months to 14-15 months (50, 51). At present, there is no consensus on second line therapy in patients with metastatic TCC and as such many treatments in this setting are offered as part of registered clinical trials.

Models of bladder cancer development

Currently available guidelines for the management of bladder cancer revolve around histopathological classification. Although this is necessary there is a divergent natural history of bladder cancers with seemingly identical histological appearance. Greater understanding of the molecular pathways that underpin the development and progression of bladder cancers will not only improve

comprehension of the biology and prognostication of patients with the disease but also lead to the development of new therapies.

For many years, a two pathway model for tumour development has been proposed, in which the majority of tumours develop either through hyperplasia, to form papillary, low grade Ta lesions or dysplasia, through CIS to T1 disease and eventually muscle invasive disease (52). In this two pathway model, the molecular alterations implicit in both arms have become increasingly well understood. In the development of low-grade papillary lesions, mutations in FGFR3, resulting in RAS|MAPK activation and changes in PI3K pathway, resulting in AKT activation are common. In the development of muscle invasive disease, loss of tumour suppressor genes P53, Rb and PTEN are common features, which result in genomic instability.

This model has informed us of the biology and natural history of the majority of bladder cancers. However, both high-grade (G3) pTa and pT1 lesions do not fit well into this model as they have the potential to develop muscle invasion despite an initial papillary phenotype. These lesions could arise from either increase in grade from low-grade tumours or from dysplasia and CIS. The latter is supported by recent evidence that suggests muscle-invasive lesions can arise from different heritage, with a significant proportion of tumours exhibiting FGFR3 mutations characteristic of NMIBC (52). Furthermore, recent advances in the understanding of different molecular subtypes of bladder cancer perhaps provides further

explanation of divergent natural history of the same pathological subtypes of bladder cancer. To accommodate these advances an enhanced two pathway model has been proposed by Knowles et al. 2015 (53) (figure 6).

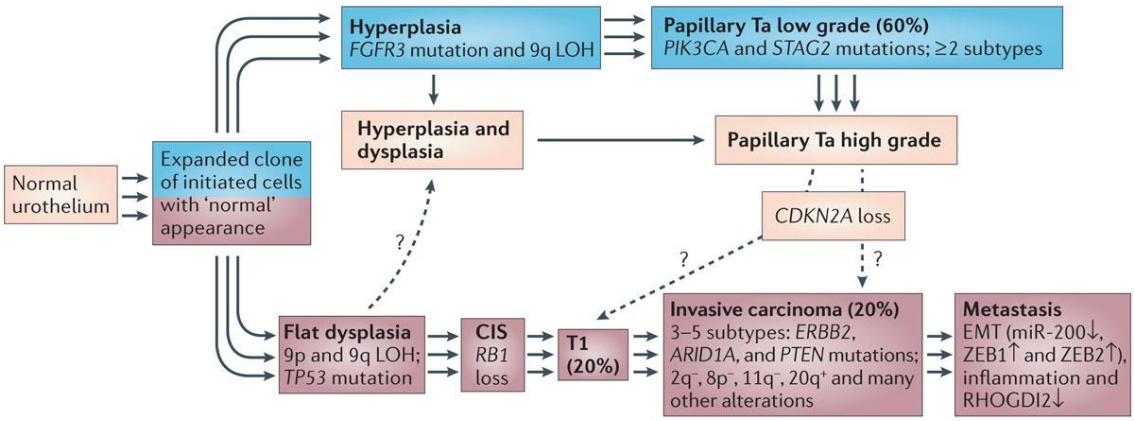


Figure 6. Enhanced two pathway model of the development of bladder cancer. Taken from Knowles et al. 2015 (53).

Gene expression profiling using microarray analysis has been used to categorise bladder cancers and has been shown to correlate with stage, grade and prognosis. Blaveri et al. (2002) constructed a 25-gene signature which accurately predicted tumour stage and poor prognosis (defined as <18 months survival) in patients with MIBC (54). Dyrkjot et al (2005) constructed a 45-gene signature of disease progression from NMIBC (55). This signature had a high positive predictive value for progression but a low negative predictive value. This, however, was probably due to a variety of treatments being used in patients in the non-progressing cohort. More recently several groups have conducted in depth analysis on the molecular classification of muscle invasive bladder cancers, based on mRNA expression and

whole exome sequencing, with the aim of unravelling the heterogeneity in clinical behaviour (56,58). This analysis has lead to the concept of different molecular subtypes of MIBC.

Damraurer et al characterized MIBC in basal and luminal subtypes which show molecular similarity to breast cancer basal and luminal subtypes (56). A seminal publication from The Cancer Genome Atlas (TCGA) group suggested 4 subtypes (I-IV) in which cluster 1 expressed luminal markers (58). Choi et al described luminal, p53 like and basal subtypes (57). Both the luminal and p53-like subtypes contained luminal markers. In studies using a luminal subtype (56,57), tumours expressed markers of differentiation, frequently had papillary morphology and also FGFR3 upregulation and mutation. Overall this may suggest an alternative lineage, where MIBC occurs as a result of previous NMIBC rather than progression from CIS. All studies had tumours expressing basal markers and in these studies those with basal markers had a worse prognosis than tumours in other categories. Further analysis of the tumours from the TCGA cohort has evaluated the overlap of the three main molecular categories (figure 7). In view of the molecular heterogeneity amongst MIBC, the authors suggest at least 3 categories should be used in any future analysis.

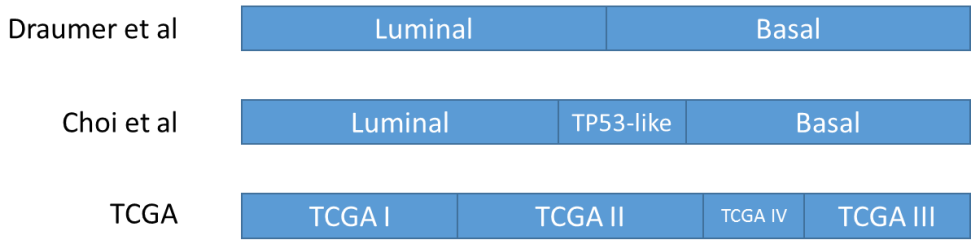


Figure 7. Schematic representation of the overlap between different molecular classification systems of MIBC. (TCGA – The Cancer Genome Atlas)

Debate remains as to how to integrate this information with conventional staging and grading as some reports suggest that some transcriptional subtypes are independent of conventional histological strategies (59). Although conventional pathological categorisation does not reliably prognosticate for some disease subtypes, it is clearly informative to a certain extent. As such, it is likely that such molecular subtyping will be additive rather than replace conventional pathological categorisation.

The TCGA group have also conducted DNA based genome wide analysis on bladder tumours. Information on copy number alterations and mutations has lead to the identification of three pathways, frequently dysregulated in bladder cancer. These include cell cycle regulation, PI3K signalling and chromatin remodelling. Of particular interest in this work is the confirmation of PI3K signalling dysregulation. AXL activation has been frequently shown to signal through the PI3K/AKT axis and this study confirms the need for its further study in bladder cancer.

Tyrosine kinases: Structure and function

Tyrosine kinases are a subclass of protein kinase that, once activated, catalyse the transfer of a phosphate group to a tyrosine residue on protein substrates. This process is fundamental to signal transduction between and within cells. This, therefore, implicates tyrosine kinases in the regulation of a wide-ranging list of cellular functions. Tyrosine kinases can be further sub-classified according to their cellular location being either receptor tyrosine kinases (RTK) or cytoplasmic tyrosine kinases.

All receptor tyrosine kinases contain an extracellular ligand binding domain, transmembrane helix and cytoplasmic protein tyrosine kinase domain. All known RTKs, apart from the insulin receptor, exist as monomers. Upon ligand binding they dimerise which results in autophosphorylation of the cytoplasmic domains. Such dimerisation, with downstream activation, can be achieved through ligand-independent mechanisms. These include receptor overexpression, with trans cellular binding, cross-talk with other receptors or increased phosphatase activity.

Tyrosine phosphorylated sites in the cytoplasm act as binding sites for a variety of signalling proteins. Such proteins contain domains which include SH2 (Src homology 2) or PTB (phosphotyrosine binding). SH2 domain–mediated binding of signalling proteins to tyrosine autophosphorylation sites provides a mechanism

for assembly and recruitment of signalling complexes by activated receptor tyrosine kinases (60) (figure 8). Downstream signalling/activity is dependent upon both the number and concentration of RTK, concentration of protein kinase activity and the ability to recruit molecules with the SH2 and PTB domains. PI3K is an RTK substrate, with SH2 domain (in the p85 subunit) that binds to activated RTK and ultimately activates AKT, which in turn activates downstream pathways involved in cell survival. Ras is activated by several RTK. SH2 containing SOS binds Grb2. This SOS/Grb2 complex binds to activated RTK thus associating SOS with the cell membrane where it can stimulate Ras. Although several effector proteins can bind Ras, a well described axis is interaction with Raf and ultimately activation MEK and ERK (MAPK). ERK activation is involved in a range of processes including metabolic regulation, cell cycle, cell migration, cell shape, proliferation, cell survival and differentiation (61)

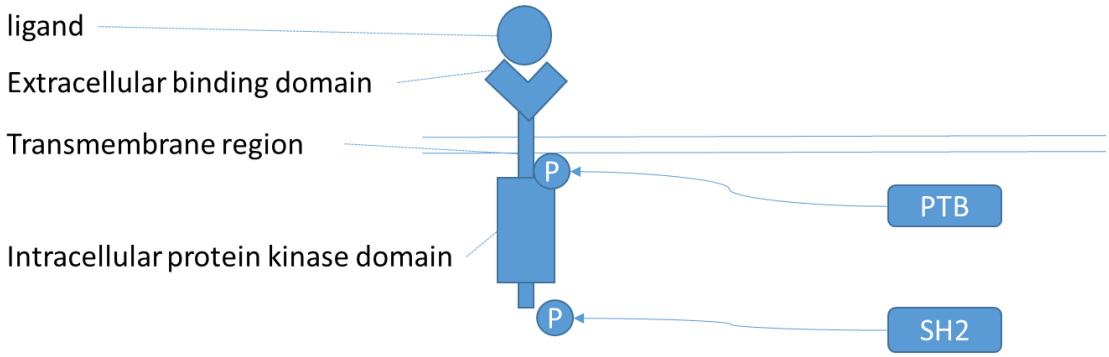


Figure 8. Schematic representation of receptor tyrosine kinase. Activation, through ligand binding and dimerization leads to phosphorylation of the intracellular domain which in turn allows binding of molecules containing PTB and SH2.

Signal Transducers and Activator of Transcription (STAT) contain an SH2 domain which binds to phosphorylated tyrosine residues on RTKs, similarly to PI3K. In the case of STAT, this leads to dimerization, followed by nuclear migration and activation of transcription. Constituent STAT3 activation has been implicated in oncogenic transformation and will be further discussed with its relevance to bladder cancer.

Termination of Tyrosine Kinase signalling and activation

When under normal, physiological controls, RTK signalling is attenuated by binding of inhibitory ligands, hetero-oligomerization with receptor mutants,

inhibition by phosphorylation on non-tyrosine residues, activity of tyrosine phosphatases or receptor endocytosis.

Tyrosine Kinase inhibitors and clinical practice

Given their role in a range of cellular functions, which, when dysregulated characterise cancer development, RTK have been investigated as potential therapeutic targets over recent years. Studies across different cancer types, including several RTK targets have been undertaken, which have led to a number of tyrosine kinase inhibitors entering routine clinical practice. Examples of TKI in routine clinical practice are given in the table 6.

Table 6. Tyrosine Kinase Inhibitors in routine clinical practice.

Agent	Disease	Target(s)	FDA/EMA approval
Imatinib	CML GIST	BCR-Abl	2002
Bosutinib	CML	BCR-Abl	2012
Lapatinib	Breast cancer	HER2 EGFR	2007 2008
Gefitinib	NSCLC	EGFR	2003
Erlotinib	NSCLC	EGFR	2004
Sorafenib	Renal Cell Carcinoma Hepatocellular Carcinoma Thyroid cancer	VEGFR PDGFR	2005
Sunitinib	Renal Cell Carcinoma GIST	PDGFR VEGFR KIT	2006

FDA – Food and Drugs Administration

EMA – European Medicine Agency

CML – Chronic Myeloid Leukaemia

NSCLC – Non-small Cell Lung Cancer

GIST – Gastrointestinal Stromal Tumour

AXL: Current perspective

AXL structure

AXL is one of three members of the TAM (Tyro3, AXL, Mer) receptor tyrosine kinase sub-family. Each member has a conserved extracellular domain containing two immunoglobulin-like domains and two fibronectin 3 domains (62) (figure 9). The intracellular kinase domain varies between members. AXL contains 999 amino acid residues with a predicted size of 110 KDa, however, actual molecular weight, given post-translational modification such as glycosylation and acetylation, is around 140kDa. *AXL* was first discovered as a transforming gene in patients with chronic myeloid leukaemia (CML) (63) and later cloned from patients with CML (64). AXL is expressed ubiquitously in humans (65) and its quiescent functions include clearance of apoptotic cells, maintenance of natural killer cell function and platelet aggregation although a wider range of functions is expected (62).

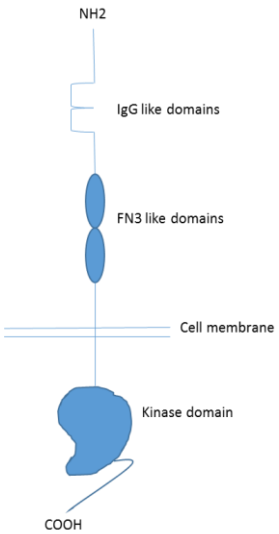


Figure 9. AXL protein structure with 2 extracellular IgG like domains, 2 fibronectin 3 - like domains and an intracellular kinase domain.

AXL activation

The mechanisms that result in AXL activation can be considered as either ligand-dependent or ligand-independent. Gas6, a vitamin K dependent protein with sequence homology to protein S, was first identified as the ligand for AXL in 1995 (66) and is involved in typical ligand-receptor activation of AXL. Gas6 protein is coded for by the GAS6 gene. Ligand-independent activation occurs through either homotypic dimerization, heterotypic dimerization, cross-talk with other receptors or transcellular activation (figure 10). Activation results in autophosphorylation of tyrosine residues in the cytoplasmic domain, which then leads to phosphorylation of other substrates or constitutes a docking site that recruit signalling molecules containing phosphotyrosine-binding domains. The findings of contemporaneous

studies of AXL signalling in different diseases, *in vitro* and *in vivo* models are discussed below.

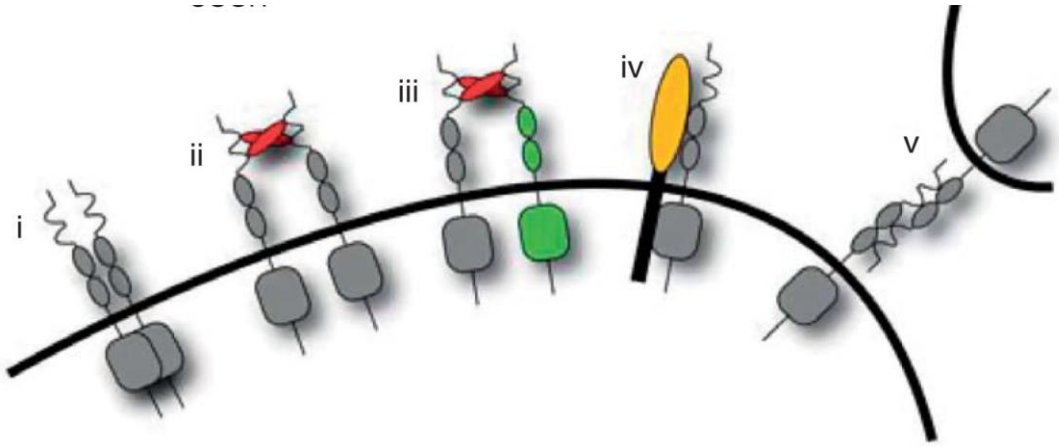


Figure 10. Mechanisms resulting in AXL activation. Taken from Linger et al. (62) (i), ligand-independent dimerization, (ii) ligand-dependent dimerization, (iii) heteromeric dimerization of two different TAM, (iv) heterotypic dimerization with a non-TAM receptor, and (v) trans-cellular binding of extracellular domains. GAS 6 is indicated as the red ligand.

AXL activation and signalling *in vitro* and in pre-clinical models

Across both a panel of mesothelioma cell lines and biopsy specimens, AXL was over-expressed (67,68) in comparison with normal mesothelial cells. Both cells transfected with shRNA to AXL and those treated with the pre-clinical AXL inhibitor, DP-3975 (Diciphera Pharmaceuticals (Lawrence, KS)) were promoted toward G0/G1 phase of the cell cycle, showed reduced AXL-PI3K interaction, AKT

activation, S6 activation and cell migration as assessed by scratch assay. Furthermore, phosphorylation of downstream molecules was found, through transfection with shRNA to GAS6 and subsequent administration of exogenous Gas6, to be Gas6 dependent. However, in this analysis, AXL inhibition with DP3975 produced only a modest reduction in cell survival. This effect was enhanced with inhibition of other receptor tyrosine kinases, including EGFR and MET, with a heat shock protein 90 inhibitor. This suggests that, in this mesothelioma model, single RTK inhibition is insufficient to ameliorate growth and survival mechanisms. The implication being that, in the clinical setting, specific AXL inhibition may produce less benefit than non-specific multi kinase inhibition.

In melanoma cell lines and tumour tissue samples, AXL mRNA levels were increased (69). Such AXL over-expression also correlated with mutations in known proto-oncogenes, which confer poor prognosis in melanoma patients, namely BRAF and NRAS. Stimulation of AXL with Gas6, initiated phosphorylation of AKT rather than ERK. AXL positive melanoma cell lines were also found to express GAS6 suggesting autocrine activation in this model. This, and indeed the majority of studies into AXL activation suggest that the transforming potential of AXL is mediated through overexpression as opposed to receptor mutation. However, manufactured C-terminal truncation mutations through cDNA retroviral incorporation, in the AXL receptor have, however, been shown to transform cell behaviour (70) via activation of the AXL receptor.

El Sayadi et al. (71) found, in leimoyosarcoma tumour lysates, that both AXL and Tyro3 were present in 12 of 13 samples and co-expressed with GAS6 in 8 of 13 samples, suggesting autocrine or paracrine signalling. In this study, AXL activation, with either co-expression of AXL and GAS6 or stimulation with exogenous Gas6 resulted in AKT and ERK1/2 phosphorylation. Cell viability and AKT phosphorylation were reduced by incubation with foretinib, a multikinase inhibitor of VEGFR, MET and AXL.

In B-cell chronic lymphocytic leukaemia (CLL), a disease characterized by proliferation and defective apoptosis of B-cell lymphocytes, AXL was constitutively active. This was determined using p-AXL expression on western blotting in both peripheral blood samples and CLL cell lines (72). In contrast, in other haemopoetic cell lines (multiple myeloma and amyloidosis) AXL was expressed but not constitutively active. Furthermore, AXL phosphorylation correlated with phosphorylation of and complexes with non-receptor kinases PI3K, PLC gamma 2 and Lyn, Syk and ZAP70 (72). The precise mechanism for constitutive AXL activation was not confirmed in this study and although bi-directional regulation of non-receptor kinases on receptor tyrosine kinases has been observed, this study showed that AXL activation was upstream of src family kinases (SFK) activation. Inhibition of AXL phosphorylation with R428, an AXL specific small molecule inhibitor, reduced the phosphorylation of the mentioned non-receptor kinases and also induced apoptosis in leukaemic B cells (72).

AXL was similarly constitutively phosphorylated and activated in the acute myeloid leukaemia (AML) cell line MV4-11 and human AML blasts (73). AXL signalling, through phosphorylation of FLT3 was abrogated using either soluble fusion protein, with structure similar to AXL extracellular domain, or foretinib. This effect on survival was shown, through flow cytometry, to be dependent on cell cycle arrest (increase in S phase) and, through annexin V-FITC labelling, due to induction of apoptosis. Abrogation of AXL activity led to a reduction in phosphorylation of FLT3 in FLTS-ITD+ (Fms-like tyrosine kinase 3 Internal Tandem Duplication, an aggressive subtype) positive cell lines and human blasts. In a severe combined immunodeficiency mouse model, AXL-fc (fusion protein) also reduced the proliferation of grafted human FLT3-ITD+ blood cells and overall survival in these mice.

Paccez et al (74) identified AXL expression in prostate cancer cell lines and prostate cancer. AXL was shown to signal through activation of the PI3K/AKT axis in addition to overlapping signalling with STAT 3. Inhibition of AXL resulted in a reduction in circulating Interleukin (IL-6) and also phosphorylated STAT3, which in turn resulted in a reduction in proliferation in prostate cancer cell lines.

AXL in bladder cancer

To date, there are two published studies of the role of AXL in bladder cancer. Sayan et al. (75), in our own laboratory in Leicester, found through investigating the role of the transcription factor activator protein one (AP-1), a known target of MEK|ERK1/2 activation, that invasive bladder cancers expressing fra-1, a constituent of AP-1 were more likely to be muscle-invasive than non-muscle-invasive. Furthermore, in fra-1 expressing cell lines, UMUC3 and J82, it was shown through qPCR that the gene for AXL was up-regulated approximately 20-fold. AXL knockdown with siRNA reduced cell invasion with no apparent effect on cell proliferation. Yeh et al. (76) identified an up-regulation of AXL and PDGFR-alpha as consequences of c-MET activation in T24 cells with inducible c-MET gene expression. This suggests crosstalk of different RTKs in bladder cancer. Furthermore, co-expression of c-MET, AXL and PDGFR-alpha resulted in invasion in cell lines and poor prognosis in a series of 65 patients. Interestingly, there was incrementally worse survival with co-expression of AXL, PDGFR and c-MET in this series.

Unpublished data, accessed via oncomine.org, supports a role for AXL in Epithelial Mesenchymal Transition (EMT) and invasion. EMT is a process by which epithelial cells lose cell-cell adhesion and gain migratory properties which ultimately result in a mesenchymal phenotype. This process is important in normal growth but is

frequently dysregulated in cancer progression. Stranskyet al. (77) characterised genetic variants between NMIBC and MIBC. In this analysis, AXL was overexpressed in MIBC and both the gene for Vimentin and ZEB2, markers of mesenchymal phenotype, were in the top five genes co-expressed with AXL (figure 11).

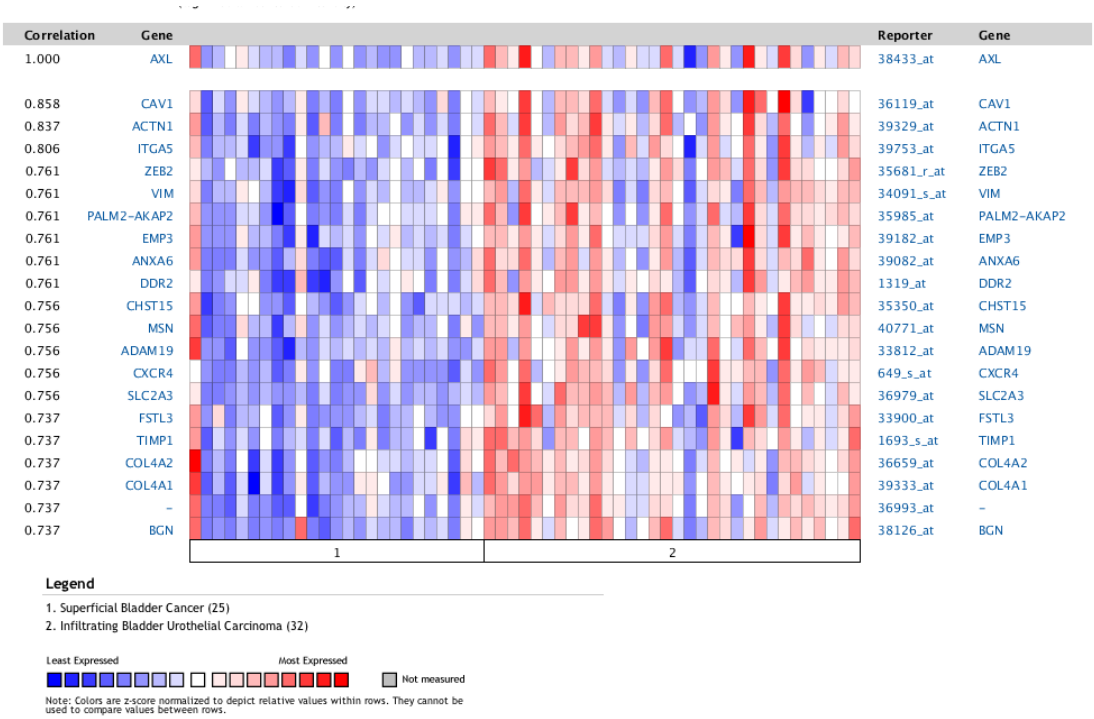


Figure 11. Data from Stransky et al (77) detailing co-expression of vimentin and ZEB2, markers of EMT in association with AXL.

AXL in treatment resistance

In addition to mounting evidence for a role in invasion, AXL expression and activation has been implicated in resistance to both conventional chemotherapy and also modern TKI therapy. Examples of this are discussed below.

TKI monotherapy has frequently been associated with rapid response in a range of cancers. Unfortunately, this is often followed by a similarly rapid cancer progression. To address this rebound phenomenon in adrenocortical cancer Lin et al. (78) developed a Sunitinib resistant cell line through chronic exposure (20nM for 48hrs). Sunitinib is a multikinase inhibitor of PDGFR, VEGFR, RON and cKIT, licensed for the use in renal cell carcinoma and GIST. Resistant cells were found to have up regulation of a number of TKs including AXL and ERK1/2. Indeed, ERK was the most up regulated of these kinases and specific ERK inhibition, with PD98059, reduced cell proliferation by approximately 65%. In addition to further implicating AXL activation as a pathway to treatment resistance, this study also highlights that activation of compensatory oncogenic signalling pathways can be through the same downstream molecules i.e. VEGFR signals through ERK which is eventually up regulated following sunitinib monotherapy, despite VEGFR abrogation.

In gastrointestinal stromal tumour (GIST) cell lines, imatinib a PDGFR and c-kit inhibitor, resistance was developed through chronic exposure to the drug (79). In these resistant cells a tyrosine kinase switch, with c-KIT down regulation and AXL up regulation, through increase gene copy number, mRNA level and protein expression was observed. Additionally, GAS6 was over expressed in resistant cell lines indicating autocrine AXL activation in imatinib resistant GIST cells.

In imatinib resistant CML cell lines, AXL was overexpressed and constitutively activated (80). Subsequent AXL knockdown, using siRNA, was able to restore sensitivity to imatinib. In imatinab resistant cells, following AXL knockdown a similar reduction in proliferation and apoptosis induction was observed in comparison to in imatinib sensitive cells. Interestingly inhibitors of the MEK/ERK and PKC axis were able to reduce the expression of AXL in resistant cell lines and also restore imatinib sensitivity. This suggests that MEK/ERK and PKC are implicated in the over expression of AXL observed in resistant cells and have a potential bidirectional signalling relationship with AXL, at least in this CML model.

Lay et al. (81) reported correlations between AXL expression, invasion and migration in addition to chemoresistance in an *in vitro* model consisting of progressively invasive lung cancer cell lines. In this model, downstream signalling was through PI3K/AKT and NF κB, which acts downstream of AKT, rather than, ERK or MEK. Furthermore, in this analysis, sulfasalazine, a non-steroidal anti-inflammatory agent routinely used in inflammatory bowel disease and rheumatoid arthritis, which acts to inhibit NF κB, reversed AXL-dependent chemoresistance and reduced cell invasion. The effect of sulfasalazine was, however, not specific to NF KB and the inhibitory effect was also seen on ERB-B2 and EGFR phosphorylation which may have contributed to the observed changes.

In another lung cancer study, aimed at better predicting response to targeted therapies and identifying future therapeutic targets, Byers et al. (82) constructed

an epithelial mesenchymal transition (EMT) gene signature. In mesenchymal cell lines, AXL was significantly over expressed compared to epithelial cell lines. Furthermore, *in vitro* AXL inhibition using SGI-7079, a pre-clinical AXL inhibitor, restored mesenchymal cell lines response to erlotinib, a TKI used as second line in metastatic non-small cell lung cancer.

