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## INVASIVE BLADDER CANCER - Aspects on staging and prognosis

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Lund University, Faculty of Medicine Doctoral Dissertation Series 2006:121

# INVASIVE BLADDER CANCER – ASPECTS ON STAGING AND PROGNOSIS

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The public defense of this thesis will, with due permission from the Faculty of Medicine at Lund University, take place in Föreläsningssal 1, Lund University Hospital, on Friday, October 6, 2006, at 0900.

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Title and subtitle Invasive bladder cancer – aspects on staging and prognosis		
Abstract  <p><i>Background:</i> The difficulty of determining the prognosis for the individual patient with invasive bladder cancer is a major clinical problem. Currently, decisions regarding therapy are mainly based on tumour stage and grade, the first of which is notoriously demanding to ascertain. New molecular markers are however proposed to be of prognostic value. At present, there is considerable debate regarding the effects of delay on prognosis, lymph node staging and detection and staging of transitional cell carcinoma (TCC) involving the prostatic urethra and prostate in males. This thesis is based on investigations of the impact of diagnostic and treatment delay on prognosis in invasive bladder cancer (Papers I and II). Intra-operative sentinel node (SN) detection for lymph node staging is evaluated prospectively in Paper III, as are the incidence of TCC in the prostatic urethra and prostate and the preoperative detection of such tumour growth in Paper IV. The prognostic values of expression profiling and tissue microarray (TMA) in high-risk bladder cancer are investigated in Papers V and VI.</p> <p><i>Results and conclusions:</i></p> <ol style="list-style-type: none"> <li>1) Diagnostic delay in patients with T1 tumours might have an adverse effect on the prognosis.</li> <li>2) Treatment delay in patients with invasive bladder cancer submitted to radical cystectomy did not influence disease-specific survival or stage progression in the present study.</li> <li>3) Intraoperative SN detection is feasible during radical cystectomy and improves nodal staging.</li> <li>4) Preoperative biopsies from the prostatic urethra identified 66% of patients with TCC in the prostatic urethra and/or prostate in a prospective study investigating the prostate and bladder neck with sagittal whole-mount technique.</li> <li>5) Preoperative investigation with cold cup mapping biopsies has a low sensitivity for detection of CIS (23%) and is probably of little clinical value for identifying patients at risk of TCC in the prostatic urethra/prostate in the cystoprostatectomy specimen.</li> <li>6) Expression profiling identified a 50 gene signature predicting lymph node metastasis and survival in patients submitted to radical cystectomy.</li> <li>7) TMA-based analysis of prognostic markers in invasive bladder cancer seems to be of limited value.</li> </ol>		
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**INVASIVE BLADDER CANCER  
– ASPECTS ON STAGING AND  
PROGNOSIS**

**FREDRIK LIEBERG, M.D.**

Doctoral Thesis  
2006  
Department of Urology  
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Cover: Bladder cancer metastasis detected in a sentinel node.

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## **ABBREVIATIONS**

CIS Carcinoma In Situ

CIS pu Carcinoma In Situ in prostatic urethra

CIS pd Carcinoma In Situ in prostatic ducts or acini

CT Computerized Tomography

FGFR3 Fibroblast Growth Factor Receptor 3 (*FGFR3* gene)

H&E Hematoxylin-Eosin

IMA Inferior Mesenterix Artery

LOH Loss of Heterozygosity

MDM2 Murine Double Minute 2

MHC class I Major Histocompatibility Complex class I

MRI Magnetic Resonance Imaging

PCR Polymerase Chain Reaction

Rb Retinoblastoma Protein (*RBI* gene)

ROC-analysis Receiver Operated Curve-analysis

SAM Significance Analysis of Microarrays

SN Sentinel Node

TCC Transitional Cell Carcinoma

TMA Tissue Microarray

TURB Transurethral Resection of Bladder tumour

UC Urothelial Carcinoma

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## LIST OF PAPERS

- I. Liedberg F, Anderson H, Månsson Å and Månsson W. Diagnostic delay and prognosis in invasive bladder cancer. *Scand J Urol Nephrol* 2003; 37:396-400. Reprinted with permission from Taylor & Francis
- II. Liedberg F, Anderson H and Månsson W. Treatment delay and prognosis in invasive bladder cancer. *J Urol* 2005; 174:1777-1781. Reprinted with permission from Elsevier Science.
- III. Liedberg F, Chebil G, Davidsson T, Gudjonsson S and Månsson W. Intraoperative sentinel node detection improves nodal staging in invasive bladder cancer. *J Urol* 2006; 175:84-89. Reprinted with permission from Elsevier Science.
- IV. Liedberg F, Anderson H, Bläckberg M, Chebil G, Davidsson T, Gudjonsson S, Jahnson S, Olsson H and Månsson W. Transitional cell carcinoma in the prostatic urethra and prostate in the cystoprostatectomy specimen – incidence, characteristics and preoperative detection. A prospective study. Submitted.
- V. Lindgren D, Liedberg F, Bendahl P-O, Andersson A, Fregyiesi A, Veerla S, Lövgren K, Chebil G, Gudjonsson S, Borg Å, Fernö M, Fioretos T, Månsson W and Höglund M. Molecular profiling of high risk bladder carcinomas reveals expression profiles highly associated with *FGFR3/TP53* mutation status and a MHC class I gene-signature that predicts lymph node metastases and survival. In manuscript.
- VI. Liedberg F, Anderson H, Chebil G, Fernö M, Gudjonsson S, Höglund M, Lindgren D, Lundberg L-M, Lövgren K and Månsson W. Tissue microarray based analysis of prognostic markers in invasive bladder cancer. Accepted *Urol Oncol*.

## INTRODUCTION

Bladder cancer is the fourth most common malignancy in males in a recent report, with a median age of 72 years at diagnosis (1). Slightly more than 50% of newly diagnosed tumours are non-invasive (stage Ta), whereas approximately 40% of primary tumours are invasive, i.e. invading sub-epithelial connective tissue or muscle (stage T1 to T4) (2). High-grade invasive bladder cancer is a lethal malignancy, if untreated >85% of patients die within two years of the diagnosis (3). The treatment of bladder cancer is at present mainly based on the tumour grade and stage, and correct assessment of these parameters is thus crucial. Today in Sweden the WHO classification from 1999 describing Papillary Urothelial Neoplasm of Low Malignant Potential and cancer grades I, II and III is the most commonly used, and not the latest WHO classification from 2004 (4). Staging of bladder tumours is performed according to the TNM classification from 2002 (5) (Table 1).

**Table 1.** TNM classification 2002.

a) Urinary bladder

Ta	Non-invasive papillary carcinoma
Tis	Carcinoma in situ
T1	Tumour invades sub-epithelial 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, uterus, vagina, pelvic wall, abdominal wall T4a Tumour invades prostate, uterus, or vagina T4b tumour invades pelvic wall or abdominal wall

b) Transitional cell carcinoma of the prostate (prostatic urethra)

Tis pu	Carcinoma in situ, involvement of prostatic urethra
Tis pd	Carcinoma in situ, involvement of prostatic ducts
T1	Tumour invades sub-epithelial connective tissue
T2	Tumour invades any of the following: prostatic stroma, corpus spongiosum, periurethral muscle
T3	Tumour invades any of the following: corpus cavernosum, beyond prostatic capsule, bladder neck (extraprostatic extension)
T4	Tumour invades other adjacent organs (invasion of the bladder)

## STAGING OF INVASIVE BLADDER CANCER

### *Transurethral resection*

Endoscopic resection of bladder tumours (TURB) has evolved from the first description of endoscopic treatment of bladder tumours in 1910 by Beer (6), until the more detailed description of the technique utilizing a diathermy resectoscope in 1955 (7). Today TURB has been described as “technically safe, oncologically anything but perfect” (8). Despite this statement, TURB is the primary measure for obtaining clinical information about tumours. The purposes of TURB are threefold:

- 1) To remove the cancer, as a possibly curative procedure.
- 2) To assess endoscopically, as the resection proceeds, the depth of tumour invasion.
- 3) To obtain samples of tumour, and in high-grade cancers, the bladder walls and prostatic urethra in males, for histological examination to determine the stage and grade of the tumour.

