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Staging and tumor biological mechanisms of lymph node metastasis in invasive urinary bladder cancer

Firas Aljabery

Division of Urology
Department of Clinical and Experimental Medicine
Faculty of Health Science
Linköping University
Linköping, Sweden
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To my my lovely **Nadia**

And my sons (**Mohammed, Laith &Yousif**)

Learn from yesterday, live for today, hope for tomorrow.

The important thing is not to stop questioning.

Albert Einstein

Supervisor

Oliver Gimm, Professor, Division of Surgery, Department of Clinical and Experimental Medicine, Faculty of Medicine and Health Sciences, Linköping University, Linköping

Deputy supervisor

Staffan Jahnson, Associated professor of Urology, consultant urologist, Department of Urology, University Hospital in Linköping and Faculty of Medicine and Health Sciences, Linköping University.

Ivan Shabo, MD PhD, Consultant, Endocrine and Sarcoma Surgery Unit, Department of Molecular Medicine and Surgery, Karolinska Institution, Stockholm.

Hans Olsson, MD PhD, Consultant, Division of Pathology, Department of Clinical and Experimental Medicine, Linköping University Hospital and Faculty of Medicine and Health Sciences, Linköping University.

Peter Söderkvist, Professor, Department of Clinical and Experimental Medicine, Division of Cell Biology, Faculty of Medicine and Health Sciences, Linköping University.

Faculty opponent

Ralph Peeker, Professor, Division of Urology, Sahlgrenska University Hospital and University of Gothenburg, Sweden.

Committee Board

Ove Andrén, Professor, Division of Urology, Örebro University Hospital and Örebro University, Sweden.

Martin Johansson, Associated Professor, Division of Pathology, Malmö University Hospital and Lund University, Sweden.

Abstract

Aim: To study the possibility of detecting lymph node metastasis in locally advanced urinary bladder cancer (UBC) treated with radical cystectomy (RC) by using preoperative positron emission tomography/computed tomography (PET/CT) and peroperative sentinel node biopsy (SNB) technique. We also investigate the clinical significance of macrophage traits expression by cancer cells, M2-macrophage infiltration (MI) in tumor stroma and the immunohistochemical expression of biomarkers in cancer cells in relation to clinico-pathologic data.

Patients and Methods: We studied prospectively 122 patients with UBC, pathological stage pT1–pT4 treated with RC and pelvic lymph node dissection (PLND) during 2005–2011 at the Department of Urology, Linköping University Hospital. In the first study, we compared the results of preoperative PET/CT and conventional CT with the findings of postoperative histopathological evaluation of lymph nodes (LNs). In the second study we investigated the value of SNB technique for detecting pathological LNs during RC in patients with UBC. We also examined the significance of the primary tumor location in the bladder in predicting the site of LN metastases, and the prognostic significance of lympho-vascular invasion (LVI) and lymph node metastasis density (LNMD) on survival. In the third study, we investigate the clinical significance of macrophage infiltration (MI) in tumor stroma and macrophage-traits expression by tumor cells. In the fourth study, we investigate the cell cycle suppression proteins p53, p21, pRb, p16, p14 ^{ARF} as well as tumors proliferative protein Ki67 and DNA repair protein ERCC1 expression in cancer cells. The results were compared with clinical and pathological characteristics and outcome.

Results: Prior to RC, PET/CT was used to detect LN metastasis in 54 patients. PET/CT had 41% sensitivity, 86% specificity, 58% PPV, and 76% NPV, whereas the corresponding figures for conventional CT were 41%, 89%, 64%, and 77%. SNB was performed during RC in 103 patients. A median number of 29 (range 7–68) nodes per patient were examined. SNs were detected in 83 out of 103 patients (81%). The sensitivity and specificity for detecting metastatic disease by SNB varied among LN stations, with average values of 67% -90%. LNMD of ≥8% and LVI were significantly related to shorter survival. In 103 patients, MI was high in 33% of cases, while moderate and low infiltration occurred in 42% and 25% of tumors respectively. Patients with tumors containing high and moderate compared to low MI had low

rate of LN metastases (P=0.06) and improved survival (P=0.06), although not at significant level. The expression of different tumor suppression proteins was altered in 47-91% of the patients. There were no significant association between cancer specific survival (CSS) and any of the studied biomarkers. In case of altered p14ARF, ERCC1 or p21, CSS was low in case of low p53 immunostaining but increased in case of p53 accumulation, although not at a significant level, indicating a possible protective effect of p53 accumulation in these cases.