In an *in vitro* breast cancer model of resistance to the HER2 inhibitor, lapitinib, AXL expression was up regulated, via a modest gene copy number increase (2-fold) and hypomethylation in the *AXL* gene promoter region resulting in increased mRNA levels (83). Interestingly, there was no observed change in gene copy number, mRNA or protein levels for HER2. In these laptinib resistant cells, phosphorylation of HER2 was reduced similarly to lapatinib sensitive cells, however, the observed increased cell survival was owing to activation of the PI3K/AKT pathway and MEK/ERK activation. To provide further support of AXL-dependent lapitinib resistance, resistant cells transfected with siRNA to AXL had regained sensitivity to lapitinib with respect to cell growth/proliferation. Furthermore, treatment with foretinib restored the ability of lapatinib to modulate gene expression in lapatinib resistant cell lines. Foretinib or lapatinib alone were unable able to interrupt the interaction between AXL and the regulatory subunit of PI3K, p85 (containing SH2), which leads to AKT activation. However, in combination this pathway was abrogated. This suggests, that in the development of lapatinib resistant breast cancer cells, cross talk between HER family receptors and AXL is an essential and seemingly reversible step.

Clinical significance of AXL

Given the large *in vitro* and *in vivo* body of evidence supporting AXL expression in alignment to invasion and chemoresistance, it is unsurprising that AXL is expressed in a range of human cancers including lung, breast, colon and thyroid (84-87). AXL expression has consistently been shown to confer an increased risk of metastasis and poor survival in patients. Furthermore, increased AXL expression has been shown to be associated with chemoresistance in clinic practice (88-91).

Current status of AXL inhibitors

Clinical Trials

Foretinib, mentioned above in the context of AML (73) and breast cancer (83), has been further evaluated in a number of clinical trials.

In a phase I dose escalation study (92), including 37 patients with a variety of metastatic cancers, who had been heavily pre-treated with standard treatment, foretinib produced stable disease over a median follow up of 6 months in 74% of trial participants. All patients reported some adverse events, which included grade 1 or 2 fatigue, hypertension, nausea, diarrhea, anorexia, vomiting and headache.

However, these all resolved with either discontinuing the drug or dose reduction. Five serious adverse events included left ventricular dysfunction, pulmonary embolism, grade 3 diarrhoea and grade 4 fatigue. A maximum tolerated dose of 80mg once daily was determined, owing to grade 3 diarrhoea and hypertension amongst those taking higher doses. Less than 1% of foretinib was excreted in the urine. In a second phase I study (93) a maximum tolerated dosage of 3.6mg/kg (240mg in 70kg male) was determined when taken on five consecutive days with nine days rest in a 14 day cycle. Dose limiting toxicities included derangement in serum AST and lipase. In this study, pharmacokinetic analysis revealed a maximum concentration at 4 hours after initial dosing which declined with monoexponential decay subsequently, with a half-life of 40 hours. Urinary excretion of foretinib was <1%. This study also showed reduction in pERK and pAKT, as evaluated by immunohistochemistry, in tumour samples taken during foretinib treatment. Overall, 55% had stable disease for 10 months and 7.5% patients had partial response.

Both of the above dosage regimes (intermittent and continuous) have been evaluated in a phase II study of foretinib in patients with locally advanced, bilateral or metastatic papillary renal cell carcinoma (94). There was an overall response rate, defined as complete or partial response, of 13.5% for a median duration of 18.5 months. Patients in the intermittent dosing cohort had slight improvements in progression free survival, although this was not statistically significant. Furthermore, germline MET mutation was predictive of a response.

Additionally, foretinib has been evaluate, in similar phase II trials in gastric cancer (95) and recurrent head and neck cancer (96). Similar results, in terms of maintaining stable disease were observed. Common to all of these trials, however, is a lack of assessment of the differential response between patients whose tumours express AXL and those that do not.

Pre-clinical investigation

Holland et al. (97) investigated R428 (Rigel pharmaceuticals), a small molecule inhibitor of AXL discovered following high-throughput screening, with an IC_{50} in the nanomolar range (14nmol in HeLa cells using pAKT as a readout). In comparison, activity against other targets Mer, Tyro3, VEGFR2, HER2 was 16, 14, 34 and >100-fold less. R428 inhibited invasion of breast cancer cell lines (MDA-MB-23) but had little effect on proliferation. *In vivo* evaluation in mouse models revealed that R428 had a half-life of 4 hours (with 25mg/kg dosage) and twice daily oral dosing was sufficient to block Gas6-induced AXL phosphorylation in mouse blood. Furthermore, R428 was able to inhibit development of metastasis in mice with breast cancer xenografts. In this study, breast cancer cells were either administered heterotopically, directly intracardiac, or orthotopically, into the mammary fat pad. Tissue lysates from these mice revealed a reduction in the EMT regulator, Snail, further implicating AXL in EMT. R428 had no effect on the extent of primary tumour growth in orthotopically (mammary fat pad) administered

breast cancer cells. Taken together this suggests R428 inhibits later stages of metastasis. To add clinical relevance, the combined effect of R428 and cisplatin was evaluated and found to be additive on the delay to onset and burden of metastasis in these mice. In a separate study, R428 inhibited melanoma cell invasion through abrogation of AXL signalling (69). As previously described in melanoma, AXL phosphorylation was GAS6 dependent in this model.

Vorinostat is a histone deacetylase inhibitor, which promotes protein acetylation, modulates gene expression and induces differentiation, growth arrest and apoptosis of tumour cells. It was approved by the FDA for treatment of cutaneous T cell lymphoma in 2006. Virinostat has been shown to have a wide reaching effect on gene signature in AML cells lines and patients peripheral blood. One of the most marked effects was in down regulating the mRNA expression of AXL (98). The authors suggest that virinostat interferes with the binding of the transcription factor SP1 to promoter regions of other genes modulated by virinostat exposure. However, the precise mechanism underpinning the effects on AXL expression were not uncovered.

Two further multikinase inhibitors, with activity against AXL, which have featured in the literature are LY2801653 (99) and Cabozantinib (100). LY2801653, is a small molecule multikinase inhibitor of MET, MST1R, FLT3, AXL, MERTK, TEK, ROS1, DDR1/2 and MKNK1/2. Cabozantinib (XL184) is a multikinase inhibitor, with FDA licensed for the treatment of medullary thyroid cancer, with action

against MET, VEGFR2, RET, KIT, AXL and FLT3. To date, reports on the effect of these agents have focussed on those mediated through inhibition of kinases other than AXL. However, in such studies the IC_{50} for AXL has been repeatedly in the nanomolar range (99,100) suggesting a potential contributory role of AXL inhibition in the observed effects.

Summary

Despite a falling incidence, bladder cancer remains an important and expensive healthcare problem. All subsets of disease carry significant cost, mandated either by the requisite repeated cystoscopic follow up in NMIBC, major surgery or radiotherapy in MIBC or palliation in metastatic bladder cancer. As such, there are several milestones in the natural history of bladder cancer in which therapeutic advances will not only improve patient care but also efficiency.

The treatment of high-risk NMIBC has remained largely unchanged for many years. Close cystoscopic follow up in parallel with immunotherapy is current best practice. Despite this treatment, a significant number of patients will develop recurrence or progress to MIBC, thus requiring radical treatment. It follows, therefore, that this subset of patients represents a challenging group and an opportunity to develop novel therapies. Currently, available alternatives under investigation revolve around optimising the delivery of standard intravesical treatments either through electromotive forces or controlled hyperthermia. To

date there are no registered clinical trials of tyrosine kinase inhibitors in NMIBC, indeed the focus of such therapies in many diseases has focussed on the setting of metastatic disease.

The transforming potential of AXL appears to be consistently mediated through increased mRNA and/or protein expression level. The processes leading to AXL activation varies between disease and cell system. There are examples in the literature of GAS6-dependent (be it endocrine or autocrine), GAS6-independent and constitutive activation. The measured consequence of such activation is most frequently mediated through either PI3K/AKT activation, MEK/ERK1/2 activation or both (figure 12). Furthermore, ERK may have a bidirectional role in AXL signalling and activation. Additionally, STAT3 signalling has been shown to be a consequence of AXL activation. AXL activation appears to results in cell migration, invasion, cell survival and chemoresistance. Within the developing field of AXL inhibitors, foretinib and R428 appear to have the largest body of evidence supporting further study. Given its proximity to clinical practice, foretinib was chosen for further investigation in this study.

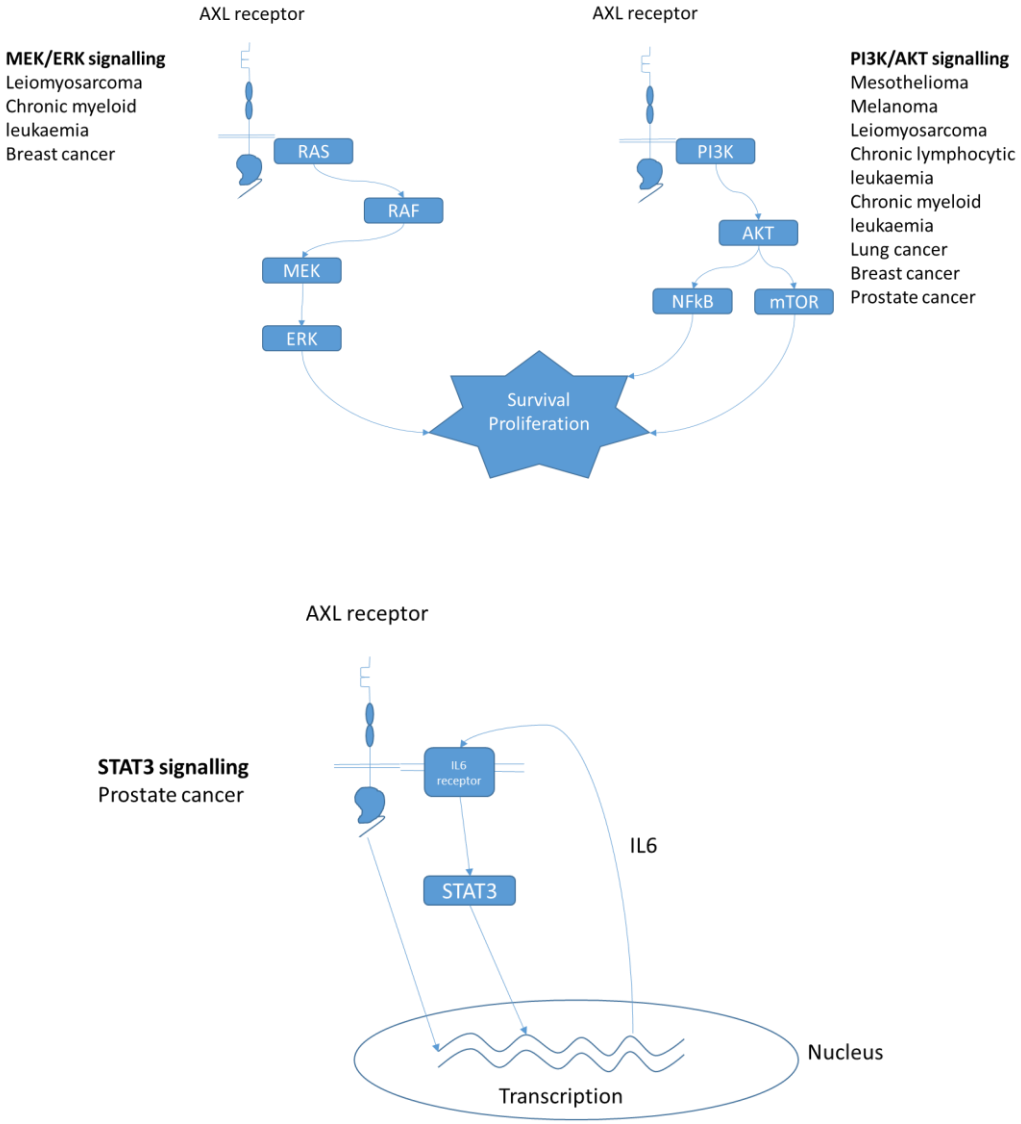


Figure 12. Schematic representation of AXL-dependant signalling pathways and different cancer types. Top; MEK/ERK and PI3K axis dependant AXL signalling. Bottom; STAT3 dependant AXL signalling

Aims and objectives

The principal hypothesis is that AXL expression correlates with invasion, metastasis and poor prognosis in bladder cancer. This is, in part, based on the growing body of AXL literature and the identification of AXL in bladder cancer cell lines exhibiting a mesenchymal phenotype. We aim to assess the expression of AXL in human bladder cancer tissue samples and correlate this expression with a range of clinical variables. In patients with bladder cancer, there is a significant fall in survival that occurs with the onset of muscle invasion and moreover metastasis. It follows therefore, that identifying whether AXL expression associates with this stage progression is a crucial step in validating AXL as a potential therapeutic target in bladder cancer. Furthermore, we aim to investigate the pathways through which AXL signals in bladder cancer cell lines and whether foretinib is able to abrogate such signalling.

Chapter 2: Materials and Methods

Human Immunohistochemistry

Patient selection

To evaluate the expression of AXL in human bladder cancers, tissue samples were used from two separate series of patients. For the cystectomy series, tissue was obtained from the University Hospitals of Leicester pathology department in accordance with the agreed standards detailed in the study protocol "Expression of Tumour Markers in Bladder Cancer" (UHL 10882). The study protocol was granted ethical approval (10/H0401/13) in 2010. For the Tissue Micro Array analysis, which was donated from the University of Newcastle-upon-Tyne, local ethical approval had been granted previously.

Cystectomy series

A non-consecutive series of 65 patients, who had previously undergone radical cystectomy at Leicester General Hospital between October 1996 and May 2008 was identified using our electronic theatre management system. Although a greater number of cystectomies were performed during this time, specimens for collection were chosen based on the adequacy of the accompanying clinical data, to allow meaningful clinical correlation. Formalin-fixed paraffin-embedded (FFPE) tissue, which had been initially used for diagnostic purposes, from these patients

was obtained from our pathology department. All patients in this series had a TURBT which confirmed either MIBC or recurrent high-risk NMIBC prior to radical cystectomy. A small proportion of patients undergoing cystectomy had no residual tumour evident on histopathological processing of the cystectomy specimen, the inference being that the entire tumour had been resected at the time of TURBT. We therefore, elected to examine tissue from the most recent TURBT prior to radical cystectomy in all cases. In addition, we identified those patients with histologically confirmed lymph node metastasis and examined this tissue also.

Tissue Microarray analysis

As part of the Bladder Cancer Database Newcastle project, Between October 1997 and December 2006 tissue was collected from over 300 patients following TURBT, across all stages and grades of bladder cancer, to construct a tissue microarray (TMA). The resultant slides were kindly donated by Professor Craig Richardson (University of Newcastle-upon-Tyne) and processed under the protocol described below. Following processing, there were 285 stained tumour cores, with complete clinical records, for further analysis.

Clinical Variables

In this study, immunohistochemistry serves both to test our initial hypothesis that AXL expression is associated with invasion, metastasis and poor prognosis and to potentially provide a basis for further in vitro study. Therefore, in addition to a qualitative analysis of AXL expression, we correlated such expression, via means of a numerical scoring system with a range of clinical variables. These include pathological stage, grade, disease-specific survival, overall survival and the need for radical cystectomy in patients initially receiving treatment for NMIBC.

Immunohistochemical methods.

Human Immunohistochemistry protocol

FFPE tissues were sectioned and prepared on vectabond slides. No sections were left for longer than 2 weeks before staining to avoid antigen degradation.

Bladder tumour and lymph node tissue was fixed in formalin and paraffin-embedded. 4μm sections were dewaxed, rehydrated and antigen retrieval was performed with microwaving for 20 minutes in 10mM citrate buffer. Endogenous peroxidase was blocked by incubation in 0.3% H2O2 for 10 minutes. After rinsing in Tris Buffered Saline (TBS) sections were incubated for 10 minutes in 1:5 normal

rabbit serum and blocking solution (TBS/ 3% Bovine Serum Albumin/ Triton X-100). Incubation with polyclonal goat IgG primary antibody to human AXL (AF-154 R&D systems, Minneapolis, US) was done at 4^{o}C overnight. After rinsing in TBS twice, secondary rabbit anti-goat IgG-horse radish peroxidase antibody (DAKO, Cambridgeshire, UK) was applied for 30 minutes. Diaminobenzidine (DAB) was used as a chromogen prior to counterstain with haematoxylin, dehydration and mounting.

Variables for optimization

Historically, the results of immunohistochemical assays have varied greatly from laboratory to laboratory. Although not excluded, such variability has been reduced by improvements in reagent quality, standardisation of antigen retrieval methods and recommendation for reference standards and controls (101-103). It is now commonplace for commercially available antibodies to be provided with suggested staining protocols. Our study used a polyclonal goat primary antibody, AF154, (R and D systems, UK) and the final IHC protocol closely reflected the manufacturer's suggestions with regards to incubation periods and reagent temperatures. However, we used in house blocking solution and DAB to reduce cost. Our secondary antibody was a HRP-linked rabbit anti goat antibody (DAKO, Cambridge, UK) the manufacturer's recommendation for usage was at a fixed dilution of 1:2000 and, therefore, was not altered in the process of optimization. As such, the most important variable to optimize was primary antibody

concentration. The manufacturer's recommendation for AF154 concentration was between 5µg/ml (1:40) and 15µg/ml (1:120). We therefore stained human bladder cancers and human stomach, the recommended positive control, across a range of concentrations which spanned below the lowest and above the highest concentration.

Image capture

All images were captured on a Leica video microscope and saved for further analysis. To maximize standardisation of obtained images, each photo was adjusted to minimize over-exposure or under-exposure, as assessed by Leica image analysis software.

Immunohistochemistry scoring

To score each section we first elected to identify regions of interest (x100 magnification) that satisfied the following criteria.

- The most invasive region of bladder cancer on the section, defined on H&E and conjunction with a consultant histopathologist.

- Positive vascular endothelium therefore indicating successful staining in that microenvironment. As such, regions with no vascular staining were ignored.

Within bladder cancer sections which contained both invasive and non-invasive tumours, the most invasive focus of tumour is the benchmark of pathological reporting and is used to guide subsequent treatment. Therefore, evaluating these areas for AXL immunostaining increases the accuracy of subsequent correlation with histopathology reports.

Following identification of these regions of interest, we examined 10 high-power fields (x400 magnification), at random, for the proportion of tumour cells taking up the stain. The score for each high-power field was calculated using the following tabulation (table 7) and aggregated to give a score between 0 and 30.

Table 7. Scoring system for cystectomy series

Score	Proportion of cells
0	0%
1	0-10%
2	10-50%
3	>50%

A variety of different scoring systems have been used to histological specimens (104-109). We elected to use a system analgous to the J-score, used to ascertain oestrogen receptor status in breast cancer (109). Although the majority of IHC

scoring systems include assessments of proportion and intensity of cell membrane staining, the J-score uses proportion alone. Clearly, a higher number should be present if more cells stain positive and a lower one with fewer cells. Given the variability in staining intensity across TURBT sections, our assumption was that, in this series, an assessment of proportion of cells taking up the stain alone would be more accurate than combining this with an assessment of intensity. Ultimately, the accuracy of any scoring method can only be validated with a sufficiently large dataset that allows a retrospective analysis once prognosis or eventual outcome is known.

In the TMA analysis, representative foci, at least with respect to macroscopic and histological appearance, of tumour had already been isolated. Multiple tumour sections are analysed on one slide, as opposed to in our cystectomy series, where whole sections were processed in several separate groups over a number of weeks. It, therefore, follows that unavoidable, albeit subtle, variation in staining protocol inherent in processing sections on different days such as variations in humidity, exposure to reagents or reagent degradation, which may affect the final appearance, would be avoided. As such, we elected to introduce a measure of staining intensity to the final TMA IHC score. Scores for each tissue core were assigned according to both the proportion of cells taking up the stain and intensity of membrane staining (table 8). This measure is analogous to the Allred staining method, employed in assessing oestrogen receptor status in breast cancer (108). In this TMA there were two sections obtained from each tumour. If only one

sample was successfully stained, then this case was disregarded from further analysis. As such, to generate a final IHC score, a mean of aggregated values was used, giving a range of 0-3.

Table 8. Scoring system for TMA series

Proportion	Score	Intensity	Score
0%	0	Absent	0
<10%	1	Weak	1
10-50%	2	Intermediate	2
>50%	3	Strong	3

Interobserver variation and validation

To improve the accuracy of our analysis, a consultant histopathologist verified the regions of interest as those representative of the most invasive cancer in each section. Additionally, we performed an assessment of interobserver agreement to support the reproducibility of our findings. Two independent scorers scored the first 35 stained sections from our cystectomy series and the results were compared using Cohens kappa coefficient (110).

Correlations with clinical variables

After sections were assigned IHC scores, a variety of associations, with the clinical parameters detailed above, were tested. For all relevant clinical variables, we undertook both univariate and multivariate analysis of the association of AXL

expression with the variables in question. For all tests a type 1 error significance level was set at 5%. Whilst it is recognised that multiple testing can lead to increased type 1 error and several authors have suggested that corrections to significance levels should always take account of this (111-113), some authors debate whether such correction is always necessary or appropriate. A Bonferroni correction, where the accepted significance level is equal to α/n, where n is the number of variables is perhaps the most widely used correction for multiple testing. Perneger VT (1998) (114) suggests that Bonferroni corrections inevitably exchange a reduced type 1 error for an increased type 2 error. Furthermore, in the context of a robust hypothesis, in this instance that AXL expression correlates with invasion, it is more appropriate not to use a Bonferroni correction. As such, we elected not to apply a post hoc Bonferroni correction.

For binary variables such as invasion, metastasis at presentation, latent metastasis and failure of standard treatment in NMIBC, binary logistic regression was used. Survival analysis was conducted using either Kaplan-Meier survival analysis or multivariate Cox regression as appropriate. To determine a role for AXL as a biomarker, we constructed a series of 2x2 contingency tables with a range of variables and calculated sensitivity, specificity, positive predictive value and negative predictive value, using Chi squared test to assess statistical significance. All statistical tests were performed on SPSS version 22 (IBM, New York).

Determining threshold values for AXL status

Given our hypothesis that AXL expression is associated with invasion, for both the cystectomy series and TMA, we plotted receiver operating characteristic curves for these variables. This allowed an assessment of how well AXL score can discriminate between muscle invasive and non-muscle invasive disease. Additionally, to determine threshold values of AXL score that would be appropriate to be considered AXL positive and AXL negative, we calculated the Youden index (115). This single statistic represents the maximum potential effectiveness of a biomarker to distinguish between disease states, in this instance non-muscle invasive and muscle invasive disease. In essence it represents the AXL score at which the sum of sensitivity and specificity is maximal. The threshold values in both the cystectomy series and TMA calculated using muscle invasion as a test variable were then used in subsequent analysis.

Mouse Immunohistochemistry

Mouse model

Historically, there has been a lack of an animal model that represents the development of CIS and subsequent progression to MIBC. Recent reports suggest that a transgenic mouse, with constitutively active STAT3, may reflect this type of bladder cancer (116). In this analysis, transgenic mice and wild-type mice were exposed to nitrosamine, a carcinogen known to induce TCC of the bladder. In the wild-type mice, once sacrificed, a variety of papillary non-invasive lesions and solid muscle invasive lesions were identified. However, in the transgenic mice at an earlier juncture they develop dysplasia, CIS and muscle invasive TCC in the absence of papillary lesions. It follows, therefore, that this is a novel *in vivo* model of CIS and muscle-invasive bladder cancer. Mice were grown and sacrificed at the host institution (Baylor Medical School, Texas, USA). FFPE sections of wild-type (n=5) and transgenic (n=5) mouse bladder were kindly supplied for further analysis. As described above the wild type mouse tumour specimens contained a mixture of papillary and muscle invasive cancers and the transgenic mouse specimens contained both CIS lesions and muscle invasive tumours. Given the small number of available sections we performed a qualitative analysis, rather than quantitative, on the stained sections.

Mouse AXL has 88% sequence homology with human AXL. It was, therefore, uncertain as to whether the protocol used in our human AXL IHC assays would provide satisfactory results. Furthermore, there are no available positive mouse controls in the literature. However, given that neither the primary nor secondary antibodies used in the protocol described were raised in mice we elected to use this as a starting point. Sections of intestine from Multiple Intestinal Neoplasia (MIN) mice, previously grown in our laboratory, were stained for AXL using our previously described protocol. MIN mice are an intestinal and colonic tumour mouse model with a truncation of the APC gene. APC is a tumour-suppressor gene, loss of which predisposes to colorectal cancer and familial adenomatous polyposis in humans. They develop hundreds of intestinal and colonic polyps during their development. We stained sections of intestine from MIN mice to evaluate the specificity of staining and also exclude significant background.

Cytoblock staining

To further confirm the expression of AXL in bladder cancer cell lines and validate our IHC and cell culture findings, we stained sections from cytoblocks of J82 and RT112 cell lines. Cells were cultured in the standard fashion until approximately $2x10^7$ cells were present in a T175 ($175cm^2$ surface area) flask (25% confluent). Cytoblocks were constructed using the Shandon™ Cytoblock™ Cell Block Preparation System (Thermoscientific, UK) and their suggested protocol (University of Leicester SOP 547 v3). This system combines the process of

centrifugation, to encourage cells down a funnel and increased cell density, and fixating grown cells to produce a formalin fixed cell button, which can be prepared for further IHC analysis.

Sections of cytoblocks were prepared and stained according to the immunohistochemistry protocol previously listed.

Cell culture studies

Preparation of foretinib

A total of 2.1mg of foretinib (GSK Brentford, UK), with a molecular weight of 632.65 $gmol^{-1}$, was supplied as a dried powder and stored at room temperature. Therefore, 2.1mg was dissolved in 330µl of 100% DMSO to give an approximately 10mM stock solution which was stored at -20^{o}C.

Cell culture general method

Ten cell lines were used in this study. J82, RT112, RT112 (E-cadherin dominant negative mutant), UMUC3 and T24 were obtained from our in-house cell line bank, stored in liquid nitrogen. These cells were initially donated from Cancer Research UK (CRUK) in 2005. HT1197, HT5637, EJ28, 253J and HT1376 were donated from Cancer Research Technologies (CRT). The medium used varied from cell line to cell line (table 10) according to recommendations from the ATCC or previous experience from CRT. The listed media all contained 10% Fetal Bovine Serum (FBS) however, for some experiments it was necessary to incubate cells in serum free medium for a period of time, so called serum-starvation. Serum starvation describes a period of cell culture in the absence of, or with minimal amounts of the relevant, growth factor containing serum. This process down regulates a variety of

intracellular signalling proteins. It is, therefore, an important step prior to stimulating a known receptor or pathway.

Table 10. Cell lines, phenotype and growth medium

Cell line	Tumour	Phenotype	Chosen growth media
J82	UC	Mesenchymal	Eagle minimum essential medium, 10% FBS
T24	UC	Mesenchymal	McCoys 5a Medium Modified, 10% FBS
RT112	UC	Epithelial	Eagle's minimum essential medium, 2mM Glutamine (glutamax), 1% NEAA, 10% FBS
UMUC3	UC	Mesenchymal	Eagle minimum essential medium, 10% FBS
HT1376	UC	Epithelial	Eagle's minimum essential medium, 1% Na Pyruvate, 1% NEAA, 10% FBS
253J	UC	Mesenchymal	RPMI, 10% FBS
EJ28	UC	Mesenchymal	Eagle's minimum essential medium, 1% Na Pyruvate, 1% NEAA, 10% FBS
HT1197	UC	Epithelial	Eagle's minimum essential medium 1% Na Pyruvate, 1% NEAA, 10% FBS
HT5637	UC	Epithelial	RPMI, 10% FBS

Cell lines were grown as a monolayer in a humidified incubator supplemented with 5% CO_2 at 37^{o}C. Trypsin-EDTA (ethylene diamine tetraacetic acid) was used to lift cells for passaging. In general cells were cultured to 70-99% confluence prior to passaging into either a larger container (T25, T75 and T175 corning plastic cell culture flasks, Sigma Aldrich) for further culture or 6cm dishes for specific experiments.

Retrieval from liquid nitrogen

Previously frozen cells were thawed in 37^{o}C water bath for 1-2 minutes. The thawed cell suspension was added to 9 mls of appropriate media then centrifuged at 1000rpm for 5 minutes. After removal of the supernatant, the remaining cell pellet was mixed with 10mls of appropriate media (table 10) and transferred to a T25 flask for further incubation.