To improve clinical staging in invasive bladder cancer, a sample with separate resectional biopsies from the tumour base including detrusor muscle is important. Random cold cup biopsies from the bladder are not generally recommended, but in case of planned radical radiotherapy or partial cystectomy the absence of concomitant carcinoma in situ (CIS) is of importance. However, the sensitivity of such biopsies is largely unknown, in one study comparing random cold cup biopsies with findings in the cystectomy specimen a 77% accuracy was found (9). These findings indicate a need for new and better methods for the detection of CIS. Fluorescence cystoscopy might represent such an improvement (10). The presence of CIS in the bladder has been presented as a risk factor for transitional cell carcinoma (TCC) in the prostatic urethra and prostate (11). The presence of TCC in the prostate and/or prostatic urethra is best determined by resectional biopsies of the floor of the prostatic urethra at 5 and 7 o'clock, extending from the bladder neck to the distal end of the verumontanum, including the underlying prostatic stroma (12). Bimanual palpation after TURB is omitted in the present TNM classification of bladder tumours, though advocated by some urologists as an investigation that can provide important prognostic information (13) (14). Except for tumour grading, no other morphological criteria are standardized to be used as prognostic parameters, which are needed for determining the optimal choice of treatment in invasive bladder cancer. However, depth of lamina propria invasion in T1 tumours (15, 16) and angiolymphatic invasion in muscle-invasive patients treated with radical cystectomy are proposed to have independent prognostic value in multivariate analyses in retrospective samples (17-19).

### *Imaging*

Ability to distinguish organ-confined muscle invasive disease (clinical stage T2a or T2b) from non-organ-confined disease (clinical stage T3a or higher, and/or lymph-node positive disease) prior to cystectomy would add significant prognostic information, and could also influence the use of neo-adjuvant chemotherapy. With or without the aid of radiological investigations (i.e. computerized tomography (CT) or magnetic resonance imaging (MRI)), the risk of clinical under-staging of local extension of the tumour is high; 41%–74% (20–25), and the mean percentage of local under-staging in these studies is 53%. Normal-appearing regional lymph nodes at a preoperative CT of the abdomen are associated with 24% unsuspected nodal metastases in a large cystectomy series (26), further exemplifying the great difficulty in distinguishing organ- versus non-organ-confined disease preoperatively.

CT obtained before cystectomy is of limited value, as suggested by one study, in which information altering the planned surgical management was found in only 4% in a consecutive series (27). However, MRI with dynamic contrast administration has been suggested to be superior to CT, particularly in detecting extravesical tumour extension (28, 29). One prospective study even claims that the MRI tumour stage confers additional prognostic information to clinical stage and grade in predicting disease relapse and death after radiotherapy (30). New technical improvements of MRI equipment might improve the staging ability, and 82% accuracy has recently been reported (31). Adding ferumoxtran-10 might also further improve radiological nodal staging (32), but high expectations on the accuracy of MRI staging have to be validated in prospective studies with survival as endpoint.

## TREATMENT OF INVASIVE BLADDER CANCER

### *Radical cystectomy for invasive bladder cancer*

The standard treatment for muscle-invasive bladder cancer is radical cystectomy. Overall survival at five years is around 60% (26, 33, 34). The decrease in morbidity and mortality in recent years has made radical cystectomy a viable treatment option also for patients above 75 years of age with co-morbidities (35, 36). A decrease in peri-operative mortality has also been described recently when radical cystectomies are performed in high-volume centres (37–39) by surgeons frequently performing the procedure (40). McCabe and co-workers recommend that a hospital should perform at least 11 radical cystectomies/year to achieve optimum outcomes (41). Advances in minimally invasive surgery have stimulated the development of laparoscopic radical cystectomy with advantages such as reduced blood loss and decreased analgesic requirement, although long-term oncologic data are awaited (42).

Bladder preservation protocols in the treatment of invasive bladder cancer have been questioned (43), but combining transurethral resection with cisplatin-based chemotherapy and/or radiation has shown almost as good survival figures as in radical cystectomy series (44). The finding that 30% (45) and 33% (46) of patients shed cancer cells to pelvic lymph nodes determined by real-time reverse transcriptase-polymerase chain reaction (PCR), despite the presence of benign lymph node histology, clearly underlines the need for better prognostic instruments selecting patients for bladder preservation.

### *Neo-adjuvant and adjuvant treatment of bladder cancer*

To increase survival after radical cystectomy, adjuvant and neo-adjuvant strategies have been explored in prospective randomized trials. The advantages of neo-adjuvant regimens include in vivo drug sensitivity testing during evaluation, possible down-staging, delivering chemotherapy without the burden of concomitant postoperative recovery and leading to early treatment of possible micro-metastases. The available definitive pathological staging speaks in favour of adjuvant protocols, decreasing risks of over-treatment with neo-adjuvant chemotherapy in patients with organ-confined disease. Due to the lack of large randomized studies with clearly favourable outcomes, meta-analyses have been performed both with available adjuvant (47) and with neo-adjuvant (48, 49) studies. The increase in absolute survival was 9%, 6.5% and 5%, respectively, with a higher level of evidence for neo-adjuvant regimens mainly due to a larger number of patients in these studies. Still, the general applicability of trials with intense chemotherapy after radical cystectomy must be questioned, as

being fit enough for a chemotherapy protocol generates eligibility bias and is in itself a good prognostic factor in invasive bladder cancer (50). The quality of the surgery performed in such studies also influences the outcome, as described by Herr and co-workers. In a multivariate analysis of patients randomized to neo-adjuvant chemotherapy, the improvement in survival for neo-adjuvant chemotherapy could only be shown for patients submitted to a lymph node dissection extended to the iliac bifurcation as compared to a limited dissection or no lymphadenectomy at all (51).

## PROGNOSIS

### *Delayed diagnosis and treatment*

Delayed treatment of a disease is due to diagnostic delay and treatment delay. The diagnostic delay can be divided into patient's delay, i.e. the time lag from the patient's first awareness of symptoms until the first medical consultation, and doctor's delay, i.e. the time lag from that consultation until the establishment of a correct diagnosis. In an early study in bladder cancer, it was suggested that a delay in diagnosis and treatment might adversely affect prognosis in terms of patient survival (52). This general hypothesis has not been confirmed subsequently (53, 54), and in one study even a trend towards shorter survival was seen among patients with a short diagnostic delay (55). In a more recent study of diagnostic delay in invasive tumours (T1–T4), a short diagnostic delay was associated with better prognosis in T1 tumours but not in muscle-invasive tumours (56). A recent prospective study comparing patients with bladder cancer detected through haematuria home screening and patients presenting in standard clinical care showed a reduction in bladder cancer mortality, possibly due to a short diagnostic delay in the screened group (57). The effectiveness of haematuria clinics has also been discussed in terms of diminishing both diagnostic and treatment delay (58, 59). Same-day diagnostic service for new cases of haematuria has successfully been organized at some hospitals in the UK, resulting in shorter hospital-based delay and an increase in the number of diagnostic cystoscopies performed within 4 weeks (60).