Conclusion: PET/ CT provided no improvement over conventional CT in detection and localization of regional LN metastases in bladder cancer. It is possible to detect the SN but the technique is not a reliable for perioperative localization of LN metastases; however, LVI and LNMD at a cut-off level of 8% had significant prognostic values. MI in the tumor microenvironment but not CD163 expression in tumor cells seems to be synergistic with the immune response against urinary bladder cancer. Our results further indicate that altered p53 might have protective effect on survival in case of altered $p14^{ARF}$, p21, or ERCC1 indicating an interaction between these biomarkers.

Populärvetenskaplig sammanfattning på svenska

Stadieindelning och tumörbiologiska mekanismer för lymfkörtelmetastas vid lokal avancerad urinblåscancer

Urinblåscancer är den nionde vanligaste cancer sjukdom världen över. Den orsakar mer än 150 000 dödsfall årligen i världen. Inväxt av blåscancer till urinblåsans muskel förekommer hos cirka 30 % av alla patienter. I Sverige ökar antal nyupptäckta fall av urinblåscancer långsamt och var antalet nya fall 2544 och sjukdomsdödligheten var cirka 600 fall per år enligt Nationellt Kvalitetsregister för Urinblåsecancer. Trots framsteg inom diagnos och behandling av urinblåsecancer är den 5-åriga sjukdomsspecifika överlevnaden låg vid lokalt avancerad urinblåsecancer och dödligheten har inte förbättrats avsevärt under de senaste 30 åren.

I de flesta fall sker spridning av tumören genom lymfbanor till lymfkörtlarna som dränerar urinblåsan i bäckenet. Lymfkörtel metastaser hos patienter med blåscancer är av stor betydelse för prognosen och är avgörande för behandlingsplanering.

Det är mycket viktigt att identifiera lymfkörtlarna med cancer innan operation eller i samband med operationen för att operera bort dessa och planera för kemoterapi i samband med operation. Det finns inga effektiva och säkra metoder för att upptäckta lymfkörtlar med metastas före operation och det saknas kunskap om tumörkarakteristika som säkert kan indikera spridning till lymfkörtlar liksom kunskap om tumörbiologiska mekanismer för denna spridning. Nya undersökningsmetoder och en förståelse för tumörbiologiska mekanismer för tumörspridning behöver utvecklas.

I denna avhandling studeras nya undersökningsmetoder för att påvisa tumörspridning till lymfkörtlar och förändringar i tumören som kan indikera ökad risk för spridning till lymfkörtlar och som kan förklara mekanismer för sådan spridning genom studier av ett patientmaterial bestående av 122 patienter med lokalt avancerad cancer i urinblåsa som behandlats 2005-2011 vid Urologiska kliniken, Universitetssjukhuset i Linköping.

I den första studien jämförde man resultaten av ny röntgen metod (PET/CT) med den gamla metoden som är konventionell CT undersökning av buk och bröstkorg. I den andra studien undersökte man en teknik för att upptäcka den första lymfkörteln som tumörens sprids till

(den så kallade portvaktskörteln) under operationen och man undersökte även om andelen lymfkörtlar med tumör och om lokalisationen av lymfkörtlar till den tumörbärande sidan av blåsan eller även till motsatta sida hade samband med tumörkarakteristika och överlevnad. I den tredje studien undersöker man immunologiskt aktiva celler (makrofager) i nära kontakt med tumörceller och om sådana makrofager även förenats med tumörceller (så kallad cellfusion). Man undersökte om förekomst av makrofager eller om förekomst cellfusion hade samband med tumörkarakteristika och överlevnad. I den fjärde studien undersöker man proteiner i tumörcellerna som kan påverka celldelning och i vissa fall reparera skador på arvsmassan (ett gemensamt namn för dessa proteiner är biomarkörer). Man undersökte om förekomst av 7 olika biomarkörer hade samband med tumörkarakteristika och överlevnad.