Freezing for further investigation

To maintain stock levels, cells were cultured with the intention of storage in liquid nitrogen for further use. Cells were passaged, ultimately into a 175 cm^{3} flask. Cells were lifted with trypsin-EDTA, re-suspended in 10mls of growth medium prior to centrifuge at 1000 rpm for 5 minutes. The resulting supernatant was re-suspended in 1ml of medium and cell number counted using a standard haemocytometer. Approximately one million cells were transferred to 0.5ml eppendorf containers and centrifuged at 1000rpm for 5 minutes. Cell pellets were then re-suspended in 1ml of freezing medium (80% growth medium, 10% FBS and 10% DMSO). They were then transferred to an initial freezer at -80^{o}C overnight prior to transfer into liquid nitrogen.

Preparation of cell lysates

For all in vitro experiments, the final cell passage was onto 6cm well plates. Relevant protein expression was assessed using lysates from cell culture monolayers. Cells were twice rinsed in PBS prior to exposure to 800µl of lysis buffer (117).

The amount of protein, and hence concentration, in each lysate was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific). Five microlitres of protein lysate were added to 200µl of working reagent in 96 well plates. The plate was then incubated for 30 minutes at 37^{o}C to allow time for colour change to take place. In parallel, a series of bovine serum albumin (BSA) known standards was assayed in a similar fashion. Absorbance was evaluated using a Genios Microplate Reader. A curve of the BSA standards was plotted and hence protein concentration of test lysates could be determined.

After protein concentration was confirmed 1µl of a 1:1 mixture of 0.002% bromophenol blue and 5% 2-mercaptoethanol was added to each lysate. Following this each lysate was boiled for 10 minutes at 96^{o}C and once cooled underwent a 5-second burst of ultrasonication. This served to reduce the viscosity of the sample and ease subsequent loading into stacking gels. At this stage, lysates were frozen pending further analysis.

To standardise the amount of protein in each running lane, a variable amount of lysate and lysis buffer was used to make a final volume of 20µL. In order to maximise the amount of protein analysed 20µl of the lysate with lowest protein concentration had no additional lysis buffer added. Lysates with higher protein concentrations would be supplemented with lysis buffer, to a volume of 20µl, dependant on the concentration of protein. For instance, if a lysate had a protein concentration twice that of the lowest lysate concentration in a particular experiment, then half the volume of the higher concentration lysate would be used and the remaining volume required to bring the total up to 20µl would be made up of lysis buffer.

Western blotting

In this study we used a 5% polyacrylamide stacking gel above a 10% polyacrylamide running gel. Gels were assembled in a standard electrophoresis chamber (Mini-PROTEAN® Tetra Cell, BIO-RAD, UK) and covered in a running buffer. Two microliters of a 1:1 mixture of 0.002% bromophenol blue and 5% 2-mercaptoethanol was added to each thawed lysates prior to boiling at 96^{o}C for a further 5 minutes prior to loading into the stacking gel. Twenty microlitres of lysate, with standardised protein concentration, was loaded in each lane. One lane was dedicated to a molecular weight marker, in which, 7µl of Pageruler Plus (ThermoScientific, UK) was loaded. A 120V potential difference was applied

across the gel for 60 minutes or until the blue colouring had reached the bottom of the gel, indicating the protein mixture had traversed the gel appropriately.

For protein blotting we used a polyvinylidine flouride (PVDF) membrane (Immobillion-P membrane, Merck Millipore, Germany), which was activated with methanol, to enhance protein binding, prior to securing in the tanks.

The final transfer setup included 3 soaked pieces of filter paper either side of the gel/membrane complex, all secured and immersed in transfer buffer in a Criterion™ Blotter (BIO-RAD, UK) tank. Given the relatively high molecular weight of AXL, we elected to use a prolonged transfer time overnight in the presence of low current and potential difference (22V).

Successful transfer was confirmed with Ponceau stain (Sigma-Aldrich, Germany). After successful staining was confirmed membranes were rinsed with distilled water.

Membranes were trimmed and divided into relevant sections prior to incubation in blocking solution for one hour. Following blocking, excess solution was removed and membranes were incubated with primary antibody at $4^{o}C$ overnight. Each antibody was diluted to the appropriate concentration in the appropriate diluent according to manufacturer's recommendation (table 11). Membranes were then rinsed in 0.1% tris buffered saline and tween 20 (TBS-T) and agitated for 10

minutes three times over prior to incubation with secondary antibody for one hour at room temperature. Finally, the excess secondary antibody was removed by rinsing membranes in 0.1% TBS-T for 10 minutes twice over.

As in our immunohistochemistry studies, the secondary antibody was linked to HRP. In this instance, HRP was used to induce a chemiluminescent reaction as opposed to a chromogenic one. 1ml of chemiluminescent substrate (SuperSignal™ West Pico PLUS Chemiluminescent Substrate) was applied to each membrane for one minute prior to shaking off the excess and securing between two sheets of acetate. In darkroom conditions, the membranes were exposed to X-ray film (Kodak BioMax Light Film, Amersham Biosciences, Buckinghamshire, UK) prior to development. The duration of exposure required to produce interpretable results on the X-ray film was variable. The principal reason for this was the exponential decay of signal strength with time produced by the chemiluminescent substrates used. Therefore, the first film was developed after an initial exposure of 10 seconds. If the signal strength was either insufficient or too strong then further exposures of longer or shorter duration were developed.

The intensity of observed bands was quantified using image J (www.imagej.net) analysis software and reported values were standardised to the intensity of alpha tubulin signals.

Table 11. Antibodies used in western blotting

*All secondary antibodies supplied by DAKO, Cambridge, UK unless indicated

Antibody	**Genus**	**Manufacturer**	**Concentration and diluent**	**Corresponding secondary antibody**
AXL AF154	Polyclonal goat IgG	R and D Systems	1µg/ml in 5% non-fat dry milk and 0.1% TBST	DαG-HRP (Santa Cruz)
AKT	Rabbit monoclonal IgG	Cell Signaling	1µg/ml in 5% non-fat dry milk and 0.1% TBST	GαR-HRP
pAKT (Ser473)	Rabbit monoclonal IgG	Cell Signaling	1µg/ml in 5% non-fat dry milk and 0.1% TBST	GαR-HRP
pAKT (Thr308)	Rabbit monoclonal IgG	Cell Signaling	1µg/ml in 5% non-fat dry milk and 0.1% TBST	GαR-HRP
pERK1/2 (Thr202/Tyr204)	Rabbit monoclonal IgG	Cell Signaling	1µg/ml in 5% non-fat dry milk and 0.1% TBST	GαR-HRP
pMEK	Rabbit monoclonal IgG	Cell Signaling	1µg/ml in 5% non-fat dry milk and 0.1% TBST	GαR-HRP
α tubulin	Mouse monoclonal antibody	Cell Signaling	1µg/ml in 5% non-fat dry milk and 0.1% TBST	GαM-HRP

D=donkey, M=mouse , G=goat, R=rabbit

Individual experiments

Determination of AXL expression

The 10 cell lines were grown as described above and passaged into 6cm dishes. Once between 70% and 100% confluent, cells were lysed with 800ul of laemmli buffer. Protein levels were quantified and standardised. The final lysates were processed alongside 7µl of marker and resultant membranes stained for AXL expression.

Stimulation of cells with GAS6

Both J82 (AXL positive) and RT112 (AXL negative) cells were each passaged into 4 separate 6cm dishes following standard culture to 70% confluence in T-175 flasks. Following rinsing with PBS twice, culture medium was replaced with serum-free medium and incubated overnight. Human recombinant GAS6 (R&D, Abingdon, UK) was added to give an overall concentration of 2µg/ml in the serum free medium. Cells were lysed at 4 time points (t=0, t=15 minutes, t=30 minutes and t=120 minutes). Lysates were processed in the usual fashion and expression of AXL, total AKT, $pAKT_{S473}$, $pAKT_{T248}$, pMEK and pERK1/2 were assessed using western blotting as described.

Transfection with siRNA to AXL

Cells were grown in the standard manner to a population of 8 million. Four cell pellets, each with 2 million cells, were mixed with 60ul of transfection buffer and 3 microlitres of siRNA or control scatter siRNA in a electroporation cuvette (0.2cm bio rad). A Gene PulserXcell electroporation system was used to apply a single pulse (250mV, 250F). The contents of the cuvette were then resuspended in culture medium and transferred to 6cm culture dishes. After further culture for 48 hours, cells were lysed and western blotting to detect AXL, AKT, $pAKT_{S473}$, $pAKT_{T248}$, pMEK and pERK1/2 followed.

Inhibition of AXL with foretinib

To assess the AXL-specific effects of foretinib, we used both GAS6 stimulated cells and quiescent J82 cells. Following standard culture and passage into 4 separate 6cm dishes, J82 cells were serum starved overnight prior to incubation with 10nM foretinib or control (DMSO) for 2 hours. Growth medium in one foretinib-containing and in the control culture dish was then supplemented with sufficient human GAS6 to give a concentration of 2µg/ml. After 5 minutes cells were lysed in the described fashion.

We then evaluated the effect of increasing concentrations of foretinib on J82 cells. Cells were serum starved overnight, prior to incubation with varying concentrations of foretinib or DMSO as a control (5mM, 10mM, 25mM and 50mM) for 2 hours. Recombinant human GAS6, giving a final concentration of 2µg/ml was added to the growth medium and foretinib solution. After 5 minutes, cells were lysed for further analysis.

Reagents and Solutions

Immunohistochemistry

Blocking solution (20mls) - 20mls TBS / 3%BSA (0.6g) / 100µl 20Triton-X

Cell culture

Lysis buffer (0.125M TrisHCL, 2% SDS, 10% glycerol)

Western blotting

5% Stacking Gel (3ml) - 0.5ml 30% acrylamide mix, 0.38ml 1.0M Tris pH 6.8, 0.03ml 10% SDS, 0.03ml 10% ammonium persulfate (APS) and 0.003ml TEMED, 2.06ml deionized water

10% Resolving Gel (10ml) - 4.1mls deionized water, 3.3ml 30% acrylamide solution, 2.5ml 1.5M Tris-HCL pH 8.8, 0.1ml 10% SDS, 0.1ml APS, 0.01ml TEMED

Running buffer (1L) - 3.02g Tris-base, 18.8g glycine and 10ml 10% SDS made up to 1L with deionised water

Transfer buffer (stock buffer solution) (1L) - 58.1g Tris-base, 29.3g glycine, 37.5ml 10% SDS made up to 1L with deionised water

Transfer buffer (final solution) (1L) - 100mls stock solution, 200mls methanol 700mls deionised water

blocking solution - 5% non-fat dry milk powder, 0.1% TWEEN 20, Tris buffered saline (TBS) or BSA solution depending on antibody

Chapter 3: Results

Human Immunohistochemistry

Preliminary Results

Optimisation of primary antibody concentration

The variation in staining with varying primary antibody concentration is shown in figure 13-15. Our interpretation was that the most intense staining, in the absence of background was achieved at a concentration of 5µg/ml (1:40). Furthermore, a typical section of human stomach is shown and compared to the expected staining pattern, according to the manufacturers (figure 16).

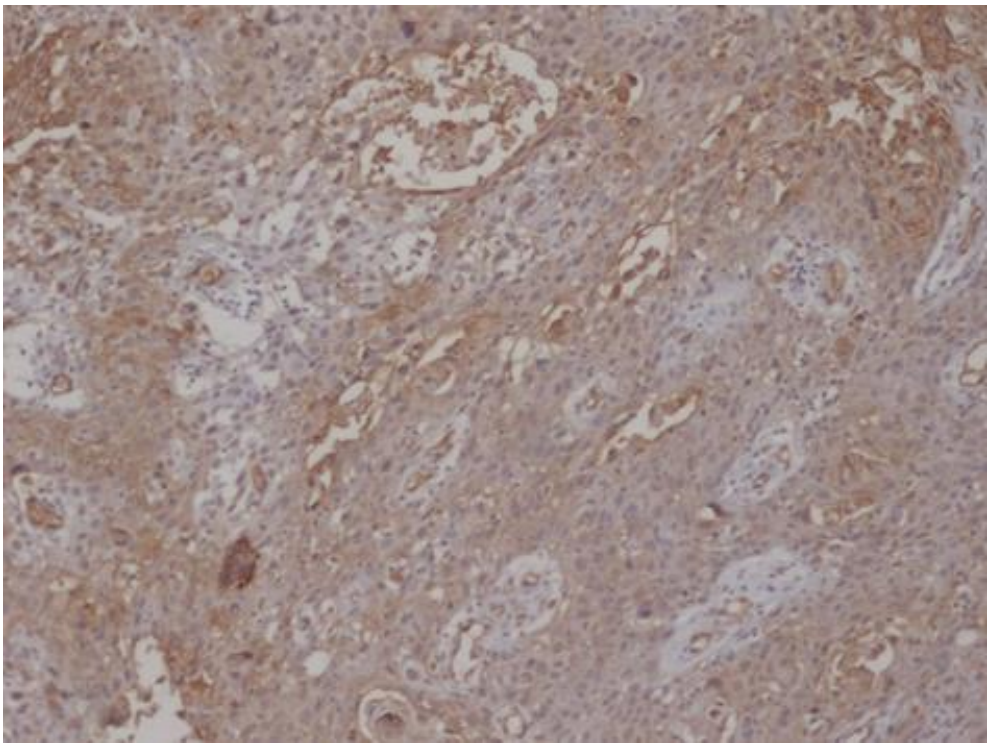


Figure 13 – AXL immunostaining at AF154 concentration of 20µg/ml (1:10)

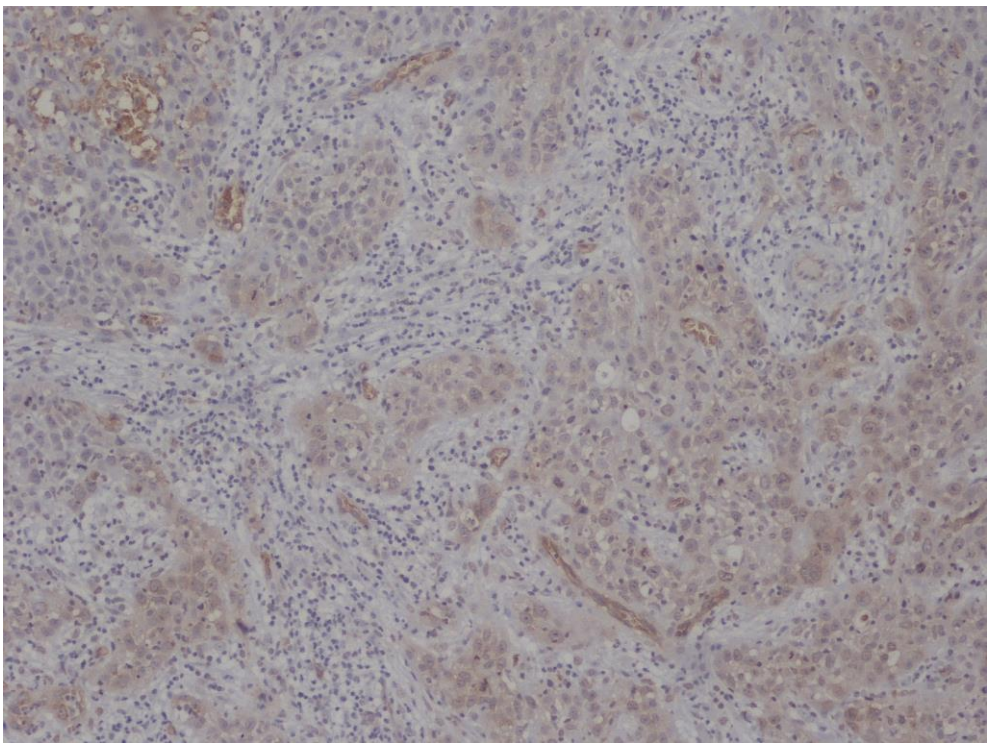


Figure 14 – AXL immunostaining at AF154 concentration of 5µg/ml (1:40)

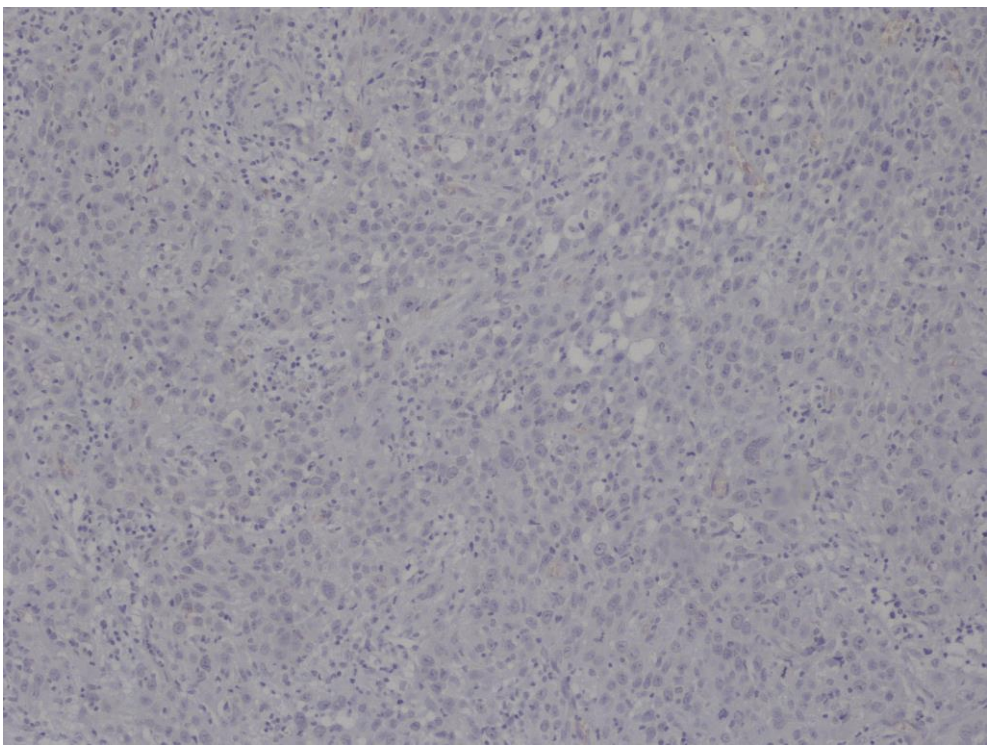


Figure 15 – AXL immunostaining at AF154 concentration of 1µg/ml (1:200)

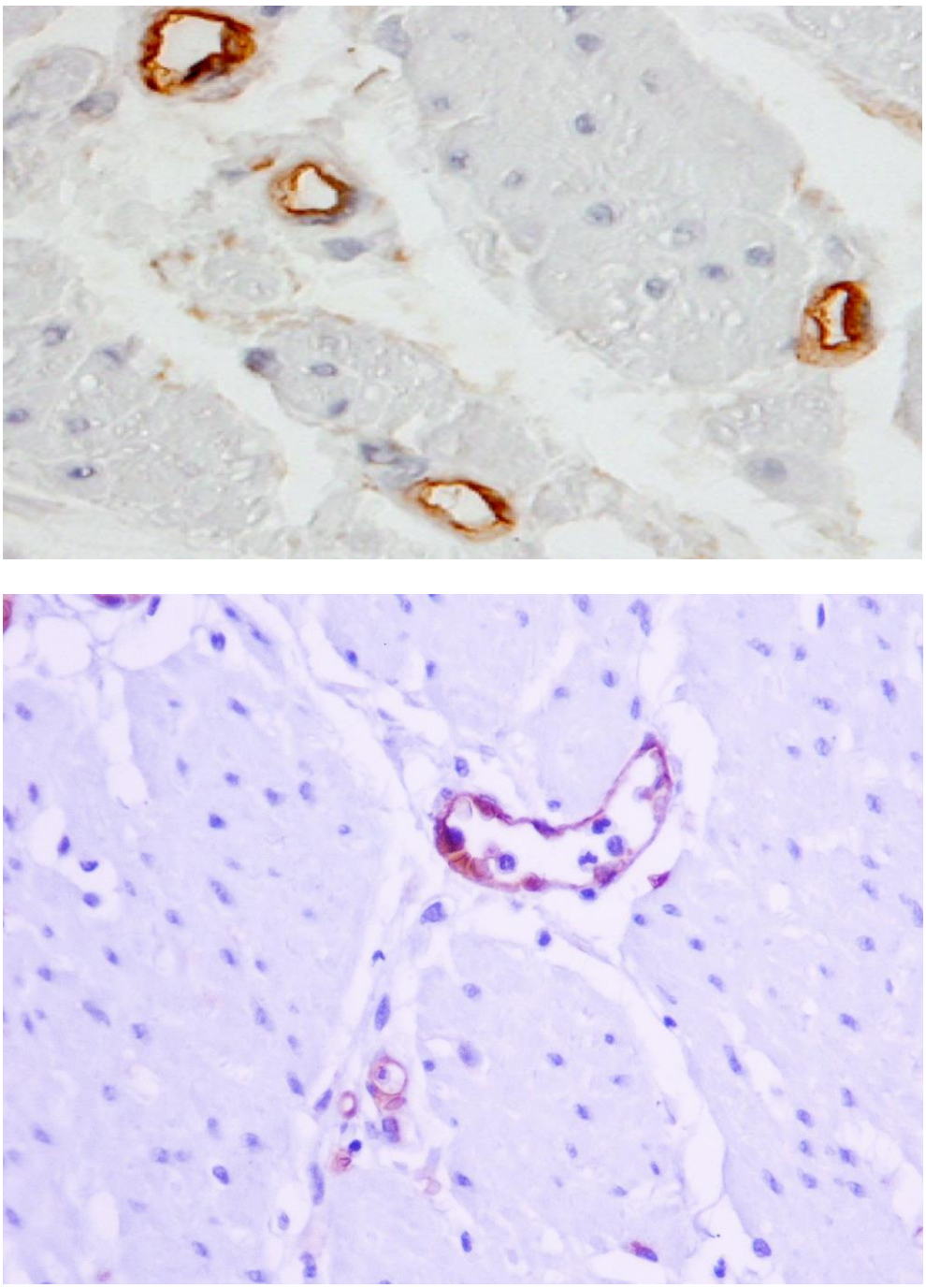


Figure 16. Comparison of AXL staining of the manufacturers recommended positive control, with reference image(top) and our staining at 5µg/ml (1:40) (bottom).

Cystectomy Series: Qualitative analysis

Our initial investigation revealed that, when present, AXL staining was distributed in the cell membrane almost exclusively (figure 17). However, the staining pattern was often heterogeneous within each section (figure 18). Explanations for this include variations in the way samples were initially fixed in formalin, tumour heterogeneity with different sub-clones of cells and protein expression and variations in the technique of applying reagents to each section. Heterogeneity within each section, regardless of the cause, poses problems with accurately scoring the stained sections. Determining whether a negative region within a section is negative because there is no AXL expression or a technical artefact becomes difficult. Each batch of processed slides contained one positive control (human stomach). However, we also observed that in all stained sections, vascular endothelium was consistently positive (figure 19). That is, the staining in vascular endothelium was similar, regardless of whether the adjacent section contained AXL positive tumour, AXL negative tumour or normal urothelium.

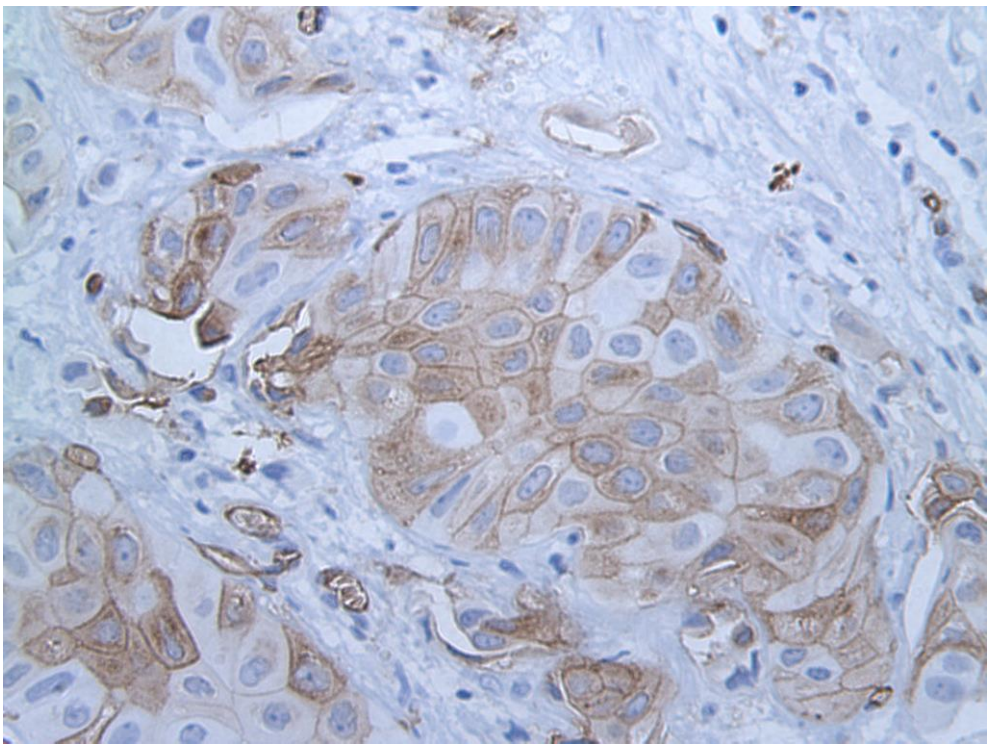


Figure 17. Predominant AXL membrane staining in MIBC

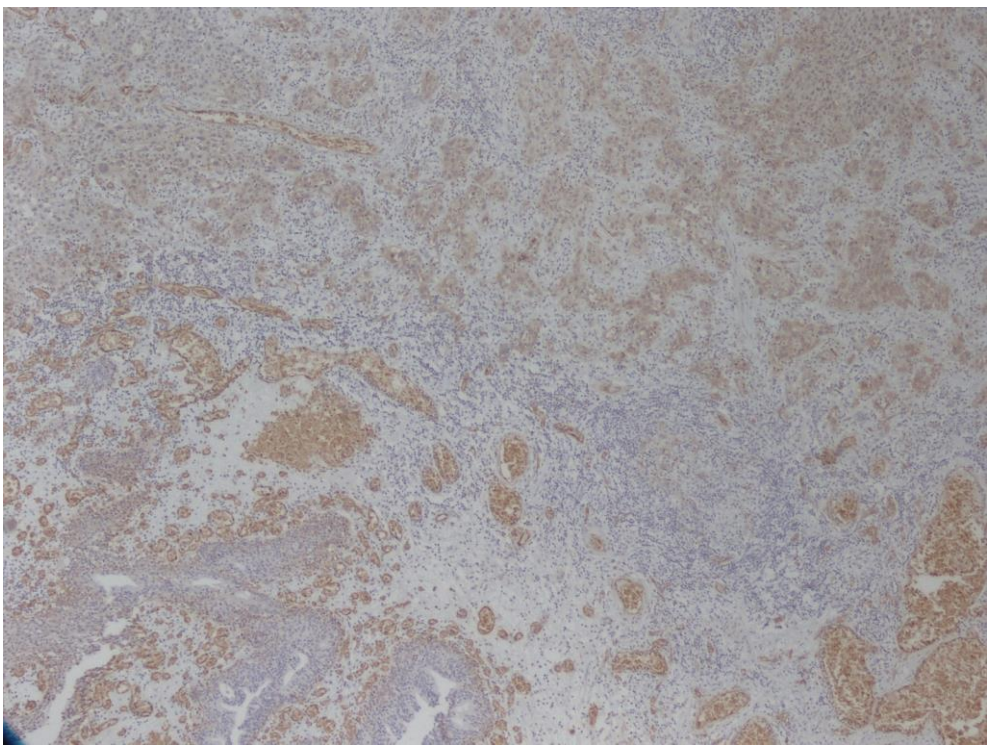


Figure 18. Heterogeneity, with regards to AXL staining across entire MIBC section