Regarding treatment delay in invasive bladder cancer, i.e. time from diagnosis to treatment (cystectomy or radiotherapy), this seems largely to be due to hospital routines, and median delays from 33 to 63 days have been reported (61–65). Muscle invasion in bladder cancer has been characterized “as a major signal of an impending lethal event” (66), and thus, early treatment seems logical. Even if only early invasion is present, as in T1 disease, delayed treatment with radical cystectomy might reduce survival (67, 68). Five current publications suggest that a long delay from diagnosis until cystectomy influences outcome, with regard to pathological tumour stage (62) and even disease-specific survival (61, 63, 65, 69).

### *Lymph node metastasis*

Apart from the pathological stage of the bladder tumour, the presence or absence of lymph node metastases is the most important determinant of survival in bladder cancer patients undergoing radical cystectomy (70). Preoperative nodal staging with CT is unreliable, with a high (21%) false-negative rate observed (27). Only a few investigations have appraised positron emission tomography (PET) and its capacity

to detect lymph node metastases in bladder cancer, and the results have been largely disappointing (71). However, some investigators (32) have recently claimed that new ferumoxtran-enhanced MRI imaging for nodal staging offers 91% sensitivity and a 98% negative predictive rate. Nonetheless, open surgery is still the standard for nodal staging, although one report has indicated that limited laparoscopic lymphadenectomy offers equivalent efficacy and a shorter postoperative stay (72). In 1950, Leadbetter (73) described a technique for “regional gland dissection” that is still in use today. The optimal extent of the lymph node dissection for accurate staging, the curative potential of the method and the prognosis of lymph-node positive disease are matters of debate, which are discussed in a recent review (74).

*Limited lymph node dissection* is an extirpation of the lymphatic tissue in the obturator fossa (i.e. between the obturator nerve and the external iliac vein), which provides only about 10 nodes for examination (75). Another method of limited dissection, usually referred to as a *conventional pelvic dissection* (76) or a *standard dissection* (77), includes the area stretching laterally as far as the genito-femoral nerve and posterior to the internal iliac vessel. Different reports indicate that surgeons using this template have removed from 8 to 26 nodes (77, 78). Thalmann et al. (79) have employed an approach that also includes pre-sacral nodes medial to the internal iliac vessels and common iliac nodes, as far up as the ureteral crossing. Pre-sacral lymph node metastases represent 8% of all lymph node metastases in a recent multicentre mapping study (80), and another study identified pre-sacral lymph node metastases in 5% of the patients (81). The commonly called *extended dissection* (78, 80, 82, 83) includes all lymphatic tissue to the aortic bifurcation, as in the regional gland dissection described by Leadbetter (73), or even up to the inferior mesenteric artery (IMA). Extending the dissection to the aortic bifurcation increases the number of nodes that are harvested, and yields reported in the literature vary between 15 and 40 nodes (77, 81, 84–86). Corresponding results in series using the IMA as the upper limit of dissection are 43 (mean) and 56 (median) nodes (78, 80, 83). There is, however, substantial inter-surgeon variability in terms of lymph node retrieval. Leissner and co-workers (85) found significant variation in the number of nodes obtained (range 10–21) by sixteen surgeons who used the aortic bifurcation as the upper limit of dissection.

#### *Sentinel node detection*

The sentinel node (SN) concept was originally suggested by Gould 1960 in parotid cancer (87), but has been further developed and applied in different malignancies since then. Knowledge of the pathway for spread of tumour cells is the basis for the sentinel node (SN) concept. According to this view, tumour cells metastasizing via the lymphatics will enter the SN, the first node of the regional lymph node basin, before they disseminate sequentially to other lymph nodes. The SN is

specific for each individual patient, and can be identified either preoperatively by lymphoscintigraphy or intra-operatively by dye detection or the use of a gamma-detecting probe following administration of a radioactive tracer close to the tumour. SN detection allows identification of a small volume of representative nodal tissue for thorough pathological evaluation. Detection of micro-metastases can be improved by ultra-staging, which entails analysis of the SN by extended serial sectioning combined with immunohistochemical techniques (88). A small pilot study has recently demonstrated that SN detection in patients with invasive bladder cancer is feasible (89).

#### *Transitional cell carcinoma in the prostate*

Transitional cell carcinoma (TCC) can spread from the bladder to the prostatic urethra and the urothelium of the prostatic ducts or originate there (primary TCC of the prostate). However, the latter is a rare event (90). The incidence of TCC in the prostatic urethra and prostate in conjunction with an invasive bladder tumour is higher, but reported figures in cystoprostatectomy specimens vary between 12% and 48% (91, 92), the higher incidence detected utilizing whole-mount pathological analysis. Hence, there are diverging reports on the incidence of TCC in the prostatic urethra and prostate. The significance of TCC in the prostatic urethra/prostate for the prognosis of the disease, associated increased risk of urethral recurrence and its

**Table 2.** Results of conservative treatment of superficial TCC in the prostatic urethra/prostate with or without transurethral prostate resection.

Author	Year	Prostatic resection prior to BCG	Outcome (complete response) (%) CIS pu	Outcome (complete response) (%) CIS pd	Outcome (complete response) (%) CIS pu/CIS pd
Hillyard (150)	1988	No	6/8 (75%)		
Bretton (151)	1989	1–3 loopfuls deep	10/19 (53%)	3/4 (75%)	
Schellhammer (152)	1995	No	8/10 (80%)	4/7 (57%)	
Palou (153)	1996	No			14/18 (78%)
Canda (154)	2004	No			7/12 (58%)
Palou (155)	2006	No		9/11 (82%)	
Σ					61/89 (69%)

**Table 3.** Survival in patients with CIS pd treated with radical cystoprostatectomy.

Author	Year	Disease specific 5-year survival in patients with CIS pd % (n)
Schellhammer (91)	1977	50% (12)
Esrig (94)	1996	82% (29)
Pagano (93)	1996	50% (14) (3 year-survival)
Njinou (156)	2003	70% (10)

implications for prostate sparing cystectomy are also matters of debate. Recent data claim that non-stromal invasion of TCC does not alter survival, whereas stromal invasion decreases survival (93, 94), but also that “non-contiguous” invasion of the prostate has better prognosis than “contiguous” (93). When TCC is superficial in the prostatic urethra (i.e. CIS pu or CIS pd), conservative treatment with BCG might be appropriate, although data with follow-up on this treatment modality are scarce (Table 2). In cases of extensive intraductal growth, radical cystoprostatectomy seems advisable as available series in the literature show that a significant proportion of the patients die from their disease despite such aggressive surgery (Table 3). Thus the influence of TCC in the prostate on survival is complex, and the prognostic significance of different degrees of prostate invasion by TCC is not taken into account in the present TNM classification with separate staging of the primary bladder tumour and TCC in the prostatic urethra/prostate, nor is the impact of different pathways (“contiguous” vs. “non-contiguous”) of prostate involvement considered. It should be observed that bladder cancer stage T4a denotes stromal invasion of the prostate (95). There are at least four suggestions available for a new TNM classification of prostatic involvement (93, 94, 96, 97), but all of them based on series with relatively small numbers of patients.