Resultaten från de olika delarbetena visar:

att PET/CT inte har bättre förmåga att upptäcka tumörspridning till lymfkörtlar jämfört med vanlig CT undersökning,

att teknik med portvaktskörtel inte har god förmåga att upptäcka spridning till lymfkörtlar och att andel lymfkörtlar med tumör mindre än 8 % ger bättre överlevnad och att tumör i lymfbanor medför ökad risk för tumör i lymfkörtlar och död i tumörsjukdom

att makrofager i anslutning till tumör men inte cellfusion motverkar spridning till lymfkörtlar och död i tumörsjukdom,

att kombinationer av vissa biomarkörer kan stimulera tumörspridning och andra kombinationer kan skydda mot tumörspridning men, den samlade bilden av dessa biomarkörers gemensamma funktion är svårtolkad.

List of papers

This thesis is based on the following studies:

I. Aljabery F. Lindblom G, Skoog S, Shabo I, Olsson H, Rosell J, Jahnson S. **PET/CT versus conventional CT for detection of lymph node metastases in patients with locally advanced bladder cancer.** BMC urology. 2015;15(1):87

II. Aljabery F. Shabo I, Olsson H, Gimm O, Jahnson S**. Radio-guided sentinel lymph node detection and lymph node mapping in invasive urinary bladder cancer-a prospective clinical study.** BJU Int. 2016.

III. Aljabery F. Shabo I, Olsson H, Gimm O, Jahnson S. **M2 macrophage infiltration and macrophage traits of tumor cells in advanced muscle-invasive urinary bladder cancer.** (Manuscript)

IV. Aljabery F. Shabo I, Olsson H, Gimm O, Jahnson S. **Value of the Biological Markers P14ARF, P53, P16, P21, PRb, ERCC1 and Ki67 as Prognostic Tools in Muscle-Invasive Bladder Cancer Treated with Cystectomy.** (Manuscript)

ABBREVIATIONS IN ALPHABETICAL ORDER

AC	Adjuvant Chemotherapy
ANOVA	Analysis Of Variance
ARF	Alternate Reading Frame Protein
AUC	Area Under the Curve
CIS	Carcinoma In Situ
CSS	Cancer Specific Survival
CT	Computerized Tomography
ERCC1	Excision Repair Cross Complementing 1
FDG	18-Flouro Deoxy Glucose
FS	Frozen Section
H&E	Hematoxylin-Eosin
NIR-ICG	Near-Infrared- Indocyanine Green
LN	Lymph Node
LNMD	Lymph Node Metastasis Density
LVI	Lymph Vascular Invasion
MI	Macrophage Infiltration
MIBC	Muscle Invasive Bladder Cancer
MRI	Magnetic Resonance Imaging
NAC	Neo Adjuvant Chemotherapy
PET/CT	Positron Emission Tomography/Computed Tomography
PLND	Pelvic Lymph Nodes Dissection
RC	Radical Cystectomy
ROC	Receiver Operated Curve-analysis
PRb	Retinoblastoma protein
SN	Sentinel Node
SNB	Sentinel Node Biopsy
SNRUBC	Swedish National Register of Urinary Bladder cancer
TAM	Tumor Associated Macrophage
TMA	Tissue Microarray
TURB	Transurethral Resection of Bladder tumor
UBC	Urinary Bladder Cancer

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BACKGROUND

Introduction to bladder cancer

Urinary bladder cancer (UBC) is the most common cancer of the urinary tract after prostate cancer in male and the 7th most commonly diagnosed cancer in male population worldwide, whilst it drops to 11th when both genders are considered (1, 2). In Europe, bladder cancer accounted for about 104,400 new cases and 36,500 deaths from cancer in 2006 (3). In Sweden, the incidence of UBC is slowly increasing. In 2015 the number of new cases was 2544 and the mortality rate of the disease was about 600 cases per year according to the Swedish National Registry of Urinary Bladder Cancer (SNRUBC) (4).