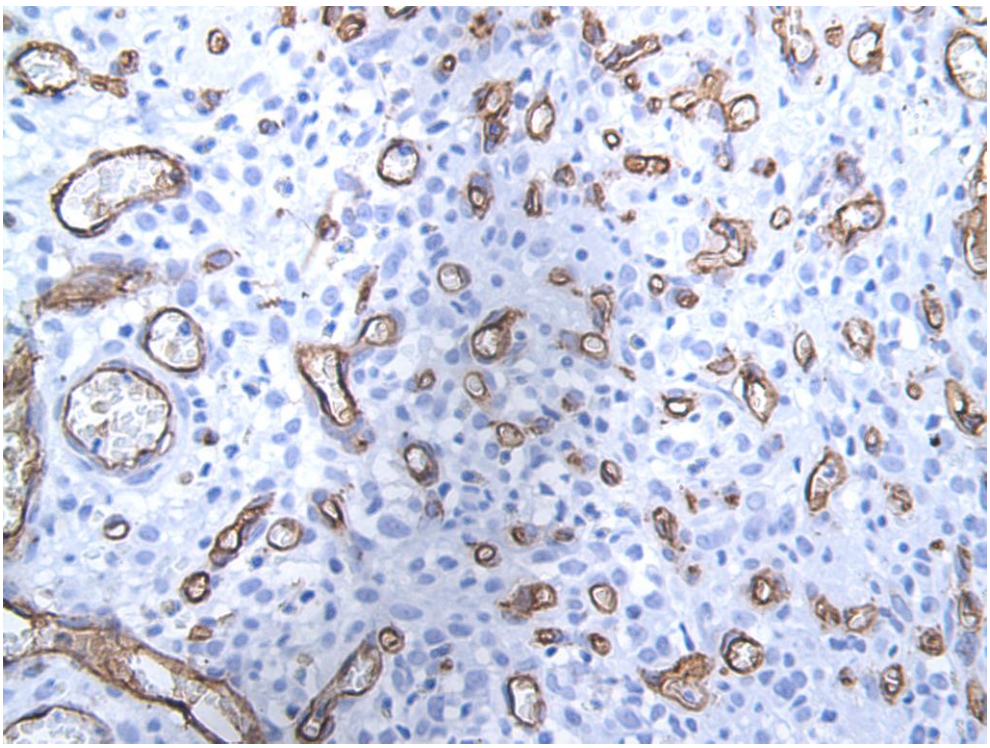


Figure 19. Consistent vascular endothelial staining in bladder cancer sections

In NMIBC, AXL expression was strongest in the basal urothelial cell layer (figure 20). In view of the recent advances in molecular subtyping of bladder cancers, this suggests that AXL may act as a marker of basal cancers, which have a worse prognosis. Although the intensity of staining in TURBT specimens was often variable, the most intense staining was observed in foci of CIS (figure 21). We found no evidence of AXL positivity in stromal cells associated with normal or cancerous urothelium apart from in vascular endothelium, which was consistent regardless of associated MIBC, NMIBC or normal urothelium (figure 19)

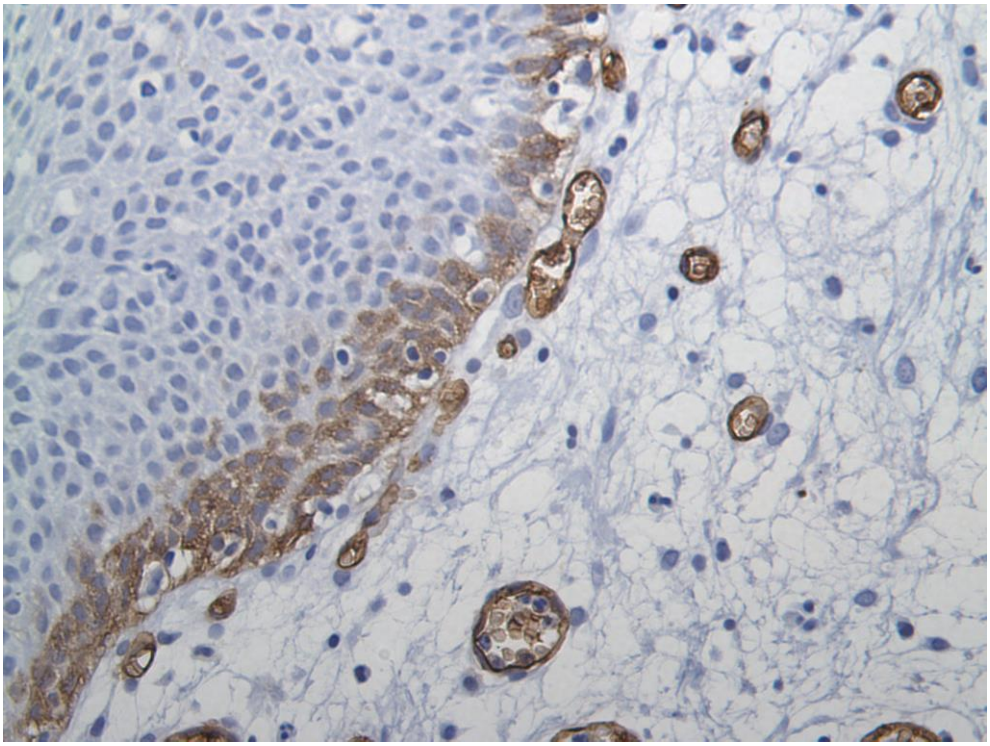


Figure 20, Basal cell distribution in NMIBC

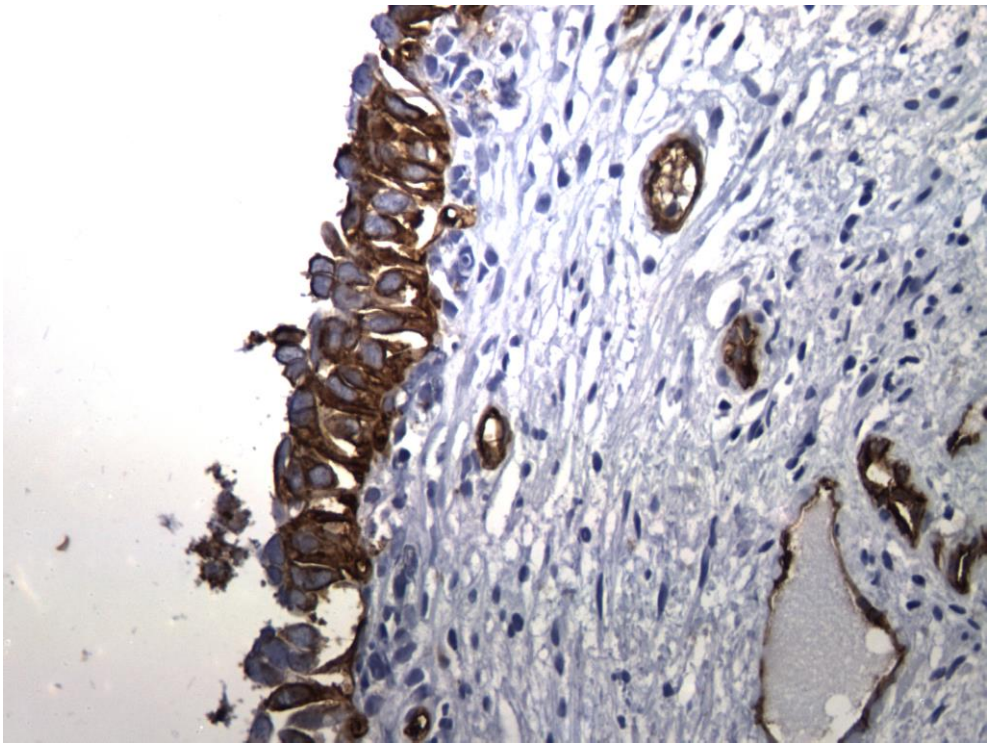


Figure 21, Strong AXL staining in CIS

Determining AXL IHC score and threshold values

Following a qualitative analysis of AXL expression, we sought to quantify AXL expression, via an IHC score discussed previously. In this analysis, the overall aim of IHC is to equate visual appearance to clinical phenotypes of disease. In this study where possible we have analysed both AXL score as a continuous variable and AXL status as a binary discrete variable.

The patient characteristics and spread of AXL scores in this cystectomy series are given in table 12 and figure 22. No patients in this series had neoadjuvant chemotherapy prior to cystectomy as this was not considered standard practice at the time.

Table 12. cystectomy series patient characteristics

Mean age (range)		65.4 (41,78)
Male gender (%)		45 (69.2%)
NMIBC	pTa	6 (28.6%)
	pT1	13 (61.9%)
	CIS	7* (33.3%)
	High grade/G3 (%)	15 (71.4)
MIBC	pT2	21 (47.7%)
	pT3	14 (31.8%)
	pT4	9 (20.5%)
	CIS	7 (15.9%)
	High grade/G3 (%)	42 (64.4)
Total		65

*5 patients with CIS in combination with NMIBC

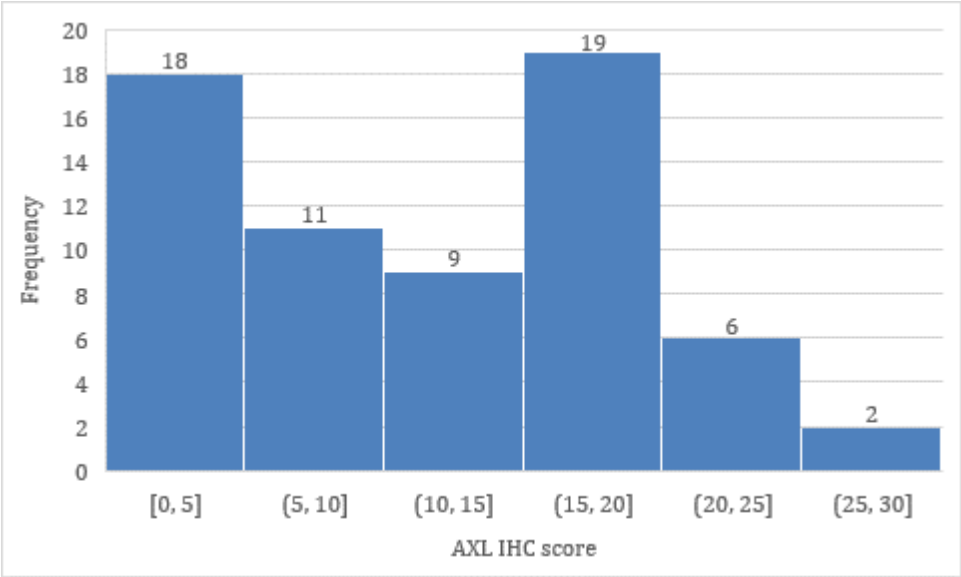


Figure 22. Histogram illustrating spread of AXL IHC scores in cystectomy series.

ROC analysis, using muscle invasion as the test variable this instance AXL score and muscle invasion were compared. The area under the curve (AUC) of 0.727 (95%CI 0.594, 0.861) (figure 23) indicates that AXL score is a good discriminator of muscle invasion. That is, sections with a higher AXL score are significantly more likely to be muscle-invasive. ROC analysis also provides a rational for the selection of threshold values for scores which could be considered positive or negative, and hence convert AXL score from an ordinal scale to AXL status, a dichotomous variable.

Table 13 and figure 24 shows the way specificity and sensitivity of AXL score varies with varying AXL IHC scores. The maximal value of the sum of sensitivity and specificity, the Youden index, gives the value which best discriminates between positive and negative test variables. In this, instance muscle invasion.

This corresponds to an AXL IHC score of 15, which was used as a threshold value for subsequent analysis.

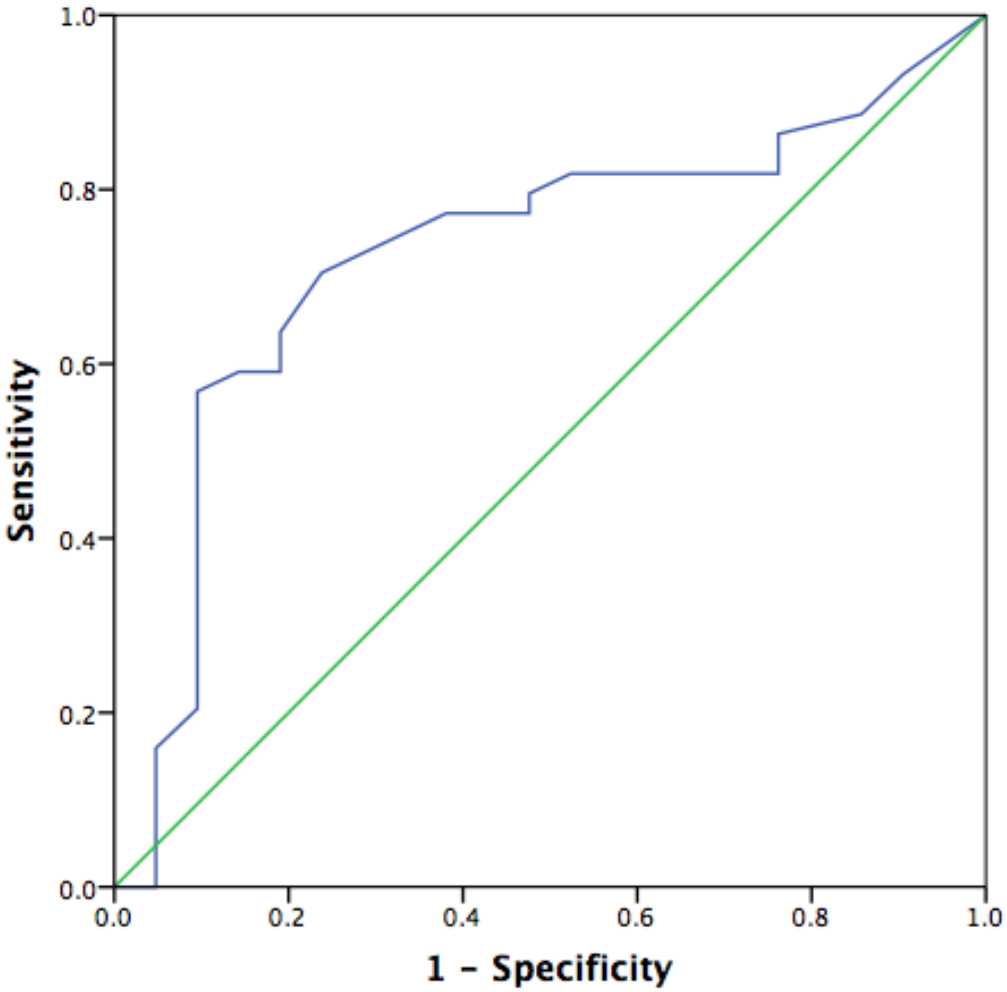


Figure 23. ROC curve comparing AXL IHC score and the presence of muscle invasion

Table 13. Sensitivity, specificity and approximation of the Youden index with varying AXL score and muscle invasion as test variable

AXL score	Sensitivity	Specificity	Sum
0.5	0.932	0.095	1.027
1.5	0.886	0.143	1.029
2.5	0.864	0.238	1.102
3.5	0.818	0.238	1.056
4.5	0.818	0.381	1.199
5.5	0.818	0.476	1.294
6.5	0.795	0.524	1.319
7.5	0.773	0.524	1.297
8.5	0.773	0.619	1.392
9.5	0.727	0.714	1.441
10.5	0.705	0.762	1.467
11.5	0.636	0.81	1.446
12.5	0.614	0.81	1.424
13.5	0.591	0.81	1.401
14.5	0.591	0.857	1.448
15.5	0.568	0.905	1.473
16.5	0.409	0.905	1.314
17.5	0.318	0.905	1.223
18.5	0.273	0.905	1.178
19.5	0.205	0.905	1.11
20.5	0.159	0.952	1.111
22	0.136	0.952	1.088
23.5	0.091	0.952	1.043
24.5	0.045	0.952	0.997
25.5	0.023	0.952	0.975
27.5	0	0.952	0.952
30	0	1	1

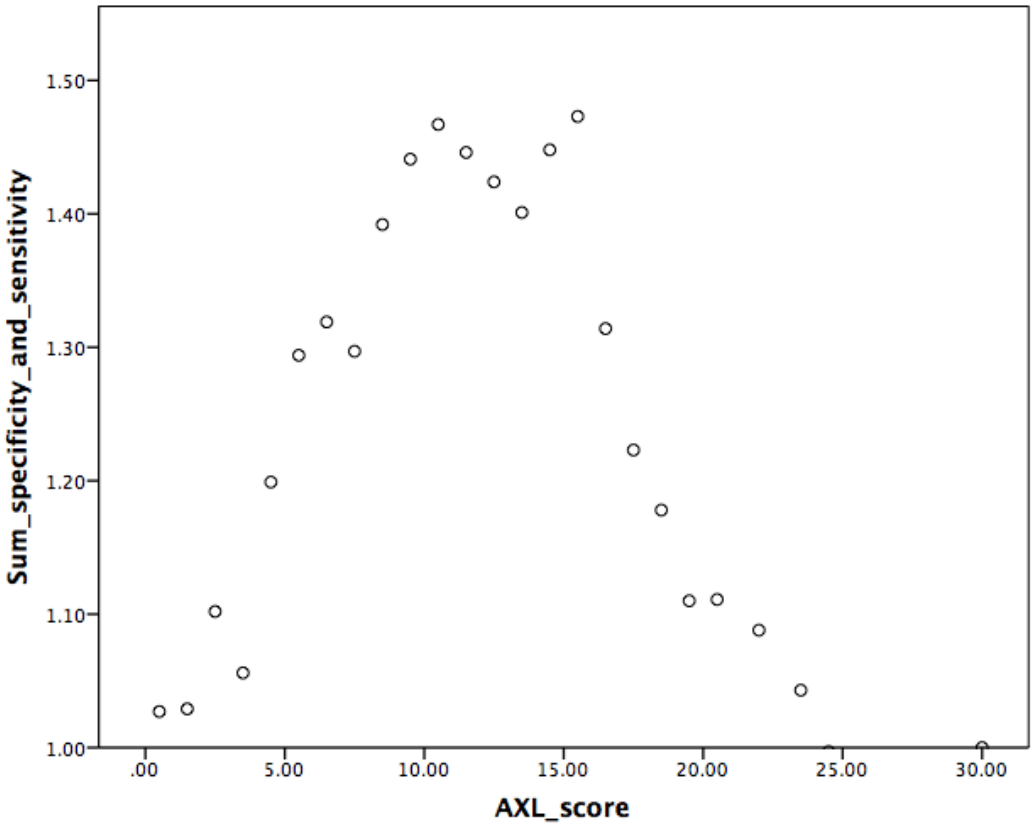


Figure 24. Approximation of the Youden index with varying AXL IHC score

Analysing whole TURBT sections from patients who subsequently underwent radical cystectomy allows a detailed qualitative analysis of the expression pattern in bladder cancer. However, this group of patients represent only a subset of all patients with bladder cancer. As such the clinical variables we were able to correlate with AXL expression, in this series were modified accordingly. We, therefore, assessed the correlation between AXL expression with the presence of muscle invasion and the presence of pre-operatively unsuspected lymph node metastasis.

AXL status and MIBC

Univariate and multivariate analysis, controlling for age, gender, grade and CIS, of predictors of invasive disease reveal that AXL positivity is associated with muscle-invasive, rather than non-muscle invasive bladder cancer (OR 3.8 and 23.3 on univariate and multivariate analysis respectively). This was the only variable that is associated with invasion on both univariate and multivariate binary logistic regression (table 14). Interestingly, male gender was also associated with invasion on multivariate analysis. This is likely to be as a result of a relatively small number of women in the included analysis (n=20). Nonetheless, it highlights the importance of controlling for gender on the subsequent analysis.

Table 14. Univariate and multivariate analysis of predictors of invasion

	Univariate analysis				**Multivariate analysis**			
Variable	**OR**	**95% CI**		**P**	**OR**	**95% CI**		**P**
Age	1.014	0.957	1.075	0.631	1.090	0.996	1.192	0.060
Gender	3.778	0.965	14.787	0.056	7.508	1.470	38.351	0.015
High grade	4.271	0.912	19.993	0.065	5.540	0.670	45.803	0.112
AXL status	3.176	1.066	9.462	0.038	23.291	2.082	260.538	0.011
CIS	0.556	0.164	1.878	0.344	0.015	0.001	0.255	0.004

AXL score was also a predictor of invasion (OR 1.110 (95% CI 1.02, 1.207 P=0.016) that is, with each point rise (from 0-30) in AXL score the chance of invasive disease increases (table 13).

Table 15. Univariate and multivariate analysis of associations with invasion

	Univariate				Multivariate			
	OR	**95% CI**		**P**	**OR**	**95% CI**		**P**
AXL score	1.113	1.029	1.204	0.007	1.110	1.020	1.209	0.016
Gender	3.778	0.965	14.787	0.056	5.383	1.067	27.163	0.042
Age	1.014	0.957	1.075	0.631	1.062	0.985	1.146	0.118
High grade	4.271	0.912	19.993	0.065	5.527	0.851	35.904	0.073
CIS	0.556	0.164	1.878	0.344	0.162	0.030	0.875	0.034

It would appear in this analysis (table 14 and 15) that CIS confers a protective association with muscle invasion. That is, the presence of CIS makes invasion less likely. This would however be an unexpected finding. It is perhaps is more in keeping with selection bias of patients undergoing cystectomy for high-risk NMIBC, which is conferred by the presence of CIS rather than indicating a protective association between CIS and invasion.

Table 16. Contingency table for AXL status and muscle invasion

	AXL positive		**AXL negative**		**Totals**	
MIBC	27	(61%)	17	(39%)	44	(68%)
NMIBC	7	(33%)	14	(67%)	21	(32%)
Totals	34	(52%)	31	(48%)	65	(100%)

Further univariate analysis in this series (Table 16) reveals that AXL positivity, as a test to predict invasion, has a sensitivity of 61.4%, specificity of 66.7%, positive predictive value of 79.4% and negative predictive value of 45.2%.

AXL status and metastatic disease

In patients with MIBC, AXL positivity was associated with preoperatively unsuspected lymph node or visceral metastasis (OR 5.5 (CI 1.0134, 29.8508) P=0.0482 Chi squared test) following complete pathological staging. AXL status, as a biomarker of unsuspected lymph node metastasis has a sensitivity of 84.6%, specificity of 50%, negative predictive value of 86.7% and positive predictive value of 45.8% (table 17).

Similarly, AXL score was significantly associated with metastatic disease (OR 1.134 (95% CI 1.002, 1.282 P=0.046) on univariate analysis.

On multivariate analysis, controlling for age, gender, grade, stage and concomitant CIS neither AXL status, nor score, reached statistical significance in terms of predicting metastatic disease. However, in this series there were 39 patients with MIBC who underwent lymphadenectomy and this number is likely to be insufficient to undertake meaningful multivariate analysis.

Table 17. AXL status and metastatic disease

	AXL Positive		**AXL negative**		**Totals**	
Metastatic MIBC	11	(85%)	2	(15%)	13	(33%)
Non-metastatic MIBC	13	(50%)	13	(50%)	26	(67%)
Totals	24	(62%)	15	(38%)	39	(100%)

CIS sub-analysis

Fourteen patients in this series had foci of CIS available for analysis in their TURBT sections. Seven (50%) of these patients had CIS in conjunction with muscle invasive disease, 5 (36%) patients had CIS with other associated non-muscle-invasive lesions whilst 2 (14%) patients had CIS in isolation. Overall 11 (79%) exhibited AXL positive immunostaining (figure 21). Interestingly, 4 patients had AXL negative NMIBC but AXL positive foci of CIS. The potential inference being that AXL expression varies between lesions in the same patient and that the most invasive lesions or at least potentially invasive lesions are AXL positive.

Lymph node metastasis sub-analysis

Seven of 11 (63%) histologically confirmed lymph nodes available for analysis displayed AXL expression (figure). Of the 11 nodes positive for cancer, 10 primary tumours were AXL positive and one AXL negative. Of the seven AXL positive lymph nodes, all primary tumours were AXL positive.

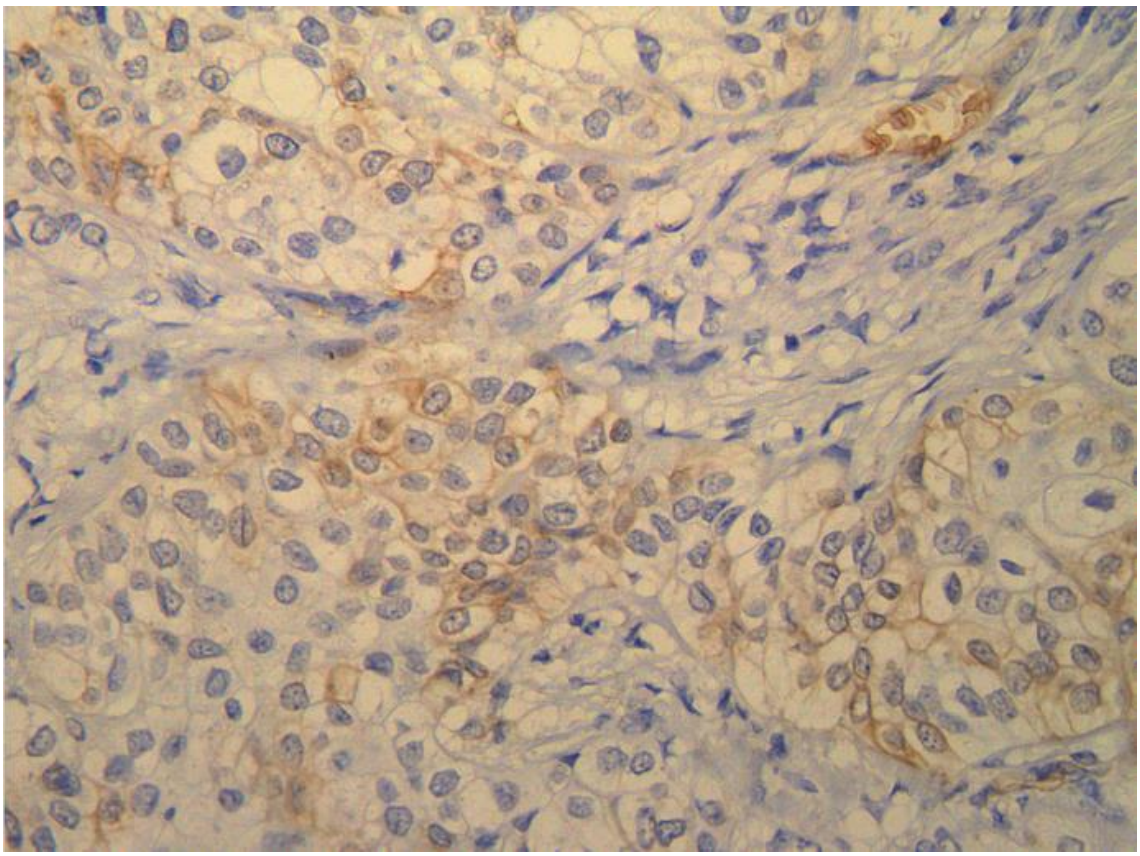


Figure 24. AXL positive metastatic lymph node

TMA analysis

Given the larger sample size, TMA analysis allows more powerful multivariate analysis in addition to further univariate analysis. For multivariate analysis it is recommended that for each measured variable there are at least 10 patients in each subgroup (118-120), hence the equivocal results in our cystectomy series. Patient characteristics and distribution of AXL scores are given in table 18 and figure 25.