Preoperative bladder tumour characteristics, such as carcinoma in situ in the bladder and bladder tumour multifocality, are described as risk factors for prostatic urethral involvement at cystoprostatectomy (11). The value of preoperative transurethral loop biopsies from the prostatic urethra has been questioned, since only 53% sensitivity for detection of prostatic stromal invasion has been reported in one study (98). Overall sensitivity (superficial and stromal involvement) for adequately obtained

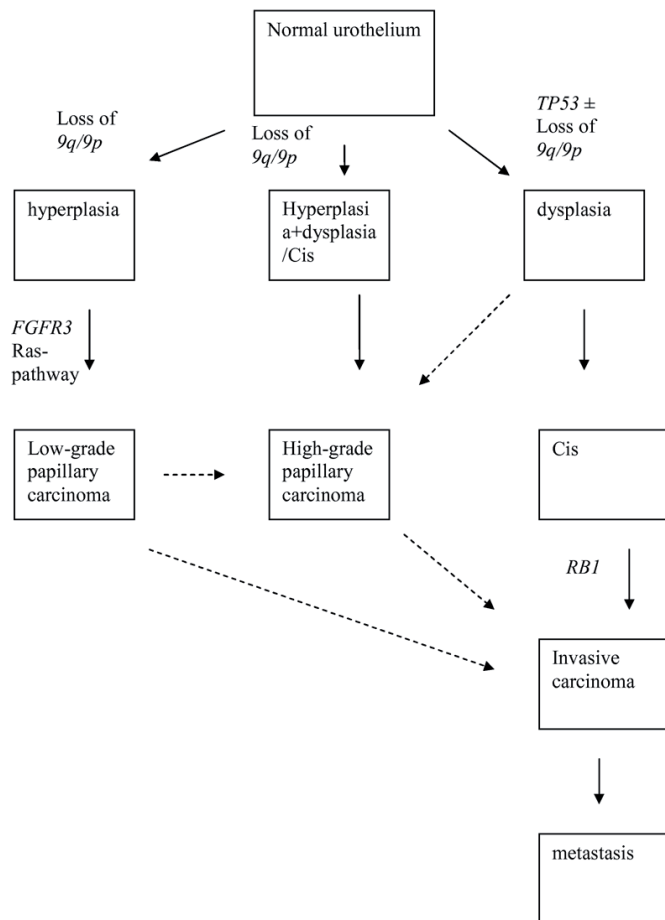
transurethral loop biopsies including the verumontanum portion of prostatic urethra (12) is probably higher, and 81% has been reported from the same series (98). Examination of frozen section specimen of the urethra has been suggested as a method for predicting urethral recurrence, but such methodology gives little information about TCC in the prostatic urethra/prostate and the method has so far only been validated in one sample (99), yet it is frequently used today.

The risk of urethral recurrence after radical cystoprostatectomy increases when TCC is present in the prostatic urethra or prostate, from 5% without involvement of the prostatic urethra/prostate, to 12% and 18% with superficial (non-stromal) and invasive prostate involvement, respectively, in a large recent sample (100). Still, some authors suggest a more liberal use of orthotopic bladder substitution despite TCC in the prostatic urethra or prostate (101). Primary cystoprostatourethrectomy is associated with substantially decreased sexual function (102, 103), which has to be balanced against the risk of urethral recurrence. To minimize the risk of incontinence and impotence, prostate sparing surgery during radical cystectomy has been suggested as an option (104). However, the finding of TCC in the prostatic urethra/prostate and/or prostatic adenocarcinoma in 74% of the prostates in one cystoprostatectomy series (92), indicates that prostate sparing radical cystectomy might be an oncologically hazardous procedure.

## NEW PROGNOSTIC METHODS

### *Genetic aspects of invasive bladder cancer*

Epithelial tumours are generally believed to progress from benign to malignant by alterations in genes of importance with regard to growth signals, angiogenesis, apoptosis and tissue invasion and metastasis, resulting in a limitless replicative potential (105). The last few years have seen an increase in information on bladder cancer genetics, epigenetics and gene expression (106–108). However, this new molecular knowledge has not yet been translated into validated predictive markers foreseeing, for example, progression of superficial to invasive disease. Urothelial



**Figure 1.** Genetic pathway of urethelial tumorigenesis. *TP53* and *RB1* have inactivating mutations and *FGFR3* has activating mutations.

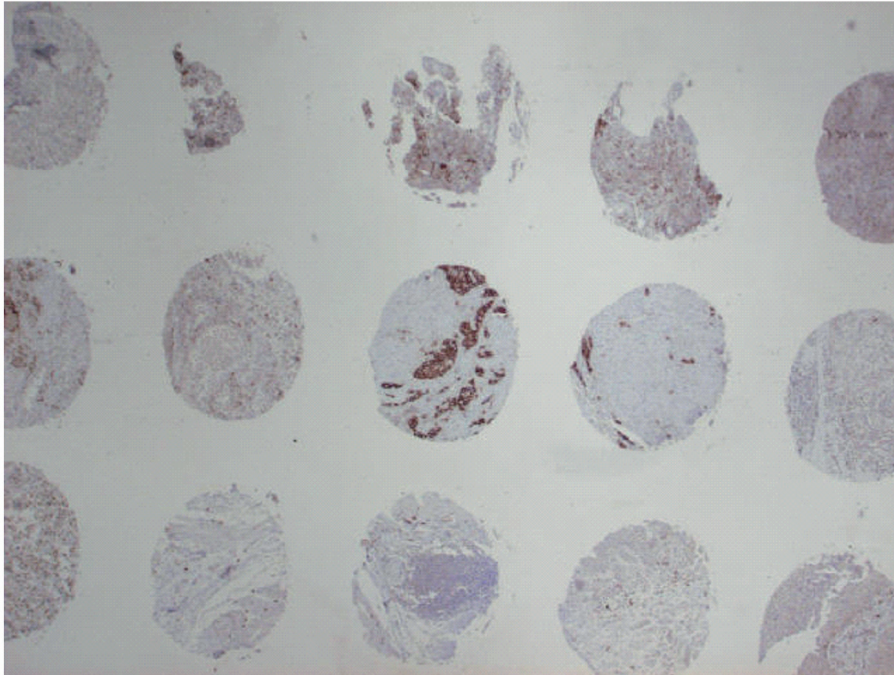
tumorigenesis in bladder cancer has traditionally been thought to occur through two, partly overlapping, different pathways characterized by genetic and epigenetic defects (109), as originally described by Koss (110). Low-grade papillary tumours frequently display a loss of chromosome 9 (111), and have gene mutations that activate the receptor tyrosine kinase-Ras pathway (exemplified by mutations of the Fibroblast Growth Factor Receptor 3 (*FGFR3*)) (112). Invasive tumours commonly have defects in the *TP53* and *RB1* function (109). A third distinct pathway for urothelial tumorigenesis has been suggested by Lee and Droller for high-grade papillary tumours via dysplasia, as in the development of CIS, and hyperplasia generating a high-grade papillary tumour that lacks *TP53* mutation (113) (Figure 1). Such molecular pathways are complex and the status of each gene or protein is influenced by several mechanisms. For example, regarding *TP53* the heterogeneity in function among different *TP53* mutations has been described (114). Over-expression of MDM2 (Murine double minute 2) which functionally inactivates *TP53* might also influence p53 status (115). The nuclear accumulation of *TP53*, as detected by immunohistochemistry, is believed to be caused by *TP53* mutations that both inactivate and stabilize the TP53 protein and has been evaluated as a prognostic marker in bladder cancer. However, the available publications show considerable differences in the prognostic significance of p53 immunohistochemistry (116). As stated by Mitra and co-workers (117), the absence of a single “gold standard” predictive molecular marker in bladder cancer is not a surprise. The cooperative promotion of tumour progression, as exemplified by additive prognostic information obtained from altered expression of *TP53* and *RB1* (118), also suggests that it is necessary to study multiple markers to better classify and determine prognosis in individual patients. Today analysis of thousands of genetic markers can be performed by expression microarray analysis (gene-expression profiling) (see below).