Radical cystectomy (RC) and pelvic lymph node dissection (PLND) is the standard treatment of localized muscle-invasive bladder cancer (5). LN metastases in patients with bladder cancer are of great importance for prognosis and is essential for preoperative and postoperative treatment planning. Hence, accurate LNs staging is mandatory in patients undergoing RC (6-9). Although the optimal range of PLND is still debated, evidence indicates that extended PLND improves survival in patients with both node positive and node negative bladder cancer (10). On the other hand, PLND is associated with increased morbidity and mortality in radical cystectomy, new methods are needed for pre- or peroperative identification of pathologic LNs in order to reduce unnecessary dissection or removal of non-pathologic LN (10).

Despite aggressive treatment, mortality rates among patients with UBC are still high (11). Understanding the biological mechanisms that cause tumor LN metastasis is of great importance to identify new prognostic indicators that can be used to assess UBC patients. The molecular and immunologic profile of UBC may be characterized so as to select patients who will benefit from different therapeutic approaches. Additionally, the available methods for staging remain inaccurate and further studies are needed to improve the staging techniques (12-18).

BLADDER CANCER STAGING AND CLASSIFICATION

TNM classification of malignant tumors is used in staging of UBC. Table 1 shows the TNM 2010 classification for UBC (3). Staging process in urinary bladder cancer include both pathological staging after transurethral resection of the tumor to identify the depth of the invasion and radiological staging to identify the local infiltration within and outside the bladder wall, regional lymph node metastasis and distant metastasis of the tumor.

Transurethral resection

Transurethral resection of bladder (TURB) is an endoscopic technique utilizing a diathermy resectoscope to enable histopathological diagnosis and staging. Bladder muscle in the resection biopsies is mandatory for correct staging. (19, 20). With larger tumors, the resection is performed in 3 levels: the exophytic part, the tumor base with underlying muscular layer and the edge of the resection area. Tumors located on the trigone or bladder neck carry higher risk of involvement of the prostatic urethra and ducts. Biopsy from the prostatic urethra at the time of primary resection is recommended. Concomitant bi-manual palpation of the bladder under anesthesia is recommended for detection of extra-vesical tumor growth and tumor fixation to surrounding anatomical structures (21).

With en bloc resection tumors of less than 1 cm in diameter can be removed completely with part of the underlying muscular layer of the bladder wall (22, 23). **Second-look resection** is usually done in the case of T1 tumors (high grade non-muscle invasive tumor) to decrease the risk of under-staging and residual disease. However, 10–30% of the tumors are found to be invasive in the muscular layer (24, 25). In a recent population-based study, 5% of T1 tumors subjected to second-look resection have muscle-invasive UBC (26).

Table 1. The TNM classification 2010 showing T staging of urinary bladder cancer.

	T — Primary Tumor	
TX	Primary tumor cannot be assessed	
T0	No evidence of primary tumor	
Ta	Non-invasive papillary carcinoma	
Tis	Carcinoma in situ: “flat tumor”	
T1	Tumor invades subepithelial connective tissue	
T2	Tumor invades muscle	
	T2a	Tumor invades superficial muscle (inner half)
	T2b	Tumor invades deep muscle (outer half)
T3	Tumor invades perivesical tissue	
	T3a	Microscopically
	T3b	Macroscopically (extravesical mass)
T4	Tumor invades any of the following: prostate stroma, seminal vesicles, uterus, vagina, pelvic wall, abdominal wall	
	T4a	Tumor invades prostate stroma, seminal vesicles, uterus, or vagina
	T4b	Tumor 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	
N2	Metastasis in multiple lymph nodes in the true pelvis	
N3	Metastasis in common iliac lymph node(s)	
	M—Distant Metastasis	
M0	No distant metastasis	
M1	Distant metastasis	

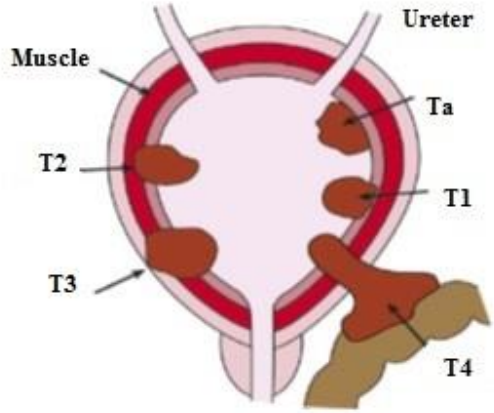

Figure 1. Urinary bladder cancer T-stage (with permission of Dr Hans Olsson, dissertation no.1335, Linköping University).