Table 18. Patient characteristics: TMA analysis

Age (years), median		73		
Male		211	74.3%	
Female		73	25.7%	
NMIBC	Stage	pTa	102	60.3%
		pT1	65	38.4%
		CIS	2	1.2%
	High-grade (G3)		43	25.4%
	Standard Therapy		155	91.7%
	Radical Cystectomy		14	8.3%
MIBC	Stage	T2	99	86.1%
		T3/T4	16	13.9%
		M1 at	62	53.9%
	High-grade (G3)		95	82.6%
	Radical Cystectomy		37	71.2%
	Radiotherapy		15	28.8%
	Neoadjuvant chemotherapy		7	16.7%

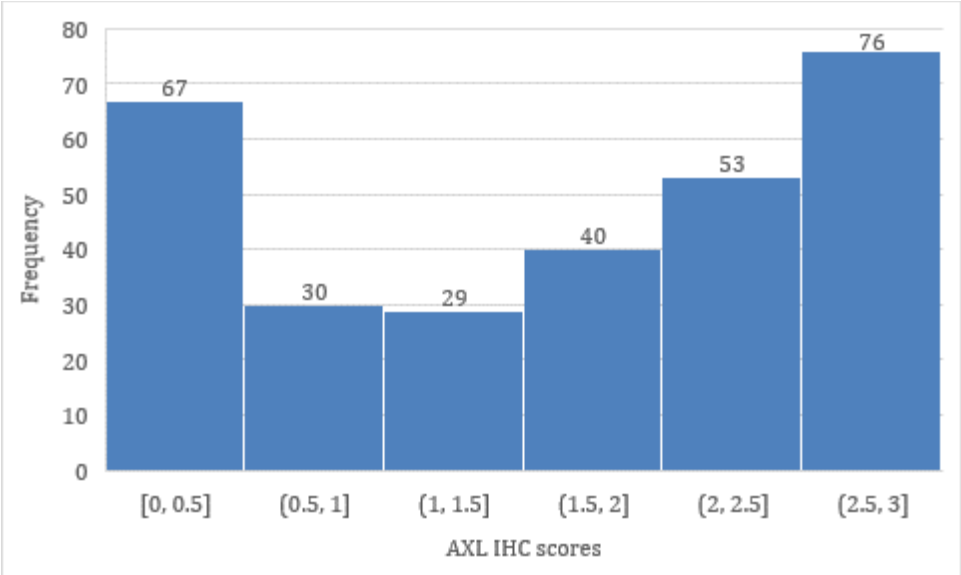


Figure 25. Histogram illustrating distribution of AXL scores in TMA analysis

Given the hypothesis of AXL expression resulting in invasion and metastasis, AXL expression was correlated with the following clinical variables;

1. The presence of muscle invasion at diagnosis
2. The presence of metastasis at diagnosis
3. The ultimate requirement of radical therapy in patients initially diagnosed with NMIBC
4. The development of latent metastasis in patients treated, with the intent of cure, for MIBC
5. Disease-specific survival in patients treated, with the intent of cure, for MIBC

As in the cystectomy series, both univariate and multivariate analysis were used. Where appropriate patient age, gender, grade (high grade), stage, prior neo-adjuvant chemotherapy, radical treatment type (cystectomy or radiotherapy) were also controlled for in multivariate analysis.

Determining threshold values for AXL status

A threshold value for AXL positivity was determined in a similar manner to that in our cystectomy series. Namely plotting of ROC curves with invasive disease as a test characteristic followed by plotting the sum of sensitivity and specificity and approximating the Youden index (figure 26, 27 and table 19). The AUC was lower

in comparison to the cystectomy series, but still significant (AUC 0.646 (95% CI 0.581, 0.710, P<0.05). The Youden index was 1.625 and therefore, values ≥ 1.6 were considered positive in the remainder of the analysis.

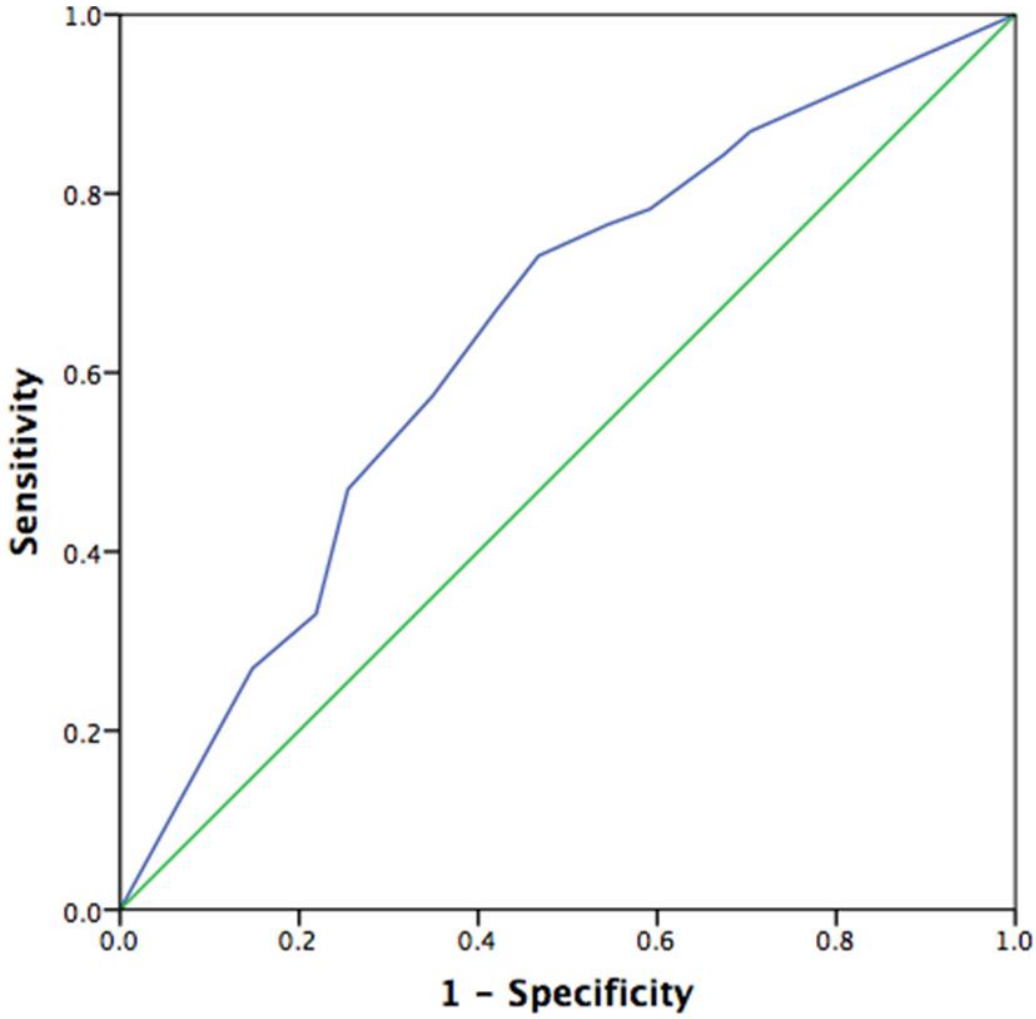


Figure 26. ROC analysis with muscle invasion as test variable

Table 17. TMA analysis: Youden index calculations

AXL score	Sensitivity	Specificity	Sum
0.25	0.896	0.237	1.133
0.625	0.87	0.296	1.166
0.875	0.843	0.325	1.168
1.125	0.783	0.408	1.191
1.375	0.765	0.456	1.221
1.625	0.73	0.533	1.263
1.875	0.67	0.58	1.25
2.125	0.574	0.651	1.225
2.375	0.47	0.746	1.216
2.625	0.33	0.781	1.111
2.875	0.27	0.852	1.122

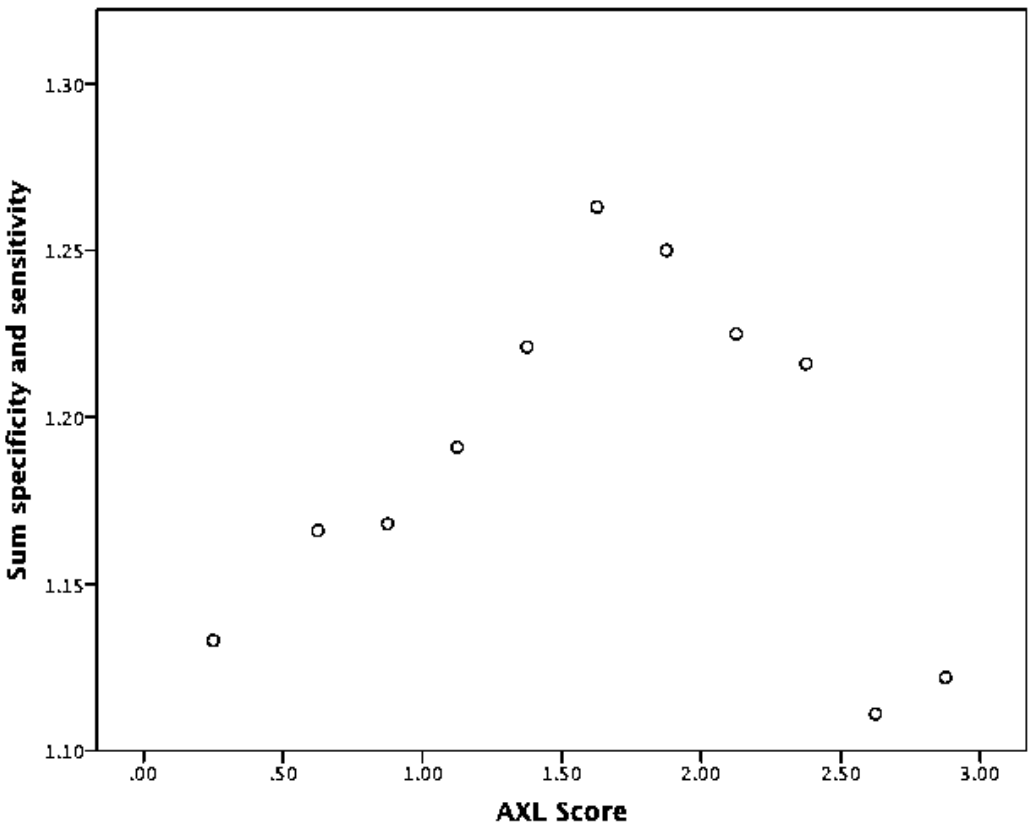


Figure 27. TMA analysis Youden index plot with muscle invasion as test variable

Muscle invasion at diagnosis

Both AXL score and AXL status were independent predictors of muscle invasion on univariate analysis (AXL score OR 1.653 (95% CI 1.302, 2.099 P<0.0001; AXL status OR 3.087 (95% CI 1.852, 5.145 P<0.0001)) and multivariate analysis (AXL score OR 1.464 (95% CI 1.098, 1.952 P<0.05) (table 20); AXL status OR 2.411 (95% CI 1.300, 4.4700 P<0.05)) when controlling for age, gender and high grade (G3) disease (table21). The association of increasing age, female gender and high-grade disease with muscle invasion is well established and supports the external validity of this study.

Table 18. Univariate and multivariate analysis of predictors of muscle invasion including AXL score

	Univariate analysis				**Multivariate analysis**			
Variable	**OR**	**95% CI**		**P**	**OR**	**95% CI**		**P**
AXL score	1.653	1.302	2.099	0.000	1.464	1.098	1.952	0.009
Age	1.027	1.005	1.049	0.014	1.026	0.998	1.056	0.073
Gender	0.509	0.298	0.870	0.014	0.414	0.203	0.844	0.015
High grade	14.805	8.112	27.023	0.000	15.123	7.957	28.741	0.000

Table 21. Univariate and multivariate analysis of predictors of muscle invasion including AXL status.

	Univariate analysis				Multivariate analysis			
Variable	**OR**	**CI**	**CI**	**P**	**OR**	**CI**	**CI**	**P**
AXL status	3.087	1.852	5.145	0.000	2.411	1.300	4.4700	0.005
Age	1.027	1.005	1.049	0.014	1.027	0.998	1.056	0.025
Gender	0.509	0.298	0.870	0.014	0.423	0.207	0.866	0.019
High grade	14.805	8.112	27.023	0.000	15.328	8.048	29.192	0.000

With regards to biomarker studies AXL status (table 20), when used to analyse invasion has a sensitivity of 54%, specificity of 47%, negative predictive value of 62.9% and positive predictive value 43.9%.

Table 22. Contingency table stratifying AXL status and muscle invasion

	AXL Positive		**AXL negative**			
MIBC	62	(54%)	53	(46%)	115	(40%)
NMIBC	79	(47%)	90	(53%)	169	(60%)
Totals	141	(50%)	143	(50%)	284	(100%)

Metastatic disease at presentation

AXL score and AXL status were associated with metastatic disease at diagnosis of bladder cancer on univariate analysis (AXL score OR 1.484 (95% CI 1.113, 1.979 $P<0.05$); AXL status OR 2.257 (95% CI 1.219, 4.178 $P<0.05$)) (table 23 and 24). However, on multivariate analysis, controlling for age, gender and high-grade

disease the significance falls to below that of conventional statistical thresholds (AXL score OR 1.208 (95% CI 0.868, 1.682 P=0.263); AXL status OR 1.387 (95% CI 0.689, 2.791 P=0.359)). The associations between metastatic disease, age, female gender and high-grade disease were all statistically significant. Explanations for the disparity between assertions from univariate and multivariate analysis include a lack of true association or a weakness in the statistical analysis. In this sub analysis the vast majority of patients had high-grade disease (n=58) in comparison to G1 or G2 disease (n=9). Having a small number of patients in this cohort would inevitably weaken this form of analysis and may account for our findings.

Table 23. Univariate and multivariate analysis of predictors of metastasis at diagnosis including AXL score

	Univariate analysis				**Multivariate analysis**			
Variable	**OR**	**95% CI**		**P**	**OR**	**95% CI**		**P**
AXL score	1.484	1.113	1.979	0.007	1.208	0.868	1.682	0.263
Age	1.058	1.028	1.088	0.000	1.063	1.028	1.099	0.000
Gender	0.490	0.268	0.897	0.021	0.559	0.273	1.145	0.559
High grade	9.032	4.236	19.256	0.000	9.186	4.122	20.472	0.000

Table 24. Univariate and multivariate analysis of predictors of metastasis at diagnosis including AXL status

	Univariate analysis				**Multivariate analysis**			
Variable	**OR**	**95% CI**		**P**	**OR**	**95% CI**		**P**
AXL status	2.257	1.219	4.178	0.010	1.387	0.689	2.791	0.359
Age	1.058	1.028	1.088	0.000	1.063	1.028	1.100	0.000
Gender	0.490	0.268	0.897	0.021	0.561	0.273	1.153	0.116
High grade	9.032	4.236	19.256	0.000	9.374	4.209	20.876	0.000

Metastatic disease following radical treatment for MIBC.

It is standard care for all patients, prior to undergoing radical treatment, with the intent of cure to have to radiological investigations aimed at excluding distant metastasis. Subsequent to radical treatment the radiological follow up of patients with bladder cancer is variable and may lead to bias i.e. patients who are more aggressively investigated are more likely to be diagnosed with metastatic disease than those who are not. Furthermore, none of the eleven patients undergoing neoadjuvant chemotherapy developed metastasis which, potentially further skews the multivariate analysis. This is indicated by the infinitely wide confidence interval observed in table 25 and 26. Unfortunately the time to metastasis was not recorded in the data collection phase and therefore not available to analyse.

Neither AXL score nor AXL status was significantly associated with the development of metastatic disease, following treatment for MIBC, on either univariate or multivariate analysis (table 23 and 24). Conversely high-grade disease appears to be associated with a lower incidence of latent metastasis, this further casts doubt of the significance of this sub analysis.

Table 25. Univariate and multivariate analysis of predictors of latent metastasis including AXL score

	Univariate analysis				Multivariate analysis			
Variable	**OR**	**95% CI**		**P**	**OR**	**95% CI**		**P**
AXL score	1.776	0.752	4.193	0.190	2.578	0.764	8.698	0.127
Age	1.034	0.958	1.115	0.393	0.959	0.851	1.082	0.499
Gender	0.156	0.037	0.653	0.011	0.139	0.022	0.895	0.038
High grade	0.162	0.036	0.734	0.018	0.179	0.024	1.326	0.092
Extravesical	1.594	0.344	7.391	0.552	1.616	0.178	14.679	0.670
Cystectomy	0.700	0.173	2.836	0.617	0.659	0.072	6.035	0.712
Neo adjuvant	0.000	0.000	.*	0.999	0.000	0.000	.*	0.999

*Indicates very wide confidence interval

Table 26. Univariate and multivariate analysis of predictors of latent metastasis including AXL status

	Univariate analysis				Multivariate analysis			
Variable	**OR**	**95% CI**		**P**	**OR**	**95% CI**		**P**
AXL status	1.800	0.338	9.581	0.491	1.754	0.215	14.346	0.600
Age	1.034	0.958	1.115	0.393	0.972	0.868	1.088	0.621
Gender	0.156	0.037	0.653	0.011	0.153	0.025	0.932	0.042
High grade	0.162	0.036	0.734	0.018	0.317	0.056	1.779	0.192
Extravesical	1.594	0.344	7.391	0.552	2.403	0.278	20.772	0.426
Cystectomy	0.700	0.173	2.836	0.617	0.852	0.092	7.929	0.888
Neo adjuvant	0.000	0.000	.*	0.999	0.000	0.000	.*	0.999

*Indicates very wide confidence interval

Table 27. Contingency table stratifying AXL status and latent metastasis in MIBC

	AXL Positive		AXL negative		Totals	
Latent metastasis	9	(82%)	2	(18%)	11	(21%)
No latent metastasis	30	(71%)	12	(29%)	42	(79%)
Totals	39	(74%)	14	(26%)	53	(100%)

AXL status as a marker for developing metastatic disease subsequent to treatment for MIBC has the following characteristics; sensitivity 82%, specificity 29%, negative predictive value 85.7% and positive predictive value of 23% (table 27).

Requirement for radical cystectomy in NMIBC

Both AXL score and AXL status were independent predictors of the need for radical treatment, or standard treatment failure, in patients initially diagnosed with NMIBC on univariate and multivariate analysis controlling for age, gender, high grade disease and stage (pTa or pT1) (table 28 and 29). pT1 disease and high-grade disease were also predictors of the need for radical treatment. This correlates with multicentre studies of risk factors for recurrence and progression (14-16). Of the 14 patients requiring cystectomy, three progressed to MIBC, 10 had high-risk recurrence despite BCG and one had an early cystectomy. The mean time between NMIBC diagnosis and cystectomy was 17 months. Increasing age was shown to be "protective" against the need for radical treatment and this is likely to reflect a higher proportion of patients that are unfit for such treatment rather than a difference in disease biology with advancing age.

Table 28. Univariate and multivariate analysis of predictors of requirement for cystectomy including AXL score

	Univariate analysis				Multivariate analysis			
Variable	**OR**	**95% CI**		**P**	**OR**	**95% CI**		**P**
AXL score	2.150	1.159	3.986	0.015	2.619	1.161	5.907	0.020
Age	0.946	0.906	0.988	0.013	0.886	0.820	0.957	0.002
Gender	3.653	0.461	28.926	0.220	3.053	0.249	37.417	0.383
High grade	9.242	2.724	31.357	0.000	11.876	1.980	71.236	0.007
Stage (pT1)	7.057	1.887	26.390	0.004	7.777	1.080	560.23	0.042

Table 29. Univariate and multivariate analysis of predictors of requirement for cystectomy including AXL score

	Univariate analysis				Multivariate analysis			
Variable	**OR**	**95% CI**		**P**	**OR**	**95% CI**		**P**
AXL status	7.881	1.706	36.405	0.008	10.520	1.905	58.094	0.007
Age	0.946	0.906	0.988	0.013	0.884	0.815	0.957	0.002
Gender	3.653	0.461	28.926	0.220	3.455	0.303	39.427	0.318
High grade	9.242	2.724	31.357	0.000	11.953	1.923	74.313	0.008
Stage (pT1)	7.057	1.887	26.390	0.004	6.448	0.927	44.860	0.060

Evaluation of AXL status as a marker of the need for radical cystectomy in NMIBC shows the following characteristics (table 30); Sensitivity 85.7%, specificity 56.8%, negative predictive value 97.7% and positive predictive value of 15.1%.

When evaluating a role for AXL status in predicting the need for radical cystectomy in high risk NMIBC the biomarker parameters are more encouraging. AXL status has a positive predictive value of 40%, negative predictive value of 8.7% sensitivity of 85.7% and specificity of 53.8%. In other words, patients with AXL positive high-risk NMIBC were 7 times more likely to undergo cystectomy than those with AXL negative tumours (95% CI 1.4, 35.5) (table 31).

Table 30. Contingency table stratifying AXL status and the need for cystectomy in NMIBC

	AXL		AXL			
NMIBC requiring cystectomy	12	(86%)	2	(14%)	14	(8%)
NMIBC not requiring cystectomy	67	(43%)	88	(57%)	155	(92%)
Totals	79	(47%)	90	(53%)	169	(100%)

Table 31. Contingency table stratifying AXL status and the need for cystectomy in high-risk NMIBC

	AXL Positive		AXL negative		Totals	
High risk NMIBC cystectomy	12	(85.7%)	2	(14.3%)	14	(26.4%)
High risk NMIBC no cystectomy	18	(46.2%)	21	(53.8%)	39	(73.6%)
Totals	30	(56.6%)	23	(43.4%)	53	(100%)

Survival analysis

In this series, 73 patients died of bladder cancer whereas 33 died of other causes and 178 were censored at the time of analysis, i.e. were still alive at the most recent recorded follow up. The median follow up was 33 months (range 0, 91). Of the 169 patients diagnosed with NMIBC, 5 died of bladder cancer. As such, this number is insufficient to draw significant conclusion. Therefore, the focus of our survival analysis is on patients diagnosed with MIBC who underwent treatment with the intent of cure and those with metastatic disease at diagnosis.

Disease-specific survival in MIBC

In patients, diagnosed with MIBC who went on to receive treatment with the intent of cure, namely radiotherapy or cystectomy, AXL score was associated with poor disease-specific survival on multivariate analysis (HR 5.255 (CI 1.228, 22.488 P=0.025)), correcting for age, gender, stage, grade, neoadjuvant chemotherapy, radiation therapy or cystectomy (table 32). That is, as AXL score rises the chance of death from bladder cancer rises by 5.255 fold. Although the hazard ratio still indicates an association with AXL positivity and poor survival, the statistical significance was lost when using AXL status rather than score (table 33).

Table 32. Disease-specific survival including AXL score

	Univariate analysis				**Multivariate analysis**			
Variable	**HR**	**95% CI**		**P**	**HR**	**95% CI**		**P**
AXL score	2.41	0.87	6.673	0.08	5.25	1.22	22.488	0.02
Age	1.07	0.98	1.159	0.09	0.99	0.88	1.114	0.87
Gender	0.28	0.07	1.000	0.05	0.37	0.08	1.673	0.20
High grade	0.39	0.11	1.1384	0.14	0.46	0.06	3.321	0.44
Extravesical	2.34	0.59	9.269	0.22	8.98	0.37	213.30	0.17
Cystectomy/radiothe	0.27	0.07	1.001	0.05	0.03	0.00	0.659	0.02
Neo adjuvant therapy	0.04	0.00	419.73	0.50	0.00	0.00	.*	0.99

*Indicates very wide confidence interval

Table 33. Disease-specific survival including AXL status

	Univariate analysis				Multivariate analysis			
Variable	**HR**	**95% CI**		**P**	**HR**	**95% CI**		**P**
AXL status	4.45	0.55	35.59	0.15	9.753	0.56	169.0	0.11
Age	1.07	0.98	1.159	0.09	0.987	0.88	1.102	0.81
Gender	0.28	0.07	1.000	0.05	0.508	0.10	2.371	0.38
High grade	0.39	0.11	1.138	0.14	0.609	0.12	3.099	0.55
Extravesical	2.34	0.59	9.269	0.22	11.33	0.82	156.4	0.07
Cystectomy/radiothe	0.27	0.07	1.001	0.05	0.079	0.00	1.107	0.06
Neo adjuvant	0.04	0.00	419.7	0.50	0.000	0.00	.*	0.98

*Indicates very wide confidence interval

Kaplan Meier survival curves (figure 28) for both AXL positive and negative MIBC patients reveals that although the difference did not reach statistical significance (P=0.124 Mantel-Cox log rank test) the five-year survival was lower in patients with AXL positive tumours (AXL positive 58.7% vs. AXL negative 80.0%). Indeed, only one patient in the AXL negative arm died indicating that the lack of statistical significance is perhaps owing to underpowering.

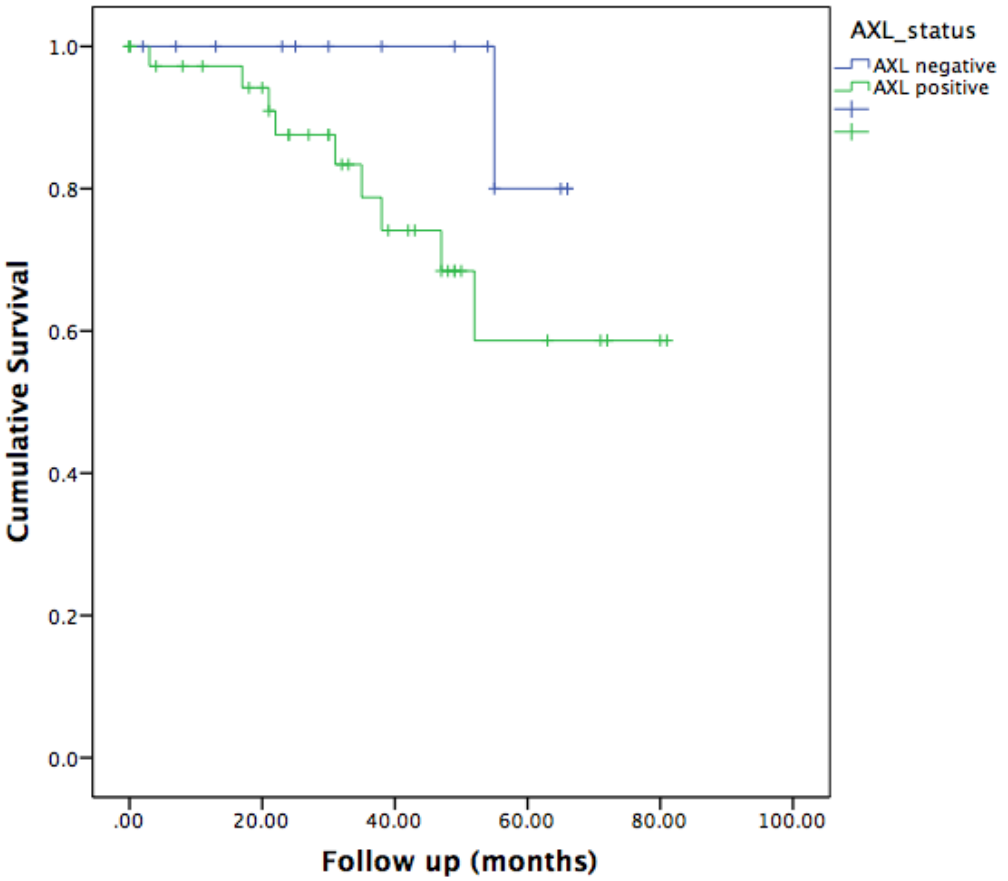


Figure 28. Kaplan Meier curve; AXL status and survival in MIBC

Survival in metastatic disease

Kaplan Meier analysis of survival in patients with metastatic disease at the time of diagnosis reveals no significant difference in survival between the two cohorts (figure 29). Mean and median survival both marginally favoured AXL negative patients (table 34) although the difference was not statistically significant. It is noteworthy, however, that in this sub-analysis it is possible that patients treated

with palliative chemotherapy would be likely to survive longer than those not. Unfortunately, this information was not gathered during the initial data collection.