#### *Global gene expression*

Gene-expression profiling has recently been used to classify bladder tumours (119–124). Gene expression profiles obtained with oligonucleotide arrays have identified a gene signature for groups of superficial tumours that did not progress (125, 126). The possibility to predict outcome in muscle-invasive tumours according to the expression of an identified set of genes has recently been suggested (122), but confirming studies are needed. Expression of a gene signature for patients at high risk of disease recurrence is currently being investigated prospectively in a breast cancer study, where patients with such high-risk profile are randomized to adjuvant treatment (127). There are also reports claiming that gene expression profiles can predict response to chemotherapy in bladder cancer patients (128).

#### *Tissue microarray*

Tissue microarray (TMA) technology was invented and developed by Kononen



**Figure 2** PTGS-2 expression in a tissue microarray section (x40 magnification).

and co-workers and has enabled simultaneous investigation of hundreds of cores of pathological tissue simultaneously (129). From the original histopathological blocks a tissue arraying instrument extracts tissue cores from previously marked areas representative of tumour tissue. The cores are placed in an empty recipient paraffin block, from which sections are later cut and studied with immunohistochemistry (Figure 2) or investigated by in situ hybridization techniques. The theoretical advantages of TMA technology are that all tissue specimens arrayed on one TMA block are analysed in an identical fashion with regard to antigen retrieval, reagent concentrations, incubation times and wash conditions. Small quantities of reagent used and expeditious investigation of several hundred tumour samples simultaneously make the TMA technology cost-effective. Validation of the TMA methodology has been performed in bladder cancer and it has been suggested that intra-tumour heterogeneity does not significantly affect the results (130). As an example of the technique, reduced expression of metastasis suppressor RhoGDI2, has been suggested to be associated with decreased survival in a cohort of 51 patients submitted to radical cystectomy investigated with immunohistochemistry utilizing the TMA technology (131). However, there are also several reports with negative findings with regard to prognostic value of markers studied in patients with invasive bladder cancer (130, 132–141).

## AIMS OF THE INVESTIGATIONS

### **Paper I**

To evaluate whether delay in diagnosis (diagnostic delay) affects disease-specific survival in invasive bladder cancer in a population-based sample with long-term follow-up.

### **Paper II**

To investigate whether treatment delay affects the disease-specific survival and/or stage progression in a series of patients who have undergone cystectomy.

### **Paper III**

To evaluate the concept of sentinel node (SN) in patients with invasive bladder cancer undergoing radical cystectomy in order to improve the lymphadenectomy and the detection of metastatic lymph nodes.

### **Paper IV**

To describe the incidence of TCC in the prostatic urethra and the prostate, to analyse characteristics of the bladder tumour with regard to risk of TCC in the prostatic urethra/prostate and to investigate the sensitivity of preoperatively obtained resection biopsies from the prostatic urethra.

### **Paper V**

To obtain a molecular description of high-risk urothelial carcinomas with expression profiling and analysis of *FGFR3* and *TP53* gene mutations and to correlate gene status with outcome.

### **Paper VI**

To investigate the expression of several potential prognostic markers with immunohistochemistry utilizing TMA technology and to correlate marker status with disease-specific survival in a cohort of patients with invasive bladder cancer submitted to radical cystectomy.

## PATIENTS AND METHODS

### Paper I

In a previous study (142), data derived from all 343 cases of bladder cancer in the Southern Swedish Health Care Region notified to the population-based Regional Tumour Register in 1988 were analysed. For 177 patients with clinical stages T1–T4, relevant variables were extracted from all available records at every level of referral, and included onset date and specific pattern of symptoms, date of first medical consultation, details of investigations, and date of diagnosis, which was defined as the date of the first positive pathologic report on a transurethrally obtained tumour specimen. Causes of death were retrieved from the Swedish Cause of Death Register for patients who had died and clinical records were again reviewed for patients who had malignancies other than bladder cancer listed as the cause of death in the Register. As the Register was available only until 1998, clinical records from January 1999 until June 2000 were reviewed to obtain causes of death for patients who had died during that time period. The main endpoint was time from diagnosis of bladder cancer to bladder cancer death with follow-up censored on 30 June, 2000, or death due to other causes before that time. The relation between tumour stage and diagnostic delay was studied using Cox regression analysis. The interaction between tumour stage and diagnostic delay was tested, and separate analyses were performed in the T1 and T2–T4 groups. The cumulative incidence of bladder cancer death was used to illustrate the effect of stage and delay.

### Paper II

The study population consisted of 141 patients with locally advanced bladder cancer submitted to radical cystectomy between 1990 and 1997 at our department. A total of 46 patients with locally advanced tumours received preoperative radiation of 20 Gy for 1 week immediately before surgery. Treatment delay was defined as the time interval between the pathology report confirming invasive disease and the performance of cystectomy, and was ascertained retrospectively from the charts. For those patients who received preoperative radiation the week before surgery, this time was included in treatment delay. Reasons for treatment delay were also retrieved from the charts. Follow-up ended in April 2003. Death from bladder cancer was the primary end point. Causes of death were obtained from the Swedish Cause of Death Register until December 2000, and after that further follow-up information was retrieved from clinical records until April 2003. Comparisons of treatment delay in groups determined by referral status and by stage progression were performed and the relative hazard of death from bladder cancer was determined by Cox regression analysis.

Treatment delay was dichotomized at 60 days, a point that is close to the median in this study. The analysis was also adjusted for potentially confounding factors such as clinical stage, age, sex, preoperative radiation and cases referred from other hospitals.

### **Paper III**

The study included 75 patients who were scheduled for radical cystectomy due to locally advanced urothelial carcinoma of the bladder. Two of the patients had clinical stage T4b disease and received neo-adjuvant chemotherapy prior to cystectomy. During the study period, 22 additional patients underwent radical cystectomy, but they were considered unsuitable for the present study for the following reasons: practical or patient-related issues (6), advanced tumour stage (7), multifocal tumours or CIS (8), and previous full-dose radiation for prostate cancer (1).

**Preoperative SN detection:** One to four days before cystectomy, a cystoscopy was performed under local anaesthesia. A 3.7 Fr Williams cystoscopy needle (Cook Urological) was used to inject 2 ml of <sup>99m</sup>Tc-nanocolloid (Nanocoll®, 35 MBq/ml) at four locations peritumorally in the detrusor muscle. The patient underwent lymphoscintigraphy approximately one hour after injection of the isotope. Scintigraphy was done in two planes, with and without a lead shield to decrease the uptake from the primary injection site.

**Intraoperative SN detection:** Immediately preceding surgery, 1 ml of <sup>99m</sup>Tc-nanocolloid (70 MBq/ml) and 1 ml of Patent Blue® were injected peritumorally into the detrusor muscle under general anaesthesia. An extended lymphadenectomy was subsequently carried out, and due to possible interference of radioactivity from the primary injection site, examination of the lymphatic tissue with a hand-held gamma probe was done *ex vivo*. Nodes that were radioactive were sent in fractions for pathological evaluation as SNs. After completion of lymphadenectomy and cystectomy, the pelvic cavity and nodal basins were investigated with the gamma probe. Remaining radioactive tissue and/or nodes in the nodal basins were identified and removed.