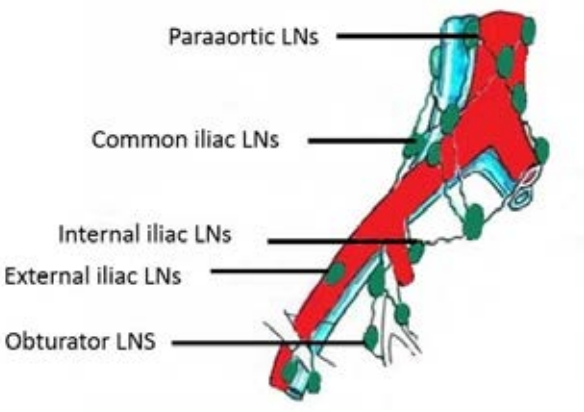

Figure 2. Anatomical regions of intra and extra pelvic lymph nodes

Table 2. The WHO 1999 and WHO 2004 grading systems

1999 WHO grading
Urothelial papilloma
Grade 1: well differentiated
Grade 2: moderately differentiated
Grade 3: poorly differentiated
2004 WHO grading
Urothelial papilloma
Papillary urothelial neoplasm of low malignant potential (PUNLMP)
Low-grade papillary urothelial carcinoma
High-grade papillary urothelial carcinoma

Imaging for staging of UBC

Magnetic resonance imaging (MRI) is reported to have an accuracy of 73–96% for primary tumor staging due to good soft tissue contrast resolution (27) . Diffusion-weighted MRI technique might show reasonable accuracy (sensitivity = 75%, specificity = 68%)for detecting metastatic lymph nodes in bladder cancer (28).

Computed tomography (CT) offers high image resolution and short investigation time which is an advantage over MRI (29). It might be useful in detecting invasion into the perivesical fat (T3b) and adjacent organ, but the sensitivity of CT in T staging varies between 55% and 92%, which limits its use in preoperative UBC staging (29-31).

Detection of LN metastasis by CT and MRI is based on size criteria, which limit their ability to identify metastasis in normal size LNs. Moreover, nodal enlargement can be due to benign disease, which impairs the specificity of CT and MRI (12, 29, 30, 32, 33).

Positron emission tomography/CT (PET/CT) enables detection of specific markers combined with simultaneous accurate anatomical depiction and might have a potential clinical use for staging metastatic BC. The optimum or specific isotope for studying bladder cancer has not yet been determined (34). As most malignant tumors are characterized by elevated glu-

cose metabolism, and therefore increased cell proliferation in tumors can be imaged by PET/CT as a higher uptake of 18-fluorodeoxyglucose (FDG) (15, 35-38). FDG is still the most widely used isotope to investigate bladder cancer (34). At the start of the present study, published series of systematic investigation of PET/CT for localization of lymph node metastases in bladder cancer were lacking.

TREATMENT OF INVASIVE URINARY BLADDER CANCER

Chemotherapy

Despite potentially curative surgery, 40–60% of patients with muscle-invasive urothelial carcinoma (stages T2-4) develop metastatic disease within 2 years (39). The 5-year survival of patients with MIBC after RC is 50% (40-43). **Neoadjuvant chemotherapy (NAC)** is the administration of chemotherapy before RC. Patients with muscle invasive bladder cancer treated with NAC show complete response rates of about 38% (44, 45). NAC does not increase the risk of postoperative complications or death (46). The administration of preoperative chemotherapy for down staging (**induction chemotherapy**) in patients with clinically node positive bladder cancer who were eligible for surgery was evaluated in many studies. After induction chemotherapy, 20-30% of patients showed complete pathologic response with subsequently significant CSS benefit after cystectomy (47-49). **Adjuvant chemotherapy (AC)** after radical cystectomy for UBC in patients with no macroscopic or radiologic evidence of residual disease was evaluated in many studies (50, 51). Patients who derived benefit from AC had a low LN density and received at least 4 cycles of treatment (50).