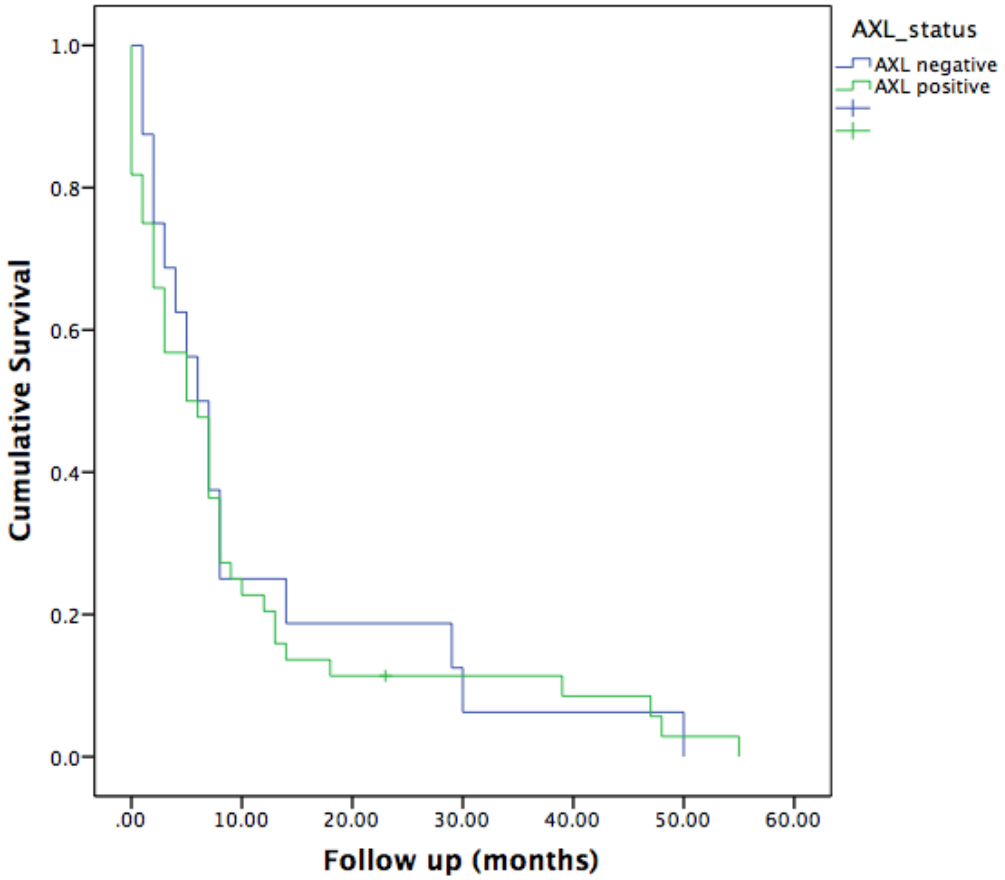


Figure 29. Kaplan Meier analysis: AXL status and survival in metastatic bladder cancer

Table 34. Mean and median survival in AXL positive and negative metastatic disease

	Mean	95% CI		Median	95% CI	
AXL positive	9.915	4.367	17.758	5	3.387	8.613
AXL negative	11.063	4.367	17.758	6	2.111	7.889

Human IHC summary

We have demonstrated that AXL expression associates with phenotypes of bladder cancer with poor prognosis. These include CIS, muscle invasion, metastasis and progression from high-risk NMIBC to MIBC. Comparison of our IHC findings with existing models of bladder cancer development (52,53) further suggests that AXL expression associates with invasive tumours (figure 30). Overall, this supports our hypothesis that AXL expression is implicated in the processes of invasion and metastasis and provides a basis for further study.

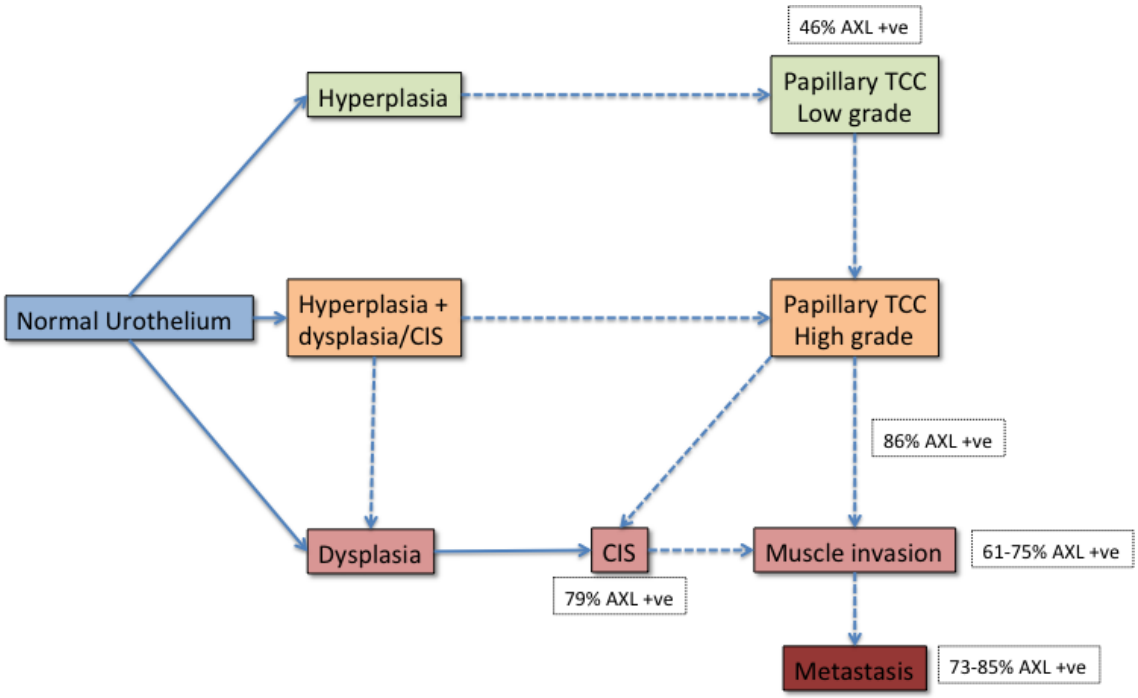


Figure 30. AXL expression at important milestones of bladder cancer development and progression.

Mouse IHC

Immunohistochemistry staining protocol development

The typical staining of MIN mice intestine is shown below (figure 31). There was specific uptake in areas of developing polyps and basal cells of intestinal glands. Additionally, there was very little background staining. As such, we concluded that the protocol used in humans provided specific staining in mice. Therefore, we used the same protocol in staining the mouse sections of bladder cancer.

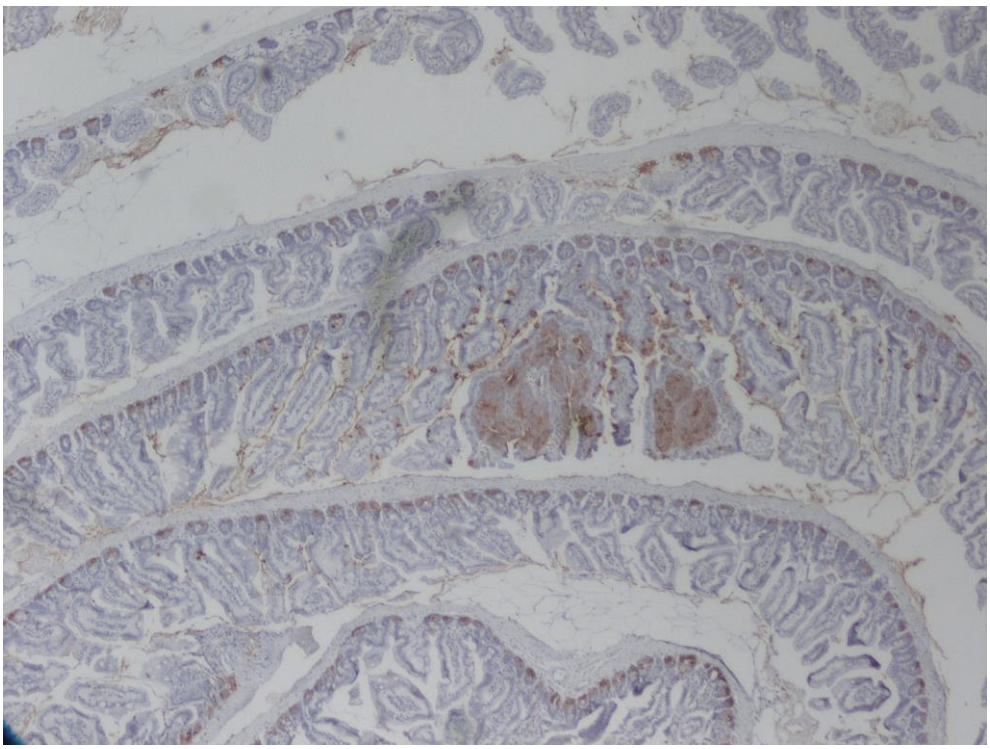


Figure 31. AXL immunohistochemical staining in MIN mice demonstrating specific staining of developing tumours and basal cell layer of intestinal glands

Mouse Immunohistochemistry Qualitative Analysis

Five FFPE sections of wild-type (WT) mouse bladder and five sections of transgenic (TG) mouse bladder were stained as described. Representative images of the different staining patterns in wild-type and transgenic mice is shown in figures 32-35. In WT mice, papillary TCC is AXL negative, although it has some uptake of stain which is more consistent with edge artefact rather than specific staining. In sections of MIBC there is a clear difference in the extent and intensity of staining between WT and TG mice. In the latter AXL staining is more intense, more extensive and more frequent (figure 34). In a similar fashion to human IHC, some of the most intense staining was observed in sections of CIS. (figure 35). Although wild type mice did develop CIS lesions, albeit later than TG mice, no wild type CIS lesions were available for analysis. Nonetheless, these findings support those of human IHC, in that AXL expression associates with muscle invasion and CIS. Furthermore, there appears to be clear value in using this mouse model to evaluate AXL inhibitors in the future.

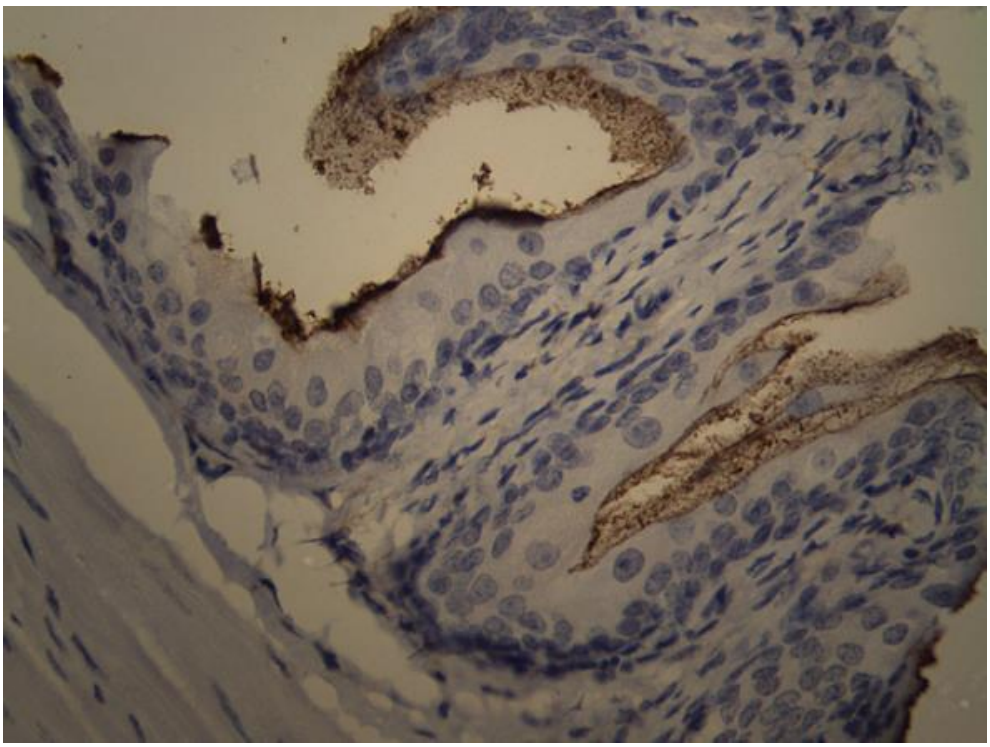


Figure 32. WT mouse papillary TCC

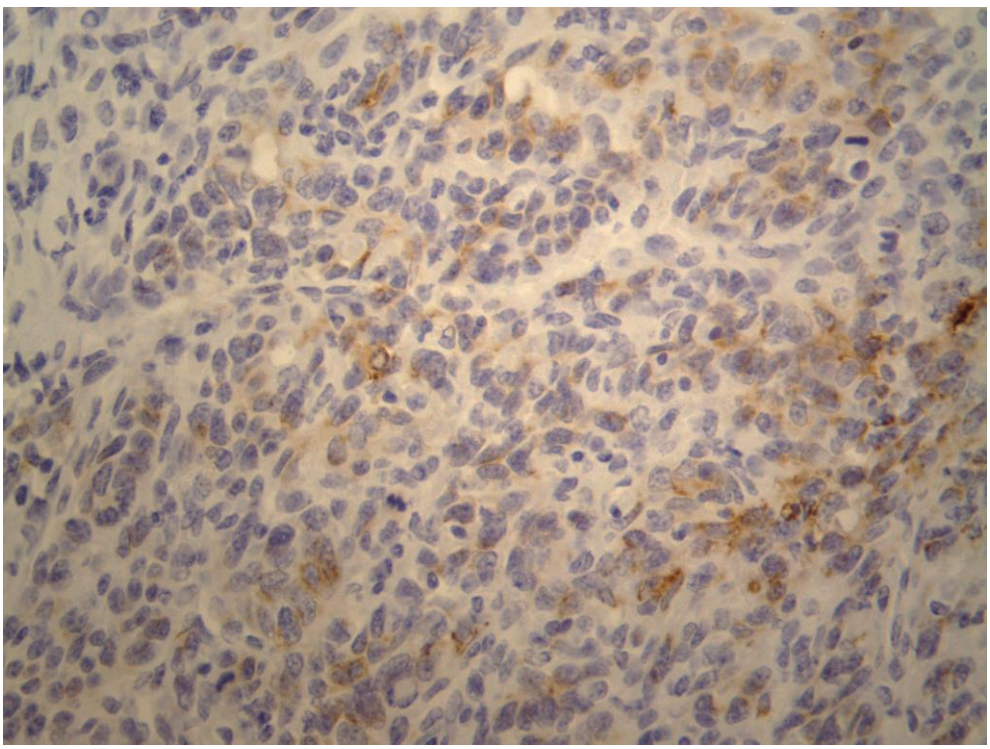


Figure 33. WT mouse MIBC

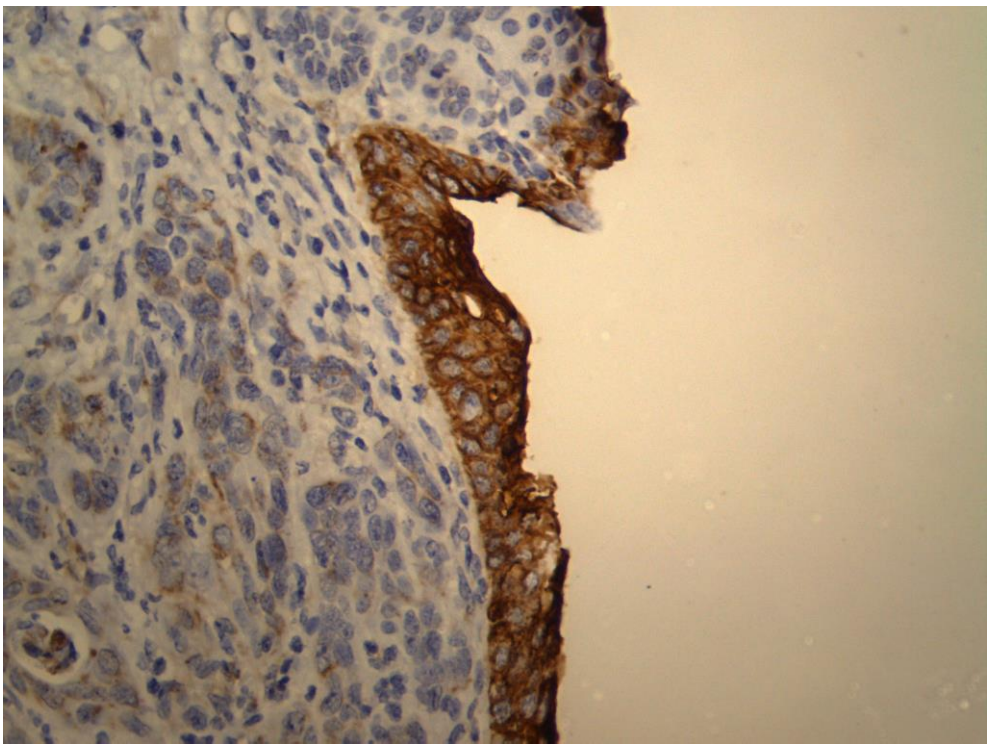


Figure 34. Transgenic mouse CIS/dysplasia

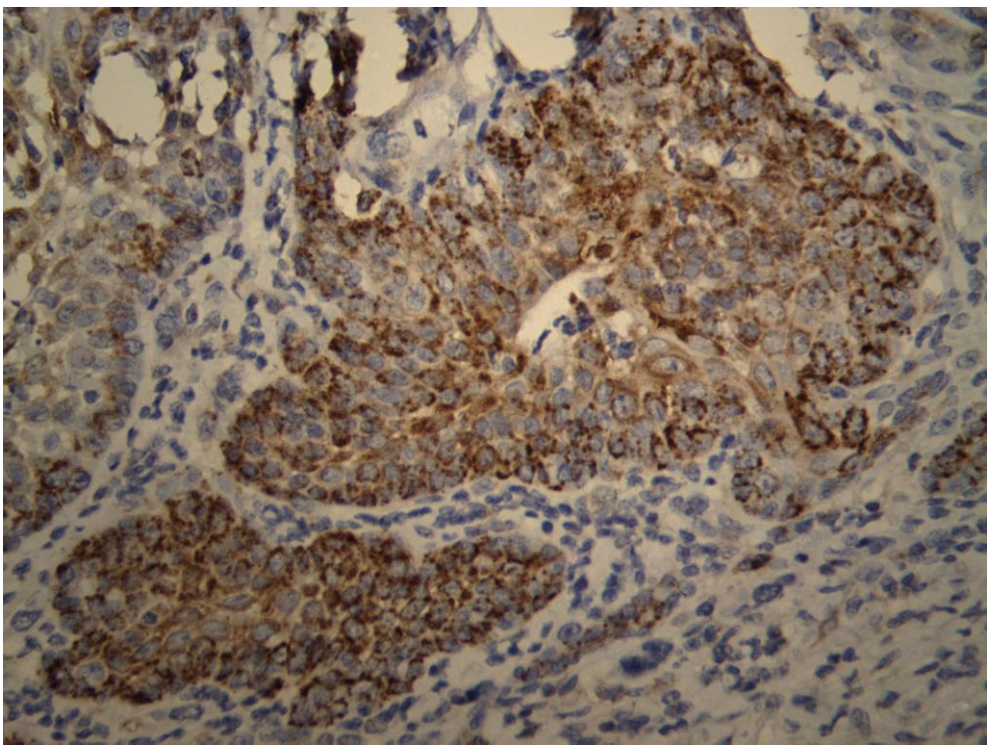
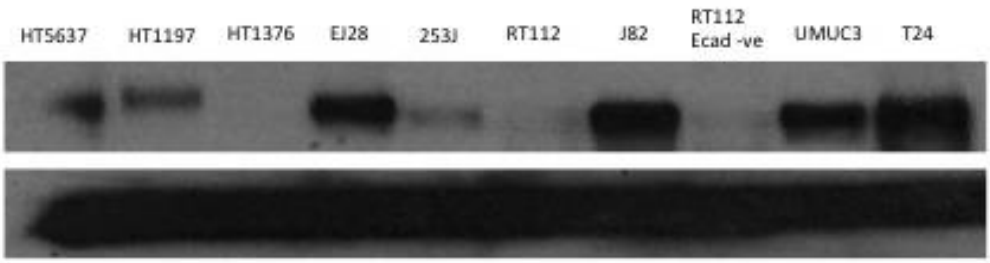


Figure 35. Transgenic mouse MIBC

Cell Culture Studies

AXL expression across a panel of bladder cancer cell lines

The greatest AXL expression was observed J82 and T24 cells whereas the lowest AXL expression was observed in RT112 and HT 1376 cells (Figure 36). J82 cells grew more quickly and with greater reliability than T24 cell and were, therefore, used for further investigation. Both RT112 and HT1376 cells were AXL negative, however, RT112 cells grew much more quickly than HT1376 and hence were selected as an AXL negative cell line to be used for further study. Furthermore, AXL expression appears to correlate with a mesenchymal rather than epithelial phenotype (table 8). This observation is supported by bladder cancer gene sequencing data, which reports co-expression of vimentin, a marker of EMT, and AXL (54,55).



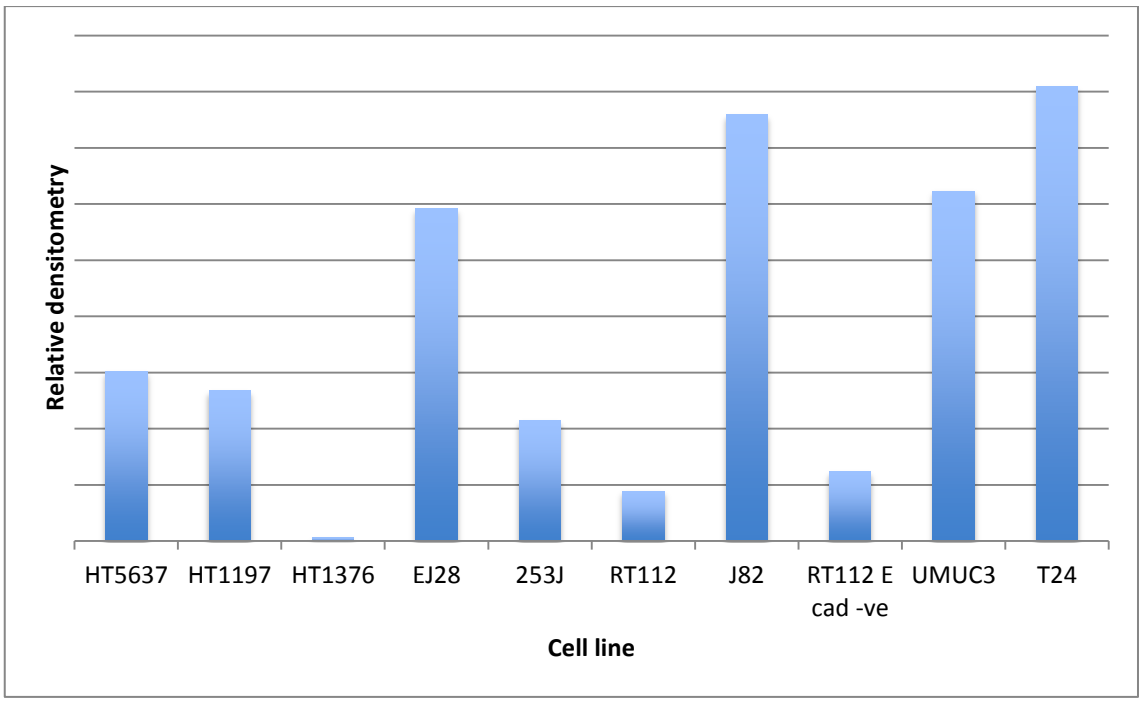


Figure 36. Top, AXL expression in Western blot analysis (top) across a range of bladder cancer cell lines with corresponding densitometry (bottom) (n=1).

The difference in phenotype between mesenchymal J82 cells and epithelial RT112 cells is clearly shown on inverse phase microscopy (figure 37). AXL IHC of J82 and RT112 cytoblocks also confirms the lack of AXL expression in RT112 cells (figure 38). We also inferred from this that our western blotting protocol was reliable despite the lack of a known negative control.

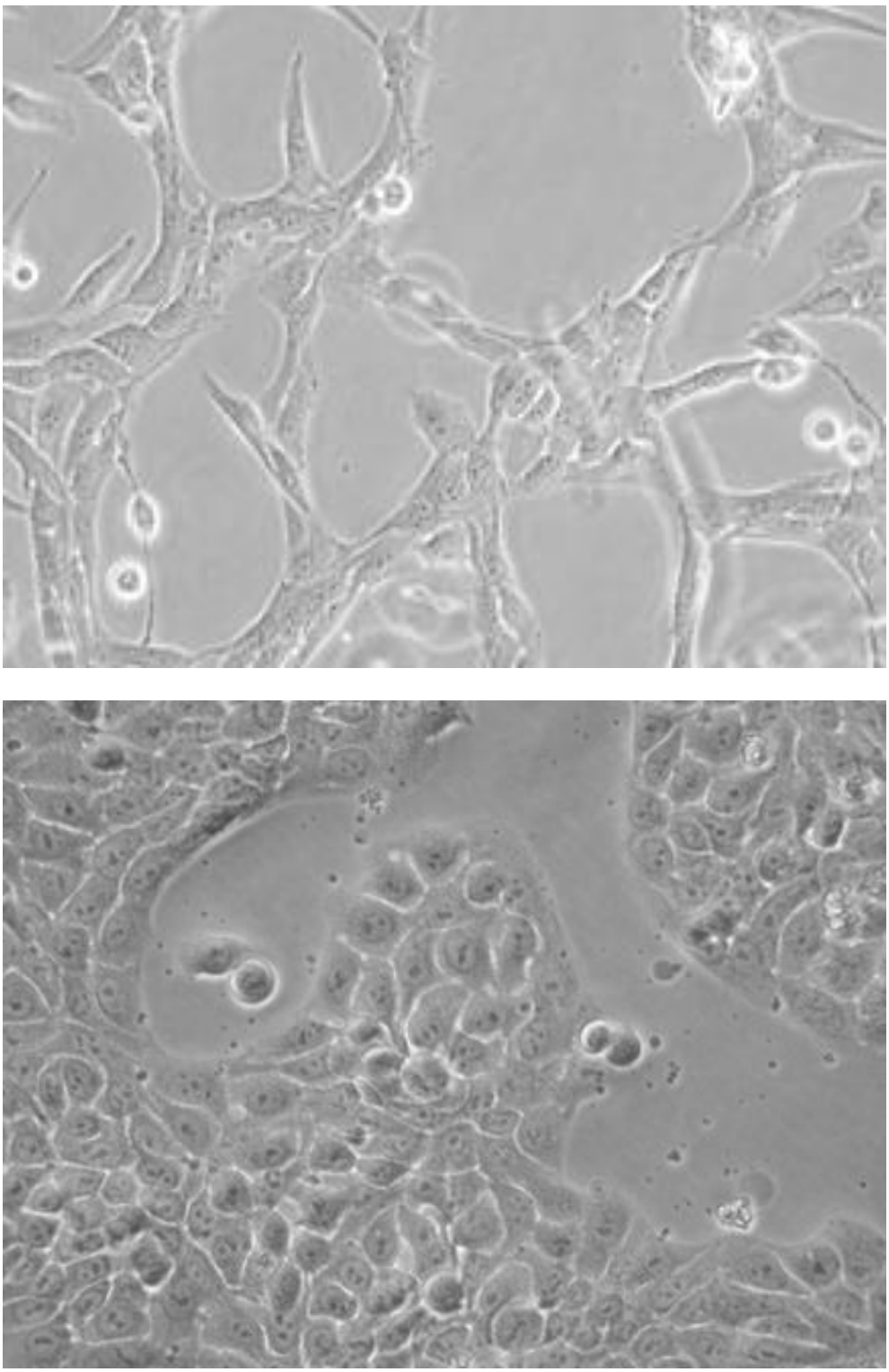


Figure 37. Inverse phase microscopy of J82 (top) and RT112 (bottom) cells.