**Histopathological evaluation:** The entire sentinel lymph nodes were paraffin-embedded, nodes larger than 4 mm were divided into 2–3 mm slices along the long axis. Each paraffin block was examined at three step-section levels separated by 150 µm. Parallel 4 µ sections were stained with hematoxylin-eosin (H&E) and immunohistochemically for cytokeratins. Sections from two or three levels were used to investigate non-SNs. Micrometastasis was defined as a lesion smaller than 2 mm, whereas a conglomerate of isolated tumour cells smaller than 0.2 mm was denoted as sub-micrometastasis.

#### **Paper IV**

We prospectively evaluated 175 consecutive male patients with a mean age of 66 years scheduled for radical cystoprostatectomy due to urothelial carcinoma at three urological departments between 2000 and 2005. During the study period another six patients were submitted to radical cystoprostatectomy, but were excluded from the study due to inadequate pathological examination of the cystoprostatectomy specimen. Twenty-six patients had previously been treated with BCG, and four had been treated with neo-adjuvant systemic chemotherapy. Patients with non-urothelial carcinoma were excluded from the study. The protocol included preoperative investigation with cold cup biopsies from the bladder (sidewalls, dome, back wall and trigone) and transurethral loop biopsies from the bladder neck to the verumontanum, obtained at the 4 and 8 o'clock positions. However, cold cup biopsies and loop biopsies from the prostatic urethra were omitted in 59 and 21 patients, respectively, mainly in referral cases. The cystectomy specimen was fixated with a Foley catheter in place distended with formalin for a minimum of 1–2 days. The bladder neck and prostate were sectioned with full thickness sagittal whole-mount technique (Figure 3). Serial 4–5 mm sagittal step sections were taken and evaluated histologically. Standardized samples from the bladder dome, sidewalls, backwall, ureters and trigone in addition to samples from macroscopic lesions were taken. The primary bladder and prostatic urethra/prostate lesions were classified according to the 2002 TNM classification. The pathway of prostatic invasion (in stages T2 and T3) was classified either as contiguous or non-contiguous tumour spread. Pre-cystectomy variables included in the analysis were previous BCG treatment and CIS. The following parameters in the cystectomy specimen were analysed: CIS (including location), bladder tumour location, uni- or multifocality, tumour size, and perineural tumour growth.

#### **Paper V**

Bladder tumours in 102 patients were sampled with cold-cup biopsy forceps from the exophytic part of the tumour and immediately transferred into transport media, transported to the laboratory and frozen. In 49 patients radical cystectomy was performed, including an extended lymphadenectomy to the aortic bifurcation, except in one patient in whom a limited dissection was performed. In 33 cases the lymphadenectomy included SN detection with examination of SN as described in Paper III. All tumour pathology was reviewed by one pathologist.

Total RNA was extracted using Trizol reagent and the integrity of the RNA samples was assessed on an Agilent 2100 Bioanalyzer. From 89/102 samples with sufficient amount of tumour material, genomic DNA was extracted using the Dneasy Tissue kit protocol. Oligonucleotide arrays were obtained from the Swegene DNA microarray resource centre, and 36288 oligonucleotides used on each slide corresponding to 18466 unique Entrez genes. Tumour sample RNA and Universal



**Figure 3.** The bladder neck and prostate are sectioned with full thickness sagittal whole mount technique. Serial 4-5 mm sagittal step sections were taken and evaluated histologically.

Human Reference RNA (Stratagene, La Jolla, CA) were differentially labelled with Cy3 and Cy5, respectively. After hybridization, the washed arrays were scanned with an Agilent G2565AA microarray scanner and images were analysed with the Genepix 4.0 software. The dataset was submitted to standard filtering before further analysis. Supervised comparisons between different group assignments were performed using Significance Analysis of Microarrays (SAM).

Mutation analyses of *FGFR3* and *TP53* were carried out on the 89 samples where genomic DNA was available as well as loss of heterozygosity (LOH) analysis for chromosome 9.

## **Paper VI**

Between 1990 and 1997 radical cystectomy was performed in 141 patients with locally advanced urothelial carcinoma (UC) at the Department of Urology, Lund University Hospital. In 133 patients tumour tissue was available for constructing the TMA block. All histopathology was reviewed by one pathologist according to TNM 2002. Lymphadenectomy was limited to the obturator fossa, and in 17 patients lymphadenectomy was omitted according to individual surgeons' preferences. Forty-three patients with locally advanced tumours received preoperative radiation of 20 Gy during a period of one week immediately preceding surgery. Eleven patients received adjuvant chemotherapy after cystectomy due to advanced pathological tumour stage. Follow-up ended in December 2005. Death from bladder cancer was the primary endpoint, and causes of death were obtained from the Swedish Cause of Death Register until December 2000, and after that further follow-up information was retrieved from clinical records until February 2006. Clinical records also substantiated data from the Cause of Death Register. All patients alive were followed at least 8 years (median 12, range 8–16). In the 43 patients who received 20 Gy prior to cystectomy and in eight patients with no tumour in the cystectomy specimen (pT0), the tissue was obtained from the transurethrally resected specimen. All the other tumours were sampled from the cystectomy specimen. At least three core biopsies (diameter 0.6 mm) were punched out from representative tumour areas in the paraffin-embedded tumour block using an arrayer (Becher Instruments, MD, USA) and were placed in a recipient paraffin array block. Putative prognostic markers selected for the analyses were chosen from different cellular processes such as cell signalling (EGFR, ERBB2), angiogenesis (VEGFC, PTGS2), cell cycle (RB1, CDKN1A, MKI67), apoptosis (TP53, AKT, PTEN), cell adhesion (CTNNA1, CTNNB1), cytoskeleton maintenance (RHOA and RHOC) and immunological response (STAT1). Immunohistochemical staining was performed in an automated immunostainer (TechMate™ 500 Plus, DAKO). At least three tissue cores from each tumour were arrayed, and when sections from the TMA blocks were stained and analysed, the extreme value ("hot spot") of the first three samples was registered. For

the 133 patients investigated, a mean number of 4 (range 2–7) displayed a complete lack of evaluable tissue for each marker studied. Two pathologists without knowledge of clinical data independently quantified the staining according to the cut-off levels used in the present study. Positive staining was visible at low magnification (x10).

## RESULTS AND CONCLUSIONS

### Paper I

The median diagnostic delay in the sample was 144 days. When the patients were stratified into groups with diagnostic delays of 0–3, 3–6, 6–12 and >12 months, those with T1 tumours in the two groups with a diagnostic delay of < 6 months showed a trend towards a decreased risk of bladder cancer death. In contrast, in patients with muscle-invasive disease, a significantly increased risk of bladder cancer death was noted for those with a diagnostic delay of < 6 months.

*Conclusions:* A trend towards better prognosis was found for patients with T1 tumours with a shorter diagnostic delay. The poor prognosis of patients with muscle-invasive disease and a short diagnostic delay suggests aggressive behaviour of the tumour and may explain the worse prognosis in these patients.

### Paper II

The median treatment delay was 49 days, but was significantly longer for the 71 cases who were referred from other hospitals (63 vs. 41 days,  $p < 0.001$ ). Treatment delay did not influence cumulative incidence of death from bladder cancer. Considering all cases, there was no significant correlation between treatment delay and stage progression. For clinical stage T2 tumours, median treatment delay was 76 days among patients with stage progression compared to 41 and 48 days for those with stage regression and stage equivalence, respectively ( $p = 0.20$ ).

*Conclusions:* Treatment delay was not found to influence disease specific-survival in the present study. Furthermore, treatment delay was not significantly longer in cases that progressed compared to those with equal or lower pathological stage in the cystectomy specimen.