Radical cystectomy

Radical cystectomy and PLND are the standard treatment of localized muscle-invasive bladder cancer. Lymph node metastasis in patients with bladder cancer is of great importance for prognosis and is essential for preoperative and postoperative treatment planning as it is an important determinant of survival. Although most patients undergoing cystectomy with lymph node dissection lack histopathological lymph node metastases, a significant percentage of them have high risk of recurrence and mortality in bladder cancer (3, 5, 52).

In male patients, complete removal of the bladder with all macroscopically visible tumors within and around the bladder includes resection of the adjacent distal ureters, and the lymph nodes corresponding to the tumor-bearing bladder. In female patients, standard anterior pelvic exenteration includes the entire urethra, adjacent vagina, uterus, distal ureters, and respective lymph nodes. In both sexes, the length of the distal urethral segment and the adjacent ureteral segments to be removed with the bladder have not been specified and depends on oncological issues such as tumor extension to the bladder neck or the prostatic urethra, or the presence of

carcinoma in situ and type of subsequent urinary diversion (52). Frozen sections of the distal urethra and the proximal ureters to be left in place are often used to determine the exact level of resection (53).

Lymph-node dissection

There are different types of PLND depending on the anatomical pelvic regions removed. The dissection includes all the fatty tissues surrounding the major blood vessels in the pelvis (Figure 2).

Conventional lymphadenectomy dissection: involves removal of nodal tissue cranially up to the common iliac bifurcation, with the ureter being the medial border, and including the internal iliac, obturator fossa and external iliac nodes, the lateral borders are the genitofemoral nerves, caudally the circumflex iliac vein, the lacunar ligament (9).

Limited lymphadenectomy: Boundaries of limited PLND include the pelvic sidewall between the external iliac vein and obturator nerves, and bifurcation of the iliac vessels to the circumflex iliac vein. It is considered to be associated with suboptimal staging (54).

Extended lymphadenectomy: Extended lymphadenectomy includes all lymph nodes in the region of the aortic bifurcation, presacral and common iliac vessels medial to the crossing ureters. Caudally and laterally are the area described for conventional lymphadenectomy (55). There are indications that extended PLND improves survival in patients with both lymph node positive and negative bladder cancer (10).

A super-extended lymphadenectomy extends cranially around the aorta to the level of the inferior mesenteric artery and around the corresponding segment of the vena cava (52). Bruins et al found that this procedure gave no further oncological benefits compared with less extensive LNs dissection. Moreover, it resulted in increased operative time but does not appear to substantially increase postoperative morbidity (54).

Per- and post-operative studies of LN and specimen processing.

Sentinel node (SN): The sentinel lymph node is considered to be the first LN into which a primary tumor drains and disseminates after that to other lymph nodes. It was originally suggested by Gould in 1960 in parotid cancer (56), and has been further developed and applied in different malignancies since then (57-62).

Sentinel node biopsy (SNB) is a surgical procedure established in clinical practice for evaluating LN metastasis in the treatment of several tumor types, such as breast cancer, melanoma and penile cancer (63-67). Successful sentinel node biopsy (SNB) and mapping of the lymphatic drainage is expected to reduce the need for extended lymphadenectomy and thereby decrease postoperative morbidity (10). In UBC, the use of SNB has been investigated in two previous studies. Sherif et al. examined the possibility of detecting SNs in 13 patients with UBC. The radioactive tracer used in this study was Albures Technetium 99, which has a large molecular weight and slow transport in the lymphatic channels and needs to be injected at least 6 hours before LN dissection. Cystectomy includes lymph node dissection of obturator fossa bilaterally and of any positive findings, guided by the preoperative lymphoscintigraphy detection and peroperative detections using gamma probe and blue dye. Sentinel nodes were detected in 11 of 13 patients (85 %). There were no false-negative SNs. The authors concluded that SNs can be identified in UBC patients with a technique based on lymphoscintigraphy and dye marker(68). In a study of 75 patients, Liedberg et al investigated the possibility of detecting SN using preoperative lymphoscintigraphy and a perioperative technique using 99m Tc nanocolloid (70 MBq/ml) and Patent Blue dye. A SN was identified in 65 of 75 patients (87%). 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%) (69).