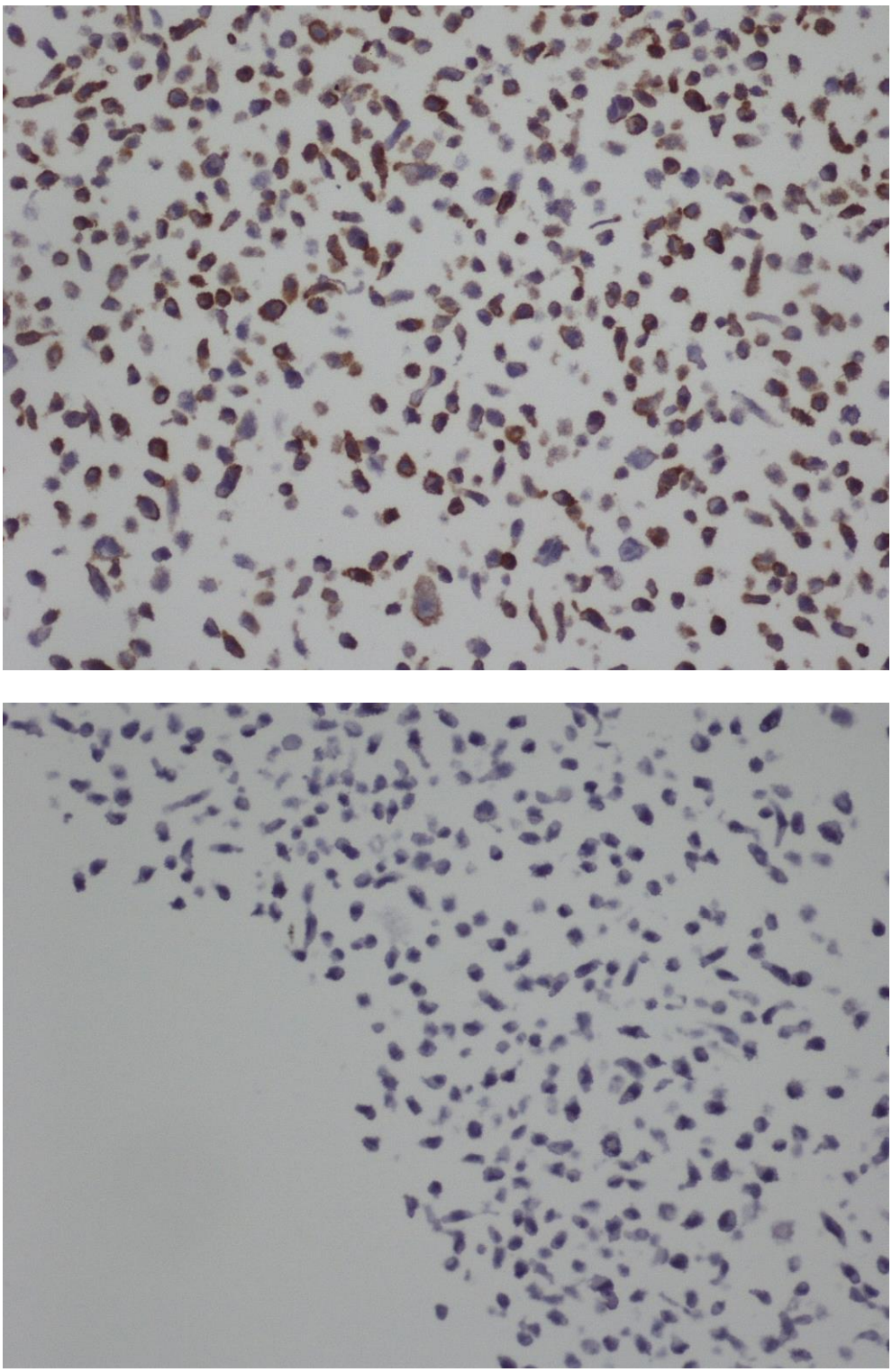


Figure 38. Cytoblock AXL staining of J82 (top) and RT112 (bottom) cells.

Stimulation of J82 and RT112 cells

Following a period of overnight serum starvation J82 cells were stimulated by exposure to GAS6. The process of serum starvation overnight appeared to have no effect on AXL expression levels. The predominant GAS6 dependent signalling appears to be through activation of AKT rather than activation of the MEK/ERK axis (figure 39). In contrast, serum starved AXL negative RT112 cells displayed little or no activation with GAS6 incubation.

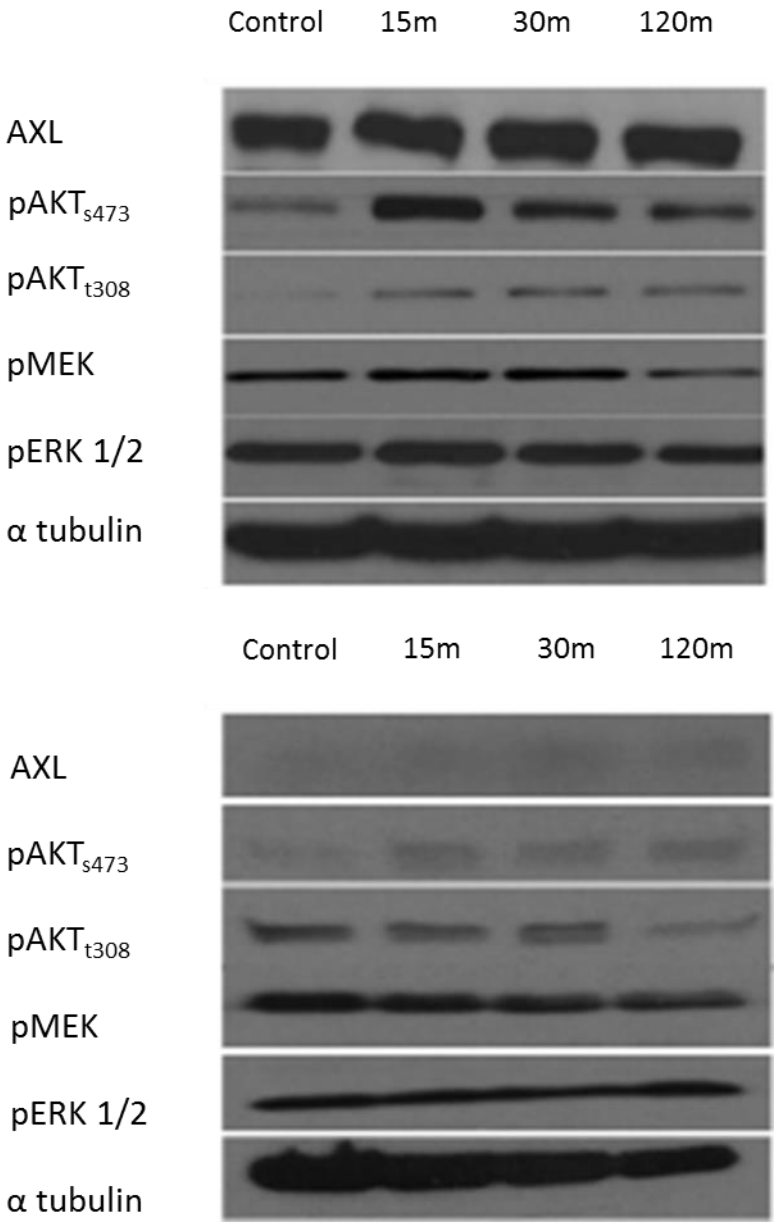


Figure 39. Effect of 2μg/ml GAS6 on expression of AXL and downstream pathways activation. J82 cells (top) and RT112 cells (bottom).

Transfection with siRNA to AXL

The effect of two different siRNA to AXL on AXL expression in J82 cells shows that knockdown of AXL was successful with both siRNA vectors (figure 40). Scatter siRNA and untreated J82 cells were used as controls. The lysates obtained were used subsequently to compare the effect on downstream markers of AXL.

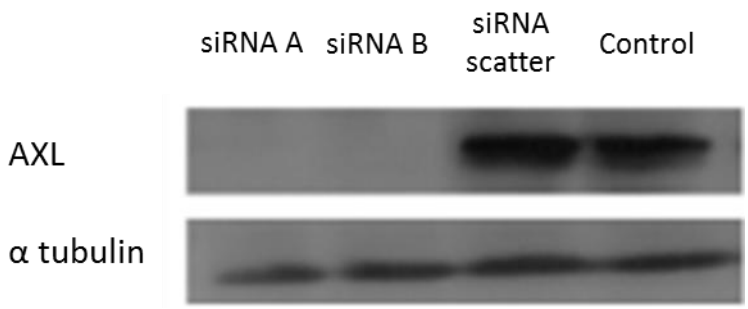


Figure 40. Effect of siRNA to AXL on AXL expression

Effect of foretinib on J82 cells

The effect of 2 hour pre-incubation with 10nM foretinib in GAS6-stimulated and quiescent J82 cells, in comparison to AXL knockdown is shown below (figure 41). Low dose foretinib is able to abrogate slightly the phosphorylation of AKT and MEK in both unstimulated and GAS6 stimulated J82 cells. Given the observed effect in unstimulated cells it is unclear whether the effect in stimulated cells is GAS6, and presumably therefore AXL, specific. Interestingly, siRNA knockdown of AXL does not significantly reduce activation of PI3K or MEK/ERK axis, indeed to a

lesser degree than serum starvation in previous experiments. However, this may be owing to the fact that subsequent to transfection cells were further incubated in serum-containing medium for a further 24 hours and reactivation of the PI3K signalling may have occurred.

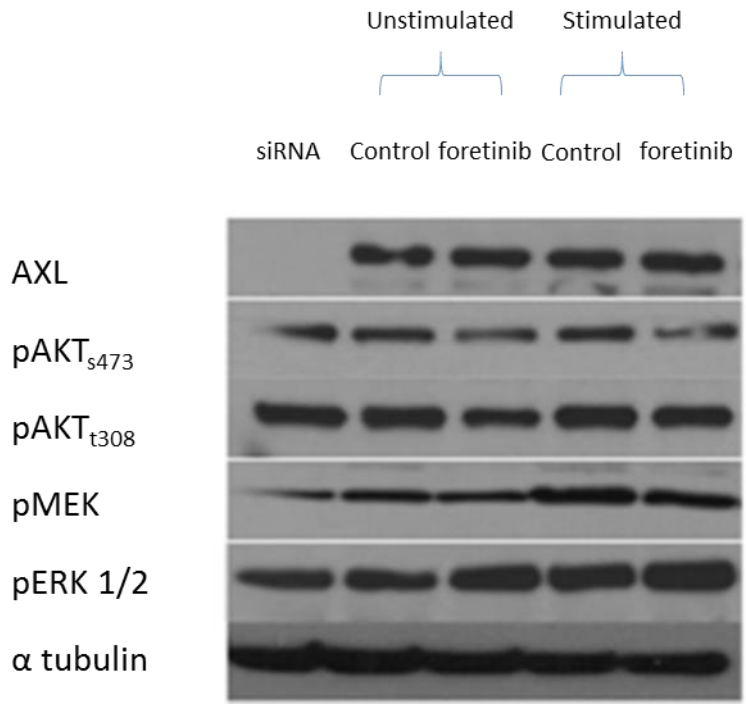


Figure 41. Effect of single dose (10nM) foretinib on downstream pathways in both stimulated (right 2 lanes) and unstimulated (left 2 lanes) (2µg/ml GAS6) J82 cells.

Effect of dose escalation of foretinib on J82 cells

Dose escalation of foretinib reveals a dose dependent inhibition of pAKT$_{S473}$ and an initial reduction in MEK and ERK phosphorylation (figure 42). However, at higher doses this effect is lost and increased expression of pMEK and pERK are observed.

This finding may implicate ERK 1/2 in bidirectional AXL signalling which has been suggested previously (72).

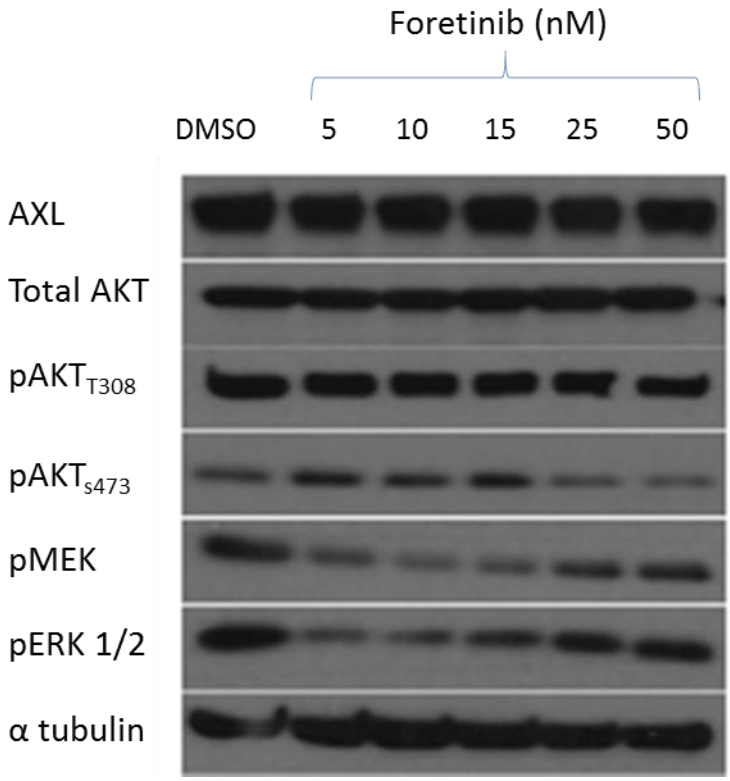


Figure 42. Effect of dose escalation of foretinib on J82 cells following stimulation with GAS6 (2µg/ml) (n=1).

Cell culture summary

Overall, we demonstrate that AXL expression varies across a panel of bladder cancer cell lines, and that the highest expression is observed in cell lines with a mesenchymal phenotype. Stimulation with GAS6 results in activation of pAKT rather than pMEK/pERK, and such stimulation can be abrogated following

preincubation with foretinib. Foretinib also appears to have a biphasic effect on pMEK and pERK expression. This suggests extra AXL-activity of foretinib in addition to the potential bidirectional signalling of MEK/ERK on upstream receptors. We do show conflicting results, in so far as AXL knockdown cells show significant levels of pAKT, pMEK and pERK expression. However, this is potentially explained by the fact that following transfection, cells were further cultured in serum containing media with the potential to activate the PI3K/AKT axis in spite of AXL knockdown.

Although foretinib is able to abrogate AXL-dependent activation of pAKT, the activation of pMEK and pERK at higher doses may preclude the further study in animals. However, we have demonstrated AXL-dependent signalling through activation of AKT. As such, our suggestion is that, although foretinib does not appear suitable for further study, alternative agents, such as R428, should be investigated.

Chapter 4: Discussion

Human immunohistochemistry

Initially, this study focused on determining AXL expression in human bladder cancer specimens with a view to establishing a role in the natural history of bladder cancers.

In NMIBC, AXL expression was strongest in the basal urothelial cell layer (figure 18b). In view of the recent advances in molecular subtyping of bladder cancers, this suggests that AXL may act as a marker of basal cancers, which have a worse prognosis. Although the intensity of staining in TURBT specimens was often variable, the most intense staining was observed in foci of CIS (figure18c). We found no evidence of AXL positivity in stromal cells associated with normal or cancerous urothelium apart from in vascular endothelium, which was consistent regardless of associated MIBC, NMIBC or normal urothelium (figure 19)

This often takes the form of either determining whether a section is positive or negative or generating a score, which lies along an ordinal or continuous scale. Each approach has their advantages. Using a binary scoring system, if validated appropriately, is more useful in clinical practice to determine if an IHC appearance conveys high or low risk for a particular disease characteristic. A continuous or ordinal scale is perhaps more useful in hypothesis generation and testing i.e. if

expression increases a particular disease characteristic becomes increasingly likely.

The process of optimizing our IHC protocol principally revolved around finding the most appropriate concentration of AXL primary antibody. This corresponded to the minimum manufacturer's recommended concentration, which gave a staining pattern on human stomach controls similar to manufacturer's reference images. Conveniently, this gave a staining pattern in bladder cancers with minimal background staining and variable intensity in AXL positive tumours. Using a standard IHC protocol and a scoring system similar to that employed in other studies (89) we have demonstrated that AXL expression associates with phenotypes of bladder cancer, which confer poor prognosis. These include, CIS, muscle invasion, unsuspected lymph node metastasis and failure of standard treatment in NMIBC. With regards to a role for AXL status as a biomarker, we have identified a number of milestones in the natural history of bladder cancer in which AXL status may be a useful measure to inform future management. In NMIBC, AXL status has a negative predictive value of 98% when applied to the eventual need for radical cystectomy. In patients undergoing radical cystectomy for MIBC, AXL status has a negative predictive value of 86% and positive predictive value of 46%, for lymph node metastasis; expressed alternatively those patients with AXL positive primary bladder tumours are over 5 times more likely to have pre-operatively unsuspected metastasis. Crucially, in the cohort of patients with high-risk NMIBC, the properties of AXL status appear to outperform the current EORTC

bladder cancer risk tables, in terms of predicting progression. AXL status has a positive predictive value of 40% in patients with high-risk NMIBC, the mean time to cystectomy being 17 months. In comparison, EORTC risk tables suggest the maximum one-year progression and recurrence rates expected in high-risk NMIBC are 17% and 61% respectively. Furthermore, most of the patients undergoing cystectomy in our series had been treated with BCG, a neglected aspect of the existing EORTC tables. Overall, this suggests AXL status could be additive to existing methods for predicting the need for radical treatment.

Although these findings will require external validation prior to influencing clinical practice, they promise to be useful in aiding decisions regarding early radical cystectomy in high-risk NMIBC and neo adjuvant therapy in patients undergoing cystectomy respectively. From a qualitative perspective, AXL expression was most intense in foci of CIS lesions but also present in most MIBC and metastatic lymph nodes in our cystectomy series. Taken together this supports the notion that AXL expression is related to the processes of invasion and metastasis in bladder cancer.

Our analysis is strengthened by the fact that it is conducted using tissue from two different units and patient cohorts. This minimizes error introduced by variation in methods of fixation and storage. Our assertion is that using sections from TURBT specimens in comparison to cystectomy sections ultimately allows a more useful analysis. Although cystectomy specimens are often easier to process and of higher quality much of the tumour tissue has been previously resected or treated

with neo-adjuvant chemotherapy. Furthermore, there would be inevitable uncertainty as to whether the observed pattern of AXL expression was present at initial diagnosis, which is the most useful juncture to influence treatment decisions.

The IHC tumour identification and scoring was validated by a consultant histopathologist. Although several automated systems for IHC interpretation exist, this is still considered the gold standard. Additionally, we have been able to examine both whole tumour sections and also representative cores of tissue in a TMA. Whole section analysis allows a thorough assessment of the distribution of AXL staining, which is predominantly in the basal urothelial layer in NMIBC and throughout the tumour in MIBC. Large number TMA analysis allows us to draw conclusions about the role of AXL in the natural history of bladder cancer using multivariate analysis with a range of disease and patient variables. Similarly, this analysis has provided further information about AXL as a biomarker for disease progression in NMIBC.

Although we have tissue from different centres, the staining protocol was performed in one centre which, to date has not been repeated independently. This potentially raises questions about the reproducibility of our findings, however this approach is commonplace in published literature and, therefore, considered acceptable. The inferences that can be drawn from IHC have inherent limitations. Although we have been able to identify increased expression levels in invasive and

metastatic bladder cancers, we cannot conclude that AXL inhibition will reverse these processes. They do, however, provide a rational for the cell culture work we have undertaken and further study.

Mouse immunohistochemistry

In IHC performed on bladder cancers from transgenic mice with constitutively active STAT3, there is a clear increase in AXL expression when compared with wild type. This supports an association between AXL and STAT3 signalling. However, the precise mechanism is not shown in the study. Interestingly, this is a pathway that has not featured heavily in the AXL related literature. More readily authors have focussed on AXL and the relationship to AKT and ERK expression (71,75,78,80). One prostate cancer study has identified a role for AXL in the regulation of STAT3 through IL-6 signalling (figure 12) (74). In this study, abrogation of AXL signalling resulted in reduced IL-6 levels and STAT3 expression. In addition to supporting potential role for STAT3 in AXL regulation in bladder cancer, our findings support the use of this mouse model in the future investigation of AXL inhibitors and bladder cancer.

Cell culture studies

In this *in vitro* analysis we have demonstrated that AXL expression levels vary between different bladder cancer cell lines. Furthermore, increased AXL expression appears to correlate with a mesenchymal phenotype, a finding supported by analysis in other cancers (67-71). Through knockdown studies in J82 cells, AXL expression has been shown to be associated with invasion in previous studies (75). Our study is additive to the literature as we have shown that stimulation, in AXL expressing J82 cells, results in phosphorylation of AKT rather than ERK. The current AXL literature in different cancers appears to show that either AKT, ERK or both are stimulated by AXL activation and this varies with the tumour type studied. Both AKT and ERK have been shown to contribute to activation of various intracellular pathways involved in signal transduction, proliferation and cell survival, which can ultimately result in a cancerous phenotype. This analysis, therefore, appears to concord with our IHC findings.

Pre-incubation with foretinib, an attractive agent given its current proximity to clinical practice and proven AXL inhibition, can abrogate GAS6-dependent AKT phosphorylation. Taken in isolation this finding would suggest merit in further study of foretinib in bladder cancer. However, we also observed that foretinib appears to have an inhibitory role on the MEK/ERK axis at lower doses, which is lost at higher doses. One possible explanation for this is a feedback mechanism,

activated by MEK/ERK knockdown, which is distinct from those studied in this thesis.

Although we have identified that stimulation of AXL activates AKT we have not shown that knockdown, via siRNA reduces AKT phosphorylation. Once transfected with siRNA to AXL, J82 cells were incubated in serum containing medium. As the PI3K/AKT axis can be activated by a number of different receptors it is possible that this results in our observed unchanged levels of pAKT.

Given the role of both AKT and ERK in a range of activities implicated in cancer development and progression our analysis does not support further work into foretinib and bladder cancer.

Conclusion

Despite changes in UK and worldwide exposure to occupational carcinogens and cigarette smoking, bladder cancer remains an important and costly disease[3]. The natural history of NMIBC and MIBC varies significantly and there is a high price, both clinically and economically on both improving available treatments in MIBC and developing therapies that can minimise the progression of NMIBC to MIBC or to the point where it cannot be safely managed endoscopically. At present, there are limited options for patients who fail standard NMIBC treatment and, therefore, this represent a challenging group. Although chemohyperthermia and EMDA are

promising avenues for further study, to our knowledge, there are currently no registered trials of TKIs in NMIBC.

Tyrosine kinase inhibitors have been investigated as adjuncts to standard therapy in bladder cancer (121). This has however focussed on a potential role for TKI as adjuncts to chemotherapy in metastatic bladder cancer or when chemotherapy fails rather than in other stages of bladder cancer.

AXL, since identification as a transforming gene in leukaemia in 1995, has become an increasingly popular molecule of interest across a range of human cancers. There are repeated reports in the literature of the association between AXL expression in cancer tissue and invasion, metastasis and poor prognosis. There are varying reports as to the downstream pathways, through which AXL signals. These centralise upon activation of either AKT or ERK. We demonstrate that in bladder cancer this appears to be through activation of the PI3K/AKT axis. In addition, it would appear that activation of the transcription factor STAT3 is implicated in the process of AXL signalling and invasion given the marked differential in AXL expression between STAT3 negative and STAT3 expressing mouse bladder cancers. This represents a novel avenue for further study and supports the use of such a mouse model in the future development of therapies for bladder cancer, which has been notoriously difficult to develop.

In conclusion, this thesis provides robust evidence for AXL as a biomarker in bladder cancer. Furthermore, we present a rationale for further experimentation into AXL as a therapeutic target in bladder cancer. Our suggested focus of such study would be aimed at ultimately evaluating the role of currently available AXL inhibitors in patients with either MIBC in conjunction with cystectomy or NMIBC with a high risk of progression.

References

1. Office of National Statistics 2014. Cancer Registration Statistics, England, 2014. Last accessed 31/5/2016 from http://www.ons.gov.uk/peoplepopulationandcommunity/healthandsocialcare/conditionsanddiseases/datasets/cancerregistrationstatisticscancerregistrationstatisticsengland

2. Griffiths TR. Action on Bladder Cancer. Current perspectives in bladder cancer management. Int J Clin Pract. 2013 May;67(5):435-48

3. Sangar VK, Ragavan N, Matanhelia SS, Watson MW, Blades RA. The economic consequences of prostate and bladder cancer in the UK. BJU Int. 2005 Jan;95(1):59-63

4. Patel MI, Bang A, Gillett D, Cheluvappa R, Smith DP. Poor survival of females with bladder cancer is limited to those aged 70 years or over: a population-wide linkage study, New South Wales, Australia. Cancer Med. 2015 Aug;4(8):1145-52.

5 Puente D, Malats N, Cecchini L, Tardón A, García-Closas R, Serra C, et al. EPICURO Study Group Investigators. Gender-related differences in clinical and pathological characteristics and therapy of bladder cancer. Eur Urol. 2003 Jan;43(1):53-62

6 Scosyrev, E., D. Golijanin, G. Wu, and E. Messing. 2012. The burden of bladder cancer in men and women: analysis of the years of life lost. BJU Int. 109:57–62.

7 Shariat, S. F., J. P. Sfakianos, M. J. Droller, P. I. Karakiewicz, S. Meryn, and B. H. Bochner. 2010. The effect of age and gender on bladder cancer: a critical review of the literature. BJU Int. 105:300–308.

8. Sobin LH, Gospodariwicz M, Wittekind C (eds). TNM classification of malignant tumors. UICC International Union Against Cancer. 7th edn. Wiley Blackwell, 2009 Dec; pp. 262-265. http://www.nicc.org/tnm/

9. Epstein JI, Amin MB, Reuter VR, Mostofi FK. The World Health Organization/International Society of Urological Pathology consensus classification of urothelial (transitional cell) neoplasms of the urinary bladder. Am J Surg Pathol 1998 Dec;22(12):1435 48

10. Sauter G, Algaba F, Amin M, et al. Tumours of the urinary system: non invasive urothelial neoplasias. In: Eble JN, Sauter G, Epstein Jl, Sesterhenn I, eds. WHO classification of classification of tumors of the urinary system and male genital organs. Lyon: IARCC Press, 2004, pp. 29-34.

11. Sobin LH, Gospodariwicz M, Wittekind C (eds). TNM classification of malignant tumors. UICC International Union Against Cancer. 7th edn. Oxford: Wiley-Blackwell, 2009.

12. Burger M, Catto JW, Dalbagni G, Grossman HB, Herr H, Karakiewicz P, et al. Epidemiology and risk factors of urothelial bladder cancer. Eur Urol. 2013 Feb;63(2):234-41

13. Maclennan GT, Kirkali Z, Cheng L. Histological grading of noninvasive papillary urothelial neoplasms. Eur Urol 2007 Apr;51(4)889-97

14. Sylvester RJ. Natural history, recurrence and progression in superficial bladder cancer. TSW Urology. 2006 (Suppl 1), 15-23.

15. Sylvester RJ, van der Meijden AP, Oosterlinck W, Witjes JA, Bouffioux C, Denis L. et al. Predicting recurrence and progression in individual patients with stage Ta T1 bladder cancer using EORTC risk tables: a combined analysis of 2596 patients from seven EORTC trials. Eur Urol. 2006 Mar;49(3):466-5

16. Millán-Rodríguez F, Chéchile-Toniolo G, Salvador-Bayarri J, Palou J, Algaba F, Vicente-Rodríguez J. Primary superficial bladder cancer risk groups according to progression, mortality and recurrence. J Urol. 2000 Sep;164(3 Pt 1):680-4

17. NICE guideline NG2 Bladder cancer: Diagnosis and management. Feb 2015

18. Soloway MS, Masters S. Urothelial susceptibility to tumor cell implantation: influence of cauterization. Cancer. 1980 Sep 1;46(5):1158-63.

19. Pan JS, Slocum HK, Rustum YM, Greco WR, Gaeta JF, Huben RP. Inhibition of implantation of murine bladder tumor by thiotepa in cauterized bladder. J Urol 1989 Dec;142(6):1589-93.

20. Brocks CP, Büttner H, Böhle A. Inhibition of tumor implantation by intravesical gemcitabine in a murine model of superficial bladder cancer. J Urol 2005 Sep;174(3):1115-8.

21. Huncharek M, McGarry R, Kupelnick B. Impact of intravesical chemotherapy on recurrence rate of recurrent superficial transitional cell carcinoma of the

bladder: results of a meta-analysis. Anticancer Res. 2001 Jan-Feb;21(1B):765-9.

22. Tolley DA, Parmar MK, Grigor KM, Lallemand G, Benyon LL, Fellows J, et al. The effect of intravesical mitomycin C on recurrence of newly diagnosed superficial bladder cancer: a further report with 7 years of follow up. J Urol. 1996 Apr;155(4):1233–1238.

23. Böhle A, Bock PR. Intravesical bacillus Calmette-Guerin versus mitomycin C in superficial bladder cancer: formal meta-analysis of comparative studies on tumour progression. Urology 2004 Apr;63(4):682-6. Discussion 686-7.

24. Sylvester RJ, van der Meijden AP, Lamm DL. Intravesical bacillus Calmette-Guerin reduces the risk of progression in patients with superficial bladder cancer: a meta-analysis of the published results of randomized clinical trials. J Urol 2002 Nov;168(5):1964-70.

25. Malmström PU, Sylvester RJ, Crawford DE, Friedrich M, Krege S, Rintala E, et al. An individual patient data meta-analysis of the long- term outcome of randomised studies comparing intravesical mitomycin C versus bacillus Calmette- Guérin for non-muscle-invasive bladder cancer. Eur Urol 2009 Aug;56(2):247-56.