### Paper III

At lymphadenectomy an average of 40 nodes (range 8–67) were removed. Thirty-two of 75 patients (43%) were lymph-node positive, and in 13 of those (41%) all lymph node metastases were located solely outside the obturator spaces. A SN was identified in 65 of 75 patients (87%). In seven of the patients, a SN was recognized when the nodal basins were checked with the gamma probe after lymphadenectomy and cystectomy. Twenty-six of the 32 lymph-node positive cases (81%) had a positive (metastatic) SN, thus the false-negative rate was 6 of 32 (19%). Five of the false-negative cases had macrometastases and/or perivesical metastases were present. In nine patients (14%) the SN contained micrometastases (< 2 mm), and in five of those subjects the micrometastasis was the only metastatic deposit.

*Conclusions:* Sentinel node detection is feasible in invasive bladder cancer, although the false-negative rate was 19% in this study. Extended serial sectioning and immunohistochemistry revealed micrometastases in SNs in nine patients, and radioguided surgery after completion of the lymphadenectomy identified SNs in an additional seven patients. We believe that the technique we used in this study improved nodal staging in these 16 patients (16 of 65 (25%)).

#### **Paper IV**

The incidence of TCC in the prostatic urethra and prostate was 29% (50/175 patients) in the cystoprostatectomy specimen. In the cystectomy specimen CIS, multifocal CIS ( $\geq 2$  locations), and tumour location in the trigone were significantly more common in cystectomy specimens with TCC in the prostatic urethra and prostate [21/50 (42%) vs. 32/125 (26%),  $p=0.045$ , 20/50 (40%) vs. 27/125 (22%),  $p=0.023$ , 20/50 (40%) vs. 26/125 (21%),  $p=0.01$ ], respectively. Preoperative resectional biopsies from the prostatic urethra in the 154 patients analysed identified 31/47 (66%) of patients with TCC in the prostatic urethra/prostate. When preoperative resectional biopsies and multifocal CIS were used as predictors in a multivariate logistic model, the model generated in a ROC curve with a sensitivity of 75% obtained a specificity of 75% in predicting TCC in the prostatic urethra/prostate.

*Conclusions:* The incidence of TCC in the prostatic urethra and prostate was 29%. Preoperative biopsies from the prostatic urethra identified 66% of patients with such tumour growth. This study suggests that preoperative investigation with cold cup mapping biopsies of the bladder probably is of little clinical value for identifying patients at risk of TCC in the prostatic urethra/prostate in the cystoprostatectomy specimen.

#### **Paper V**

FGFR3 and TP53 mutations were detected in 23/89 (26%) and 35/89 (39%) of the tumours, respectively. As TP53 can be functionally inactivated by MDM2 over-expression, the MDM2 transcription pattern was investigated as revealed by the microarray analysis. Expression of MDM2 was increased in ten tumours, none of which had TP53 mutations as compared to the other tumours. The global pattern of gene expression was similar in TP53 mutated tumours and those who were not TP53 mutated but over-expressed MDM, indicating that impairment of the TP53 pathway can be achieved by either of these two mechanisms. LOH on chromosome 9 was found in 45 of the 70 (64%) investigated cases. There was no specific change in gene expression between cases with and without LOH on chromosome 9 in these patients.

With the aim of identifying a gene signature specific for tumours associated with

lymph node metastases, a SAM analysis between node positive and node negative patients was performed. The 50 most discriminatory genes for lymph node status revealed a lower expression in node positive tumours as compared to lymph node negative tumours (Figure 4). A large proportion of the 50 genes identified were related to immunologic processes, especially to the major histocompatibility complex (MHC) class I antigen presentation machinery. To evaluate the predictive strength of the achieved 50-gene signature regarding lymph node metastasis, a leave-one-out cross-validation procedure was performed and the performance of the predictor was evaluated using ROC analysis. The area under the ROC curve is 0.77, and out of the 16 tumours with the highest expression of the 50 predictor genes, 15 were lymph node negative. In a multiple logistic regression model including clinical tumour stage dichotomized (T3/T4 vs. T1/T2) and the 50 gene signature, the odds ratio for node positivity was 0.071 (95% CI: 0.015-0.31,  $p=0.001$ ) for a patient with a gene predictor above median as compared to a patient with a predictor value below median within the same stage group (T1/T2 or T3/T4). In a multivariate survival analysis including pathological tumour stage groups (T1/T2 vs. T3/T4), lymph node status and the 50 gene predictor, the gene signature is an independent predictor of survival patient with a gene predictor above median as compared to a patient with a predictor value below median (HR 0.18 (95% CI: 0.03-1.0,  $p=0.05$ )).

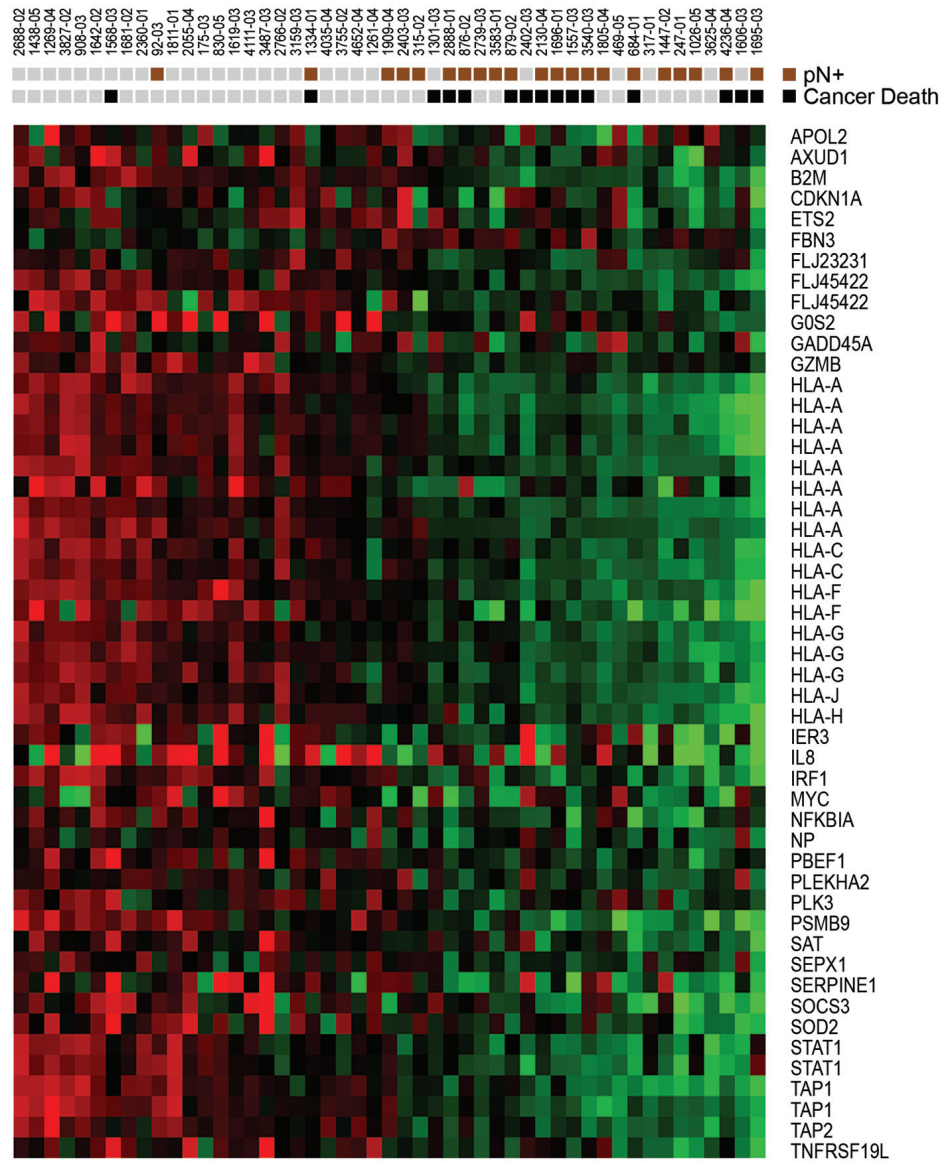
*Conclusions:* The identified 50-gene signature predicted lymph node metastasis and survival in patients submitted to radical cystectomy. Future analyses are necessary to validate the predictive strength of the identified genes in a new cohort of patients.