Frozen section (FS): perioperative FS examination of external iliac, hypogastric, and obturator lymph nodes during radical cystectomy with the intention to limit PLND has been investigated in several studies (70-73). In a multicenter study, Adsan et al tested the reliability of frozen section examination of external iliac, hypogastric, and obturator lymph nodes during radical cystectomy. The authors concluded that performing FS seems to be a reliable procedure for the evaluation of the LNs, and the information obtained with FS of the LNs can be used to determine intraoperatively the extent of LN dissection (71).

Postoperative handling of the surgical specimen

After surgical removal of the specimens (urinary bladder and LNs), the specimens were fixed with formalin for a minimum of 1-2 days. This process is essential for optimizing the quality of the surgical specimen for pathological examination. After proper fixation, macroscopic dissections (so called cutting up) followed by dehydration, paraffin embedding, sectioning and staining. The ischemic time (from surgical removal of the specimen to the time in formalin fixation) is an important factor because the degeneration of the target proteins starts directly and can cause evaluation problems, mainly concerning the immunohistochemical investigations (74). The protocols of formalin fixation have become stricter nowadays. For example, in breast cancer it is recommended immediate fixation in order to keep the ischemic time below 1 hour to preserve the estrogen receptors (75). The specimens are fixated in 4 % formaldehyde solution in 0.1 M phosphate buffer pH 7.2 (formalin) (76). The quantity of formalin is recommended to be 10 times that of the tissue to be fixed (77).

The macroscopic visible bladder tumors were cut in 4-5-mm pieces and sufficient blocks were prepared. Standardized samples from the bladder dome, sidewalls, back wall, ureters and trigone, in addition to samples from macroscopic lesions, were taken (77).

In the presence of visible tumor in a single lymph node, one routine section is required to demonstrate the tumor and its possible extra-nodal extension. In the absence of visible tumor in the lymph node, the entire node should be submitted for microscopic examination. If the node is so small that it cannot be sliced, it may be embedded as one piece (78-81).

PROGNOSIS IN BLADDER CANCER AFTER CYSTECTOMY

Clinical and pathological factors

Age, gender, performance status, tumor grade, pathological T and N category, presence of lymphovascular invasion (LVI), presence of carcinoma in situ and use of neoadjuvant chemotherapy are classical factors used in most of the available nomograms to predict outcome (82).

Pathological T category is one of the most important and independent predictors of CSS and OS in UBC patients after RC. The 5-year CSS in patients with ≤pT1, pT2, pT3, and pT4 is reported as 80–90%, 50–70%, 30–45 % and 20–35%, respectively (11, 83, 84). Increased T category is usually associated with increased incidence of LN metastasis as well as local recurrence (85).

The pathologic Tumor Grade has limited predictive value after RC which may due to the fact that most of patients undergoing RC have high-grade disease (84, 86).

Surgical Margin is considered as an independent predictor factor of local disease recurrence and CSS. It is correlated with T stage, pelvic radiation, extent of LN dissection, and surgeon experience (87).

Tumor Size was considered as an independent predictor of CSS within 10 years for 94% of patients with pT2 tumors of ≤3 cm and 68 % for pT2 tumors of >3 cm (88).

LVI occurs in 30–50% of RC specimens, correlates with poor prognosis and is well documented as an independent predictor of recurrence and CSS. It might be useful in identifying high-risk patients with negative LNs after cystectomy (89-92).

Histologic variants of UBC, such as adenocarcinoma, squamous cell carcinoma, small cell carcinoma and carcinosarcoma, were identified as an independent predictor of recurrence and CSS (93). Xylinas et al. analyzed differences between pure urothelial BC and that with variant histology in 1,984 patients treated with RC. The authors reported worse outcomes with variant groups in univariate analyses, which was not the case in multivariable analyses as T category was the strongest factor for outcome and not the histologic variant of tumor (94).