26. van den Bosch S, Alfred Witjes J. Long-term cancer-specific survival in patients with high-risk, non-muscle-invasive bladder cancer and tumour progression: a systematic review. Eur Urol 2011 Sep;60(3):493-500

27. Lerner SP, Tangen CM, Sucharew H, Wood D, Crawford ED. Failure to achieve a complete response to induction BCG therapy is associated with

increased risk of disease worsening and death in patients with high risk non-muscle invasive bladder cancer. Urol Oncol. 2009 Mar-Apr;27(2):155-9

28. The Hymn Trial - Hyperthermia Plus Mitomycin http://www.birmingham.ac.uk/research/activity/mds/trials/crctu/trials/hymn/index.aspx last accessed 1/9/2016

29. World Health Organization (WHO) Consensus Conference in Bladder Cancer, Hautmann RE, Abol- Enein H, Hafez K, Haro I, Mansson W, Mills RD, Montie JD, et al. Urinary diversion. Urology 2007 Jan;69(1 Suppl):17-49.

30. Stein JP, Lieskovsky G, Cote R, Groshen S, Feng AC, Boyd S, et al. Radical cystectomy in the treatment of invasive bladder cancer: long-term results in 1,054 patients. J Clin Oncol 2001 Feb;19(3):666-75.

31. Shariat SF, Karakiewicz PI, Palapattu GS, Amiel GE, Lotan Y, Rogers CG, et al. Outcomes of radical cystectomy for transitional cell carcinoma of the bladder: a contemporary series from the Bladder Cancer Research Consortium. J Urol 2006 Dec;176(6 Pt 1):2414-22; discussion 2422.

32. Madersbacher S, Hochreiter W, Burkhard F, Thalmann GN, Danuser H, Markwalder R, et al. Radical cystectomy for bladder cancer today--a homogeneous series without neoadjuvant therapy. J Clin Oncol. 2003 Feb 15;21(4):690-6

33. Pollack A, Zagars GZ. Radiotherapy for stage T3b transitional cell carcinoma of the bladder. Semin Urol Oncol 1996 May;14(2):86-95.

34. De Neve W, Lybeert ML, Goor C, Crommelin MA, Ribot JG. Radiotherapy for T2 and T3 carcinoma of the bladder: the influence of overall treatment time. Radiother Oncol 1995 Sep;36(3):183-8.

35. Mameghan H, Fisher R, Mameghan J, Brook S. Analysis of failure following definitive radiotherapy for invasive transitional cell carcinoma of the bladder. Int J Radiat Oncol Biol Phys 1995 Jan;31(2):247-54.

36. Näslund I, Nilsson B, Littbrand B. Hyperfractionated radiotherapy of bladder cancer. A ten-year followup of a randomized clinical trial. Acta Oncol 1994;33(4):397-402.

37 Bekelman JE, Handorf EA, Guzzo T, Evan Pollack C, Christodouleas J, Resnick MJ, et al. Radical cystectomy versus bladder-preserving therapy for muscle-invasive urothelial carcinoma: examining confounding and misclassification biasin cancer observational comparative effectiveness research. Value Health. 2013 Jun;16(4):610-8 38 Gore, JL et al. Use of radical cystectomy for patients with invasive bladder cancer. Journal of the National Cancer Institute 2010; 102(11): 802-811.

39 Koga F, Kihara K, Fujii Y, Yoshida S, Saito K, Masuda H, et al. Favourable outcomes of patients with clinical stage T3N0M0 bladder cancer treated with induction low-dose chemo-radiotherapy plus partial or radical cystectomy vs immediate radical cystectomy: a single-institutional retrospective comparative study. BJU Int. 2009 Jul;104(2):18994

40 Kotwal S, Choudhury A, Johnston C, Paul AB, Whelan P, Kiltie AE. Similar treatment outcomes for radical cystectomy and radical radiotherapy in invasive bladder cancer treated at a United Kingdom specialist treatment center. Int J Radiat Oncol Biol Phys. 2008 Feb 1;70(2):456-63

41 Munro NP, Sundaram SK, Weston PM, Fairley L, Harrison SC, Forman D. et al. A 10-year retrospective review of a nonrandomized cohort of 458 patients undergoing radical radiotherapy or cystectomy in Yorkshire, UK. Int J Radiat Oncol Biol Phys. 2010 May 1;77(1):119-24

42 van der Steen-Banasik E, Ploeg M, Witjes JA, van Rey FS, Idema JG, Heijbroek RP. et al. Brachytherapy versus cystectomy in solitary bladder cancer: a case control, multicentre, East-Netherlands study. Radiother Oncol. 2009 Nov;93(2):352-7.

43. Svatek RS, Shariat SF, Novara G, Skinner EC, Fradet Y, Bastian PJ, et al. Discrepancy between clinical and pathological stage: external validation of the impact on prognosis in an international radical cystectomy cohort. *BJU International*. 2011;107(6):898–904.

44 Hoskin PJ, Rojas AM, Bentzen SM, Saunders MI. Radiotherapy with concurrent carbogen and nicotinamide in bladder carcinoma. J Clin Oncol. 2010 Nov 20;28(33):4912-8

45 James ND, Hussain SA, Hall E, Jenkins P, Tremlett J, Rawlings C et al. BC2001 Investigators. Radiotherapy with or without chemotherapy in muscle-invasive bladder cancer. N Engl J Med. 2012 Apr 19;366(16):1477-88

46 Choudhury A, Swindell R, Logue JP, Elliott PA, Livsey JE, Wise M. et al. Phase II study of conformal hypofractionated radiotherapy with concurrent gemcitabine in muscle-invasive bladder cancer. J Clin Oncol. 2011 Feb 20;29(6):733-8

47. Sherif A, Holmberg L, Rintala E, Mestad O, Nilsson J, Nilsson S, et al. Nordic Urothelial Cancer Group. Neoadjuvant cisplatinum based combination chemotherapy in patients with invasive bladder cancer: a combined analysis of two Nordic studies. Eur Urol 2004 Mar;45(3):297-303.

48. Advanced Bladder Cancer Meta-analysis Collaboration. Neoadjuvant chemotherapy in invasive bladder cancer: a systematic review and meta-analysis. Lancet 2003 Jun;361(9373):1927 34.

49 Advanced Bladder Cancer (ABC) Meta-analysis Collaboration. Neoadjuvant chemotherapy in invasive bladder cancer: update of a systematic review and meta-analysis of individual patient data advanced bladder cancer (ABC) meta-analysis collaboration. Eur Urol 2005 Aug;48(2):202-205; discussion 205-6

50. Sternberg CN, Vogelzang NJ. Gemcitabine, paclitaxel, pemetrexed and other newer agents in urothelial and kidney cancers. Crit Rev Oncol Hematol 2003 Jun;46(Suppl):S105-S15.

51. von der Maase H, Sengelov L, Roberts JT, Ricci S, Dogliotti L, Oliver T, et al. Long-term survival results of a randomized trial comparing gemcitabine

plus cisplatin, with methotrexate, vinblastine, doxorubicin, plus cisplatin in patients with bladder cancer. J Clin Oncol. 2005 Jul 20;23(21):4602-8

52. Goebell PJ, Knowles MA. Bladder cancer or bladder cancers? Genetically distinct malignant conditions of the urothelium. Urol Oncol. 2010 Jul Aug;28(4):409-28

53 Knowles MA, Hurst CD. Molecular biology of bladder cancer: new insights into pathogenesis and clinical diversity. Nat Rev Cancer. 2015 Jan;15(1):25-41

54 Blaveri E, Simko JP, Korkola JE, Brewer JL, Baehner F, Mehta K, et al. Bladder cancer outcome and subtype classification by gene expression. Clin Cancer Res. 2005 Jun 1;11(11):4044-55.

55. Dyrskjøt L, Zieger K, Kruhøffer M, Thykjaer T, Jensen JL, Primdahl H, et al. A molecular signature in superficial bladder carcinoma predicts clinical outcome. Clin Cancer Res. 2005 Jun 1;11(11):4029-36.

56 Damrauer JS, Hoadley KA, Chism DD, Fan C, Tiganelli CJ, Wobker SE. et al. Intrinsic subtypes of high-grade bladder cancer reflect the hallmarks of breast cancer biology. Proc. Natl Acad. Sci. USA 111, 3110–3115 (2014)

57. Choi W, Shah JB, Tran M, Svatek R, Marquis L, Lee IL et al. p63 expression defines a lethal subset of muscle-invasive bladder cancers. PLoS ONE 7, e30206 (2012)

58. The Cancer Genome Atlas Research Network. Comprehensive molecular characterization of urothelial bladder carcinoma. Nature 507, 315–322 (2014).

59 Sjödahl G, Lauss M, Lövgren K, Chebil G, Gudjonsson S, Veerla S. et al. A molecular taxonomy for urothelial carcinoma. Clin Cancer Res. 2012 Jun 15;18(12):3377-86

60 Schlessinger J. Cell signaling by receptor tyrosine kinases. Cell. 2000 Oct 13;103(2):211-25

61 Davis, R.J. (2000). Signal transduction by the JNK group of MAP mode of receptor tyrosine kinase interaction. Biochem. J. 331,kinases. Cell 103, 239–252.

62. Linger RM, Keating AK, Earp HS, Graham DK. TAM receptor tyrosine kinases: biologic functions, signaling, and potential therapeutic targeting in human cancer. Adv Cancer Res. 2008;100:35-83.

63. Liu E, Hjelle B, Bishop JM. Transforming genes in chronic myelogenous leukemia. Proc. Natl. Acad. Sci. USA. 1988;85(6):1952–1956

64. O'Bryan JP, Frye RA, Cogswell PC, Neubauer A, Kitch B, Prokop C, et al. AXL, a transforming gene isolated from primary human myeloid leukemia cells, encodes a novel receptor tyrosine kinase. Mol. Cell Biol. 1991;11(10):5016–5031

65. O'Bryan JP, Fridell YW, Koski R, Varnum B, Liu ET. The transforming receptor tyrosine kinase, Axl, is post-translationally regulated by proteolytic cleavage. J Biol Chem. 1995 Jan 13;270(2):551-7

66. Stitt TN, Conn G, Gore M, Lai C, Bruno J, Radziejewski C, et al. The anticoagulation factor protein S and its relative, Gas6, are ligands for the Tyro 3/Axl family of receptor tyrosine kinases. Cell. 1995;80(4):661–670.

67 Ou WB, Corson JM, Flynn DL, Lu WP, Wise SC, Bueno R, et al. AXL regulates mesothelioma proliferation and invasiveness. Oncogene. 2011 Apr 7;30(14):1643-52.

68. Ou WB, Hubert C, Corson JM, Bueno R, Flynn DL, Sugarbaker DJ, et al. Targeted inhibition of multiple receptor tyrosine kinases in mesothelioma. Neoplasia. 2011 Jan;13(1):12-22.

69. Sensi M, Catani M, Castellano G, Nicolini G, Alciato F, Tragni G, et al. Human cutaneous melanomas lacking MITF and melanocyte differentiation antigens express a functional Axl receptor kinase. J Invest Dermatol. 2011 Dec;131(12):2448-57.

70. Burchert A, Attar EC, McCloskey P, Fridell YW, Liu ET. Determinants for transformation induced by the Axl receptor tyrosine kinase. Oncogene. 1998;16(24):3177–3187

71. El Sayadi H, Pissaloux D, Alberti L, Tabone-Eglinger S, Ranchere D, Decouvelaere AV, et al. Autocrine role for Gas6 with Tyro3 and Axl in leiomyosarcomas. Target Oncol. 2013 Jan 25.

72. Ghosh AK, Secreto C, Boysen J, Sassoon T, Shanafelt TD, Mukhopadhyay D, et al. The novel receptor tyrosine kinase Axl is constitutively active in B-cell

chronic lymphocytic leukemia and acts as a docking site of nonreceptor kinases: implications for therapy. Blood. 2011 Feb 10;117(6):1928-37.

73. Park IK, Mishra A, Chandler J, Whitman SP, Marcucci G, Caligiuri MA. Inhibition of the receptor tyrosine kinase Axl impedes activation of the FLT3 internal tandem duplication in human acute myeloid leukemia: implications for Axl as a potential therapeutic target. Blood. 2013 Mar 14;121(11):2064-73.

74 Suh YA, Jo SY, Lee HY, Lee C. Inhibition of IL-6/STAT3 axis and targeting Axl and Tyro3 receptor tyrosine kinases by apigenin circumvent taxol resistance in ovarian cancer cells. Int J Oncol. 2015 Mar;46(3):1405-11.

75. Sayan AE, Stanford R, Vickery R, Grigorenko E, Diesch J, Kulbicki K, et al. Fra-1 controls motility of bladder cancer cells via transcriptional upregulation of the receptor tyrosine kinase AXL. Oncogene. 2012 Mar 22;31(12):1493-503.

76. Yeh CY, Shin SM, Yeh HH, Wu TJ, Shin JW, Chang TY, et al. Transcriptional activation of the Axl and PDGFR-α by c-Met through a ras- and Src-independent mechanism in human bladder cancer. BMC Cancer. 2011 Apr 16;11:139.

77. Stransky N, Vallot C, Reyal F, Bernard-Pierrot I, de Medina SG, Segraves R, et al. Regional copy number-independent deregulation of transcription in cancer. Nat Genet. 2006 Dec;38(12):1386-96. Epub 2006 Nov 12. Erratum in: Nat Genet. 2008 Mar;40(3):373.

78. Lin CI, Whang EE, Moalem J, Ruan DT. Strategic combination therapy overcomes tyrosine kinase coactivation in adrenocortical carcinoma. Surgery. 2012 Dec;152(6):1045-50.

79 Mahadevan D, Cooke L, Riley C, Swart R, Simons B, Della Croce K, et al. A novel tyrosine kinase switch is a mechanism of imatinib resistance in gastrointestinal stromal tumors. Oncogene. 2007 Jun 7;26(27):3909-19.

80 Dufies M, Jacquel A, Belhacene N, Robert G, Cluzeau T, Luciano F, et al. Mechanisms of AXL overexpression and function in Imatinib-resistant chronic myeloid leukemia cells. Oncotarget. 2011 Nov;2(11):874-85.

81 Lay JD, Hong CC, Huang JS, Yang YY, Pao CY, Liu CH, et al. Sulfasalazine suppresses drug resistance and invasiveness of lung adenocarcinoma cells expressing AXL. Cancer Res. 2007 Apr 15;67(8):3878-87

82 Byers LA, Diao L, Wang J, Saintigny P, Girard L, Peyton M, et al. An epithelial-mesenchymal transition gene signature predicts resistance to EGFR and PI3K inhibitors and identifies Axl as a therapeutic target for overcoming EGFR inhibitor resistance. Clin Cancer Res. 2013 Jan 1;19(1):279-90.

83 Liu L, Greger J, Shi H, Liu Y, Greshock J, Annan R, et al. Novel mechanism of lapatinib resistance in HER2-positive breast tumor cells: activation of AXL. Cancer Res. 2009 Sep 1;69(17):6871-8.

84 Shieh YS, Lai CY, Kao YR, Shiah SG, Chu YW, Lee HS, et al. Expression of axl in lung adenocarcinoma and correlation with tumor progression. Neoplasia. 2005;7(12):1058–1064.

85 Craven RJ, Xu LH, Weiner TM, Fridell YW, Dent GA, Srivastava S, et al. Receptor tyrosine kinases expressed in metastatic colon cancer. Int. J. Cancer.1995;60(6):791–797.

86 Ito T, Ito M, Naito S, Ohtsuru A, Nagayama Y, Kanematsu T, et al. Expression of the Axl receptor tyrosine kinase in human thyroid carcinoma. Thyroid. 1999;9(6):563–567.

87 Linger RM, Keating AK, Earp HS, Graham DK. Taking aim at Mer and Axl receptor tyrosine kinases as novel therapeutic targets in solid tumors. Expert Opin Ther Targets. 2010 Oct;14(10):1073-90

88 Gioia R, Leroy C, Drullion C, Lagarde V, Etienne G, Dulucq S, et al. Quantitative phosphoproteomics revealed interplay between Syk and Lyn in the resistance to nilotinib in chronic myeloid leukemia cells. Blood. 2011.

89 Huang F, Hurlburt W, Greer A, Reeves KA, Hillerman S, Chang H, et al. Differential mechanisms of acquired resistance to insulin-like growth factor-i receptor antibody therapy or to a small-molecule inhibitor, BMS-754807, in a human rhabdomyosarcoma model. Cancer Res. 2010; 70:7221-7231.

90 Liu L, Greger J, Shi H, Liu Y, Greshock J, Annan R, et al. Novel mechanism of lapatinib resistance in HER2-positive breast tumor cells: activation of AXL. Cancer Res. 2009; 69:6871-6878.

91 Macleod K, Mullen P, Sewell J, Rabiasz G, Lawrie S, Miller E, et al. Altered ErbB receptor signaling and gene expression in cisplatin-resistant ovarian cancer. Cancer Res. 2005; 65:6789-6800.

92 Shapiro GI, McCallum S, Adams LM, Sherman L, Weller S, Swann S, et al. A Phase 1 dose-escalation study of the safety and pharmacokinetics of once-daily oral foretinib, a multi-kinase inhibitor, in patients with solid tumors. Invest New Drugs. 2012 Oct 6

93 Eder JP, Shapiro GI, Appleman LJ, Zhu AX, Miles D, Keer H, et al. A phase I study of foretinib, a multi-targeted inhibitor of c-Met and vascular endothelial growth factor receptor 2. Clin Cancer Res. 2010 Jul 1;16(13):3507-16.

94 Choueiri TK, Vaishampayan U, Rosenberg JE, Logan TF, Harzstark AL, Bukowski RM, et al. Phase II and biomarker study of the dual MET/VEGFR2 inhibitor foretinib in patients with papillary renal cell carcinoma. J Clin Oncol. 2013 Jan 10;31(2):181-6.

95 Shah MA, Wainberg ZA, Catenacci DV, Hochster HS, Ford J, Kunz P, et al. Phase II study evaluating 2 dosing schedules of oral foretinib (GSK1363089), cMET/VEGFR2 inhibitor, in patients with metastatic gastric cancer. PLoS One. 2013;8(3):e54014.

96 Seiwert T, Sarantopoulos J, Kallender H, McCallum S, Keer HN, Blumenschein G Jr. Phase II trial of single-agent foretinib (GSK1363089) in patients with recurrent or metastatic squamous cell carcinoma of the head and neck. Invest New Drugs. 2012 Aug 24. [Epub ahead of print] PubMed PMID: 22918720.

97 Holland SJ, Pan A, Franci C, Hu Y, Chang B, Li W, et al. R428, a selective small molecule inhibitor of Axl kinase, blocks tumor spread and prolongs survival in models of metastatic breast cancer. Cancer Res. 2010 Feb 15;70(4):1544-54.

98 Silva G, Cardoso BA, Belo H, Almeida AM. Vorinostat induces apoptosis and differentiation in myeloid malignancies: genetic and molecular mechanisms. PLoS One. 2013;8(1):e53766.

99 Yan SB, Peek VL, Ajamie R, Buchanan SG, Graff JR, Heidler SA, et al. LY2801653 is an orally bioavailable multi-kinase inhibitor with potent activity against MET, MST1R, and other oncoproteins, and displays anti-tumor activities in mouse xenograft models. Invest New Drugs. 2012 Dec 29.

100 Yakes FM, Chen J, Tan J, Yamaguchi K, Shi Y, Yu P, et al. Cabozantinib (XL184), a novel MET and VEGFR2 inhibitor, simultaneously suppresses metastasis, angiogenesis, and tumor growth. Mol Cancer Ther. 2011 Dec;10(12):2298-308.

101 Taylor CR. Quality assurance and standardization in the immunohistochemistry. a proposal for the annual meeting of the Biological Stain Commission, June 1991. Biotech Histotech 1992; 67:110-17.

102 Taylor CR. Report from the biological stain commission: Fda issues final rule for classification/reclassification of immunochemistry (IHC) reagents and kits. Biotech Histochem 1998;73:175-7.

103 Taylor CR. an exaltation of experts: concerted efforts in the standardization of immunohistochemistry. applImmunohistochem 1993;1:232-43.

104 Rizzardi AE, Johnson AT, Vogel RI, Pambuccian SE, Henriksen J, Skubitz AP, et al. Quantitative comparison of immunohistochemical staining measured by digital image analysis versus pathologist visual scoring. DiagnPathol. 2012 Jun 20;7:42.

105 Adams EJ, Green JA, Clark AH, Youngson JH. Comparison of different scoring systems for immunohistochemical staining. J ClinPathol. 1999 Jan;52(1):75-7.

106 van Diest PJ, van Dam P, Henzen-Logmans SC, Berns E, van der Burg ME, Green J, et al. A scoring system for immunohistochemical staining: consensus report of the task force for basic research of the EORTC-GCCG. European Organization for Research and Treatment of Cancer-Gynaecological Cancer Cooperative Group. J ClinPathol. 1997 Oct;50(10):801-

107 Choudhury KR, Yagle KJ, Swanson PE, Krohn KA, Rajendran JG. A robust automated measure of average antibody staining in immunohistochemistry images. J Histochem Cytochem. 2010 Feb;58(2):95-107

108 Allred DC, Harvey JM, Berardo M, Clark GM. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. 1998; Mod Pathol, 11:155–168

109 Kurosumi M. Immunohistochemical assessment of hormone receptor status using a new scoring system (J-Score) in breast cancer. Breast Cancer. 2007;14(2):189-93.

110 Cohen, Jacob (1960). "A coefficient of agreement for nominal scales". Educational and Psychological Measurement 20 (1): 37–46

111 Tukey JW. Some thoughts on clinical trials, especially problems of multiplicity. Science 1977;198:679-84. 2

112 Greenhalgh T. Statistics for the non-statistician. l. Different types of data need different statistical tests. BMJ 1997;315:364-6. 3

113 Bland JM, Altman DG. Multiple significance tests: The Bonferroni method. BMJ 1995;310:170.

114 Perneger TV. What's wrong with Bonferroni adjustments. BMJ. 1998 Apr 18;316(7139):1236-8

115 Youden WJ. Index for rating diagnostic tests. Cancer. 1950 Jan;3(1):32-5.

116 Ho PL, Lay EJ, Jian W, Parra D, Chan KS. Stat3 activation in urothelial stem cells leads to direct progression to invasive bladder cancer. Cancer Res. 2012 Jul 1;72(13):3135-42.

117 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970 Aug 15;227(5259):680-5.

118 Concato J, Peduzzi P, Holford TR, Feinstein AR. Importance of events per independent variable in proportional hazards analysis. I. Background, goals, and general strategy. J Clin Epidemiol. 1995 Dec;48(12):1495-

119 Peduzzi P, Concato J, Feinstein AR, Holford TR. Importance of events per independent variable in proportional hazards regression analysis II. Accuracy and precision of regression estimates. j clin epidemiology 1995 Dec;48(12):15031510

120 Peduzzi P, Concato J, Kemper E, Holford TR, Feinstein AR. A simulation study of the number of events per variable in logistic regression analysis. J Clin Epidemiol. 1996 Dec;49(12):1373-9

121 McHugh LA, Sayan AE, Mejlvang J, Griffiths TR, Sun Y, Manson MM, Tulchinsky E, et al. a dual inhibitor of ErbB-1/-2 receptors, enhances effects of combination chemotherapy in bladder cancer cells. Int J Oncol. 2009 Apr;34(4):1155-63

Abbreviations

AKT	Protein kinase B
AML	Acute myeloid leukaemia
AP1	Activator protein 1
APC	Adenometous polyposis coli
APS	Ammonium persulphate
ATCC	American type culture collection
BCA	Bicinchoninic acid
BCG	Bacillus Calmette Guerin
BS	Blocking solution
BSA	Bovine serum albumin
c-KIT	Stem cell growth factor receptor
c-MET	Hepatocyte growth factor receptor
CIS	Carcinoma In Situ
CLL	Chronic lymphocytic leukaemia
CM	Cyclophosphamide methotrexate
CML	Chronic Myeloid Leukaemia
CMV	Cyclophosphamide methotrexate vincristine
CRT	Cancer research technologies
CT	Computerised Topography
DAB	Diaminobenzidine
DDR1/2	Discoidin domain receptor family, member 1/2
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

dsRNA	double stranded ribonucleic acid
EBRT	External beam radiotherapy
EDTA	Ethylene diamine tetraacetic acid
EGFR	Epidermal growth factor receptor
EMA	European Medicine Agency
EMDA	Electromotive drug administration
EMT	Epithelial mesenchymal transition
EORTC	European Organisation for Research and Treatment of Cancer
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FDA	Food and Drugs Administration
FFPE	Formalin-fixed, paraffin-embedded
FGFR3	Fibroblast growth factor receptor 3
FLT3	Fms-like tyrosine kinase 3
FLTS-ITD	Fms-like tyrosine kinase 3 Internal Tandem Duplication
GC	Gemcytabine cisplatin
GIST	Gastrointestinal stromal tumour
Grb2	Growth factor receptor-bound protein 2
H&E	Haematoxylin and eosin
HER2	Human epidermal growth factor receptor 2
HR	Hazard ratio
HRP	Horseradish peroxidase
IC_{50}	Half maximum inhibitory concentration

IgG	Immunoglobulin G
IHC	Immunohistochemistry
IMS	Industrial methylated spirit
KDa	Kilodaltans
MAPK	Mitogen activated protein kinase
MEK	Mitogen activated protein kinase kinase
MERTK	Proto-oncogene tyrosine-protein kinase MER
MIBC	Muscle-Invasive Bladder Cancer
MIN	Multiple intestinal neoplasia
MKNK1/2	Mitogen activated protein kinase-interacting serine/threonine-protein kinase 1/2
MRI	Magnetic Resonance Imaging
mRNA	messenger ribonucleic acid
MST1R	Macrophage-stimulating protein receptor
MVAC	Methotrexate vincristine adromicin cisplatin
NF KB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHS	National Health Service
NMIBC	Non-Muscle-Invasive Bladder Cancer
NRS	Normal rabbit serum
NSCLC	Non-small cell lung cancer
OR	Odds ratio
p53	Tumour protein p53
PAGE	Polyacrylamide gel electrophoresis

PDGFR	Platelet-derived growth factor receptor
PI3K	Phosphatidylinositol 3-Kinase
PKC	Protein kinase C
PLC gamma2	1-Phosphatidylinositol-4,5-bisphosphate phosphodiesterase gamma-2
PTB	Phosphotyrosine binding
PTEN	Phosphate and tensin homologue deleted on chromosome ten
PUNLMP	Papillary urothelial neoplasia of low malignant potential
PVDF	Polyvinylidine fluoride
qPCR	Quantitative polymerase chain reaction
RAS	Rat sarcoma protein
Rb	Retinoblastoma
RNA	Ribonucleic acids
ROC	Receiver operating characteristics
RON	Recepteur d'origine Nantais kinase
RTK	Receptor tyrosine kinase
S6	ribosomal S6 kinase
SDS	Sodium dodecyl sulfate
SFK	Src family kinase
SH2	Src homology 2
shRNA	small hairpin ribonucleic acid
siRNA	small interfering ribonucleic acid
SOS	Son of sevenless

sRNA	small ribonucleic acid
STAT	Signal Transducers and Activator of Transcription
STAT3	Signal Transducers and Activator of Transcription 3
TAM	Tyro-3 AXL Mer (receptor family)
TBS	Tris buffered saline
TBS-T	Trisbuffered saline – tween 20
TCC	Transitional Cell Carcinoma
TEMED	Tetramethylethylenediamine
TG	Transgenic
TKI	Tyrosine kinase inhibitor
TMA	Tissue micro array
TURBT	Transurethral resection of bladder tumour
UHL	University Hospitals Leicester
UK	United Kingdon
V-FITC	Five-fluorescein isothiocyanate
VEGFR	Vascular epidermal growth factor receptor
WT	Wild-type
ZAP70	Zeta-chain-associated protein kinase 70
ZEB2	Zinc finger E-box-binding homeobox 2

Research publications and abstracts

Published abstracts

AXL in Bladder Cancer: A New Marker of Invasion, Metastasis and Potential Therapeutic Target. Dyer J, Ghouri S, Dormer J, Tulchinsky E, Mellon JK. British Journal of Surgery 2014;101;s4;1-73

Presentations

AXL in Bladder Cancer: A New Marker of Invasion, Metastasis and Potential Therapeutic Target. Dyer JE, Ghouri S, Dormer J, Tulchinsky E, Mellon JK.

EAU 2014, Stockholm

BAUS section of academic urology annual meeting 2014

AUA 2013, San Diego

Malcolm Copcoat Endourology and Laparoscopy meeting at the Royal Society of Medicine April 2013,

BAUS 2013 basic science section