## **Paper VI**

The median age at surgery was 66 years (range 26–82 years), and 111 of the 133 subjects (83%) were men. One hundred and six (80%) of the cystectomies were performed for muscle-invasive disease (clinical stage T2a or higher). Twenty-four of the 116 patients (21%) submitted to regional lymphadenectomy were lymph-node positive. At end of follow-up 55/133 (41%) patients were dead of disease. Decreased immunohistochemical expression of CTNNA1 and PTEN correlated with higher pathological tumour stages whereas increased AKT and ERBB2 correlated with lower pathological tumour stages. In grade three tumours increased RHOA expression was more common than in grade two. No other associations were found between the 15 factors studied and pathological stage, lymph node status or tumour grade. For none of the markers studied was a correlation found between bladder cancer death and altered marker status. Studying the 71 patients with organ-confined disease (pathological tumour stage  $\leq$  T2b) and the 82 patients who did not receive preoperative radiation or adjuvant chemotherapy displayed the same result.

*Conclusions:* The present study could not identify any prognostic factors using immunohistochemistry in conjunction with TMA technology. Whether this negative

finding is related to the group of patients or factors studied or the methodology is unclear. However, the results suggest that TMA-based analysis of prognostic markers in invasive bladder cancer in the present setting is of limited clinical value



**Figure 4.** A heat-map illustrating the 50 most discriminatory reporters for lymph node metastasis status. On top, case identifiers as well as presence of lymph node metastasis and cancer death are indicated.

## GENERAL DISCUSSION AND SUMMARY

In the present thesis data suggest that diagnostic delay in patients with T1 tumours might have an adverse effect on the prognosis (Paper I), which is in agreement with recent data in the literature (56). Still, due to the low incidence of advanced bladder cancer in screening studies (143) and the presumably short diagnostic and therapeutic time frame, as suggested by the fact that patients with muscle-invasive tumours and a short diagnostic delay had a worse prognosis, general screening of bladder cancer is not realistic. It is probably more efficient to decrease diagnostic delay with a rapid workup of patients with macroscopic haematuria, as a large proportion (15%) of these patients have bladder cancer (144). The value of general health education regarding macroscopic haematuria with the aim of decreasing patients' delay is unknown.

Treatment delay in patients with invasive bladder cancer submitted to radical cystectomy did not influence disease-specific survival or stage progression in our study (Paper II). Factors related to the study, such as short median treatment delay, relatively small sample size and confounding between short treatment delay and advanced tumours within individual clinical stages, might affect the outcome of the study. As five current publications suggest that a long delay from diagnosis until cystectomy influences stage progression (62) and even disease-specific survival (61, 63, 65, 69), it is plausible that a certain treatment delay, individual for each patient, affects the disease-specific survival despite the findings in our study. In T1 disease where only early invasion is present, delayed treatment with radical cystectomy also probably reduces survival (68, 145). The possible negative impact of treatment delay on patients' health-related quality of life is also a matter that has to be taken into consideration when the subject is further studied.

Sentinel node detection is feasible in invasive bladder cancer, as stated in Paper III, and the method improves nodal staging in addition to an extended lymphadenectomy. Extended serial sectioning identified micrometastases in 9/65 patients (14%) within SNs and radio-guided surgery identified SNs in an additional seven patients after completion of the lymphadenectomy. The clinical significance of micrometastases and even isolated tumour cells in SNs is presently not known in bladder cancer. However, the recent suggestion that even tumour cell mRNA detected with PCR in pathologically negative lymph nodes might influence survival, speaks in favour of prognostic significance of small amounts of metastatic disease (46). Micrometastases also represent an unsolved issue in breast cancer patients (146) with regard to prognostic significance of the disease. Larger series of patients with micrometastases with long-term follow-up are needed, taking other factors such as bone marrow micrometastases into account, which has been suggested to be an unrelated

prognostic event in breast cancer patients (147, 148). Multicentre studies defining which patients are suitable for SN detection will further clarify the role of the SN concept in invasive bladder cancer.

Involvement of TCC in the prostatic urethra and prostate affects prognosis in patients submitted to radical cystoprostatectomy. The relatively high incidence (29%) detected in the present study (Paper IV) suggests that adequate assessment with whole-mount technique of the prostatic urethra and prostate is important. Preoperatively obtained resectional biopsies from the prostatic urethra identified 66% of patients with such tumour growth, and should not be abandoned. However, preoperative investigation with cold cup mapping biopsies in order to detect CIS, a possible risk factor for TCC in the prostatic urethra and prostate, is probably of little value for identifying patients at risk of TCC in the prostatic urethra/prostate in the cystoprostatectomy specimen. The sensitivity of cold cup mapping biopsies from the bladder for detecting CIS was only 23% (9/39) when compared with CIS in the cystectomy specimen. This finding merits further investigation and a randomized study comparing cold cup biopsies and fluorescence directed biopsies would adequately assess the sensitivity of the two methods when compared with the incidence of CIS in the cystectomy specimen.

The prognostic value of the identified 50-gene signature (Paper V) is currently being tested in a new cohort of patients submitted to radical cystectomy, and data are not yet available. If the results in the present study can be confirmed, identifying patients at high risk of lymph node metastases before cystectomy might be important with regard to neo-adjuvant chemotherapy. The status of the 50-gene predictor could also implicate that additional therapy, i.e. adjuvant chemotherapy would be indicated for a patient with low expression of the gene predictor. Presence of stromal elements in the tumour specimen can influence the gene expression profile. Micro-dissection might eliminate this contamination. However, ischaemic damage and secondary changes in the gene expression profile occur within one hour of ischaemia (149). This suggests that transurethral tumour sampling might be better than sampling from the cystectomy specimen with micro-dissection, as ischaemia due to the surgery inevitably takes place.

The value of tissue microarray studies in invasive bladder cancer for the identification of new molecular prognostic markers seems to be questionable, as concluded in paper VI. The reason for the lack of prognostic value of the investigated factors is unclear. Intra-tumour heterogeneity might affect the outcome when using the TMA methodology, when sampling only small areas of the tumour. This might be even more pronounced in large invasive tumours as compared to smaller, more homogenous, non-invasive bladder tumours. Other possible explanations for the results in Paper VI are the immunohistochemistry in itself as a method to assess transcriptional activity,

inappropriate cut-off levels, a too small sample size and perhaps incomplete surgery leaving undetected lymph node metastases due to limited lymphadenectomy. It is possible that exploratory studies identifying prognostic markers using oligonucleotide arrays as in Paper V, where a larger number of factors are investigated, might be more useful. Moreover, as compared to immunohistochemistry, quantification of mRNA levels with, for example, oligonucleotide arrays or PCR may be done more systematically, possibly reducing divergent results from different laboratories. As an example, molecular staging using PCR-detected mRNA of Uroplakin II in lymph node samples has recently been suggested to be more predictive than conventional pathological analysis in a cystectomy series (46). A study using PCR technology to investigate bone marrow samples from a large number of patients submitted to radical cystectomy is currently underway in our department.

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