Pathological N-category is an important pathological prognostic factor after RC. The 5-year recurrence-free survival in patients with LN-positive UBC is poor, ranging from 34-43% (43, 48, 95). However, better survival has been described in patients with low-volume LN metastasis (96-98). The number and size of positive lymph nodes, extracapsular extension, number of lymph nodes, aggregate lymph node metastasis diameter, and the extent of lymphadenectomy have been investigated as critical determinants for survival in LN-positive UBC (43, 99-102). However, the quality of the PLND as measured by the number of dissected nodes with a cut-off value of 15 nodes has an impact on survival even in node negative patients (7). The concept of **lymph node metastasis density (LNMD)** has been proposed as a prognostic factor in patients with LN positive UBC. It is defined as the proportion of pathological LNs in relation to the total number of LNs removed in each patient after lymphadenectomy. In most series, a cut-off value of 20% has been reported to statistically distinguish between different outcomes. The surgical extent of lymphadenectomy, pathological evaluation of lymph nodes and the number of lymph nodes retrieved can affect the cut-off values of LNMD (96, 97). Lymph node location was not associated with worse prognosis; however, the number of positive LNs was associated with worse prognosis (95). Approximately 70–80% of patients with pathological LN develop recurrence compared with 30–40% of patients with T3-4 disease and pathologically negative LNs within 5 years of RC (6, 8, 10, 95-101, 103, 104). In this respect, prospective trials are needed to investigate more accurately the role of LNMD in patients with LN-positive UBC. The high recurrence rate seems to indicate that bladder cancer is a systemic disease even before cystectomy (105-111).

Tumor markers

Studying the molecular profiling of bladder cancer and the tumor microenvironment might offer additional methods to predict tumor prognosis and treatment outcomes. Molecular diagnostics applications are an integral part of the management of several solid tumors such as breast, colon, and lung cancer (112-114). The molecular biomarkers are a cellular, biochemical, and/or molecular structures that can be objectively measured and evaluated as an indicator of biological processes (115). A cancer biomarker is produced either by the tumor cell or by surrounding tissues in response to cancer. It can be DNA, RNA, proteins, peptides, hor-

mones, metabolites, and even biological processes such as apoptosis, angiogenesis or proliferation. Currently, insufficient evidence exists to recommend the standard use of the prognostic biomarker in clinical management of UBC. In this respect, further trials are needed to investigate more accurately the role of biomarkers in patients with UBC (3).

Tumor-associated macrophages

Tumor associated macrophages (TAM) are a heterogeneous group of non-neoplastic cells in tumor stroma. Circulating monocytes differentiate to macrophages when they enter into tissue stroma. Macrophages are classified as M1 and M2 subsets based on functional and phenotypic characteristics (116). M1 macrophages are classically activated and produce pro-inflammatory and tumoricidal cytokines, such as, IL-12, and TNF. M2-macrophages have been described as immune regulators and produce anti-inflammatory cytokines, e.g. IL-10 and TGF-β1. M2 macrophage show immunosuppressive functions including scavenging potentials and promote angiogenesis and tissue repair (117). Recent studies suggests that macrophages play a key role in regulating the metastatic potential of cancer cells (118, 119).

TAMs are considered to be M2-differentiated macrophages and promote tumor progression (120). Monocytes are actively recruited to the tumor stroma, and high infiltration of TAMs in many tumor types correlates with lymph node involvement and distant metastasis (121, 122). However, the clinical significance of macrophage infiltration in tumor stroma is still controversial. High infiltration of TAMs is correlated with poor prognosis in breast, prostatic, ovarian and cervical carcinoma (119, 122-127). In colorectal cancer, there are conflicting data about the clinical significance of macrophage infiltration, and several studies show that low macrophage density in tumor stroma is associated with an unfavorable prognosis (128).

The theory of cell fusion and macrophage traits in cancer cells

The theory of cell fusion in cancer states that cancer cells may produce hybrids with metastatic ability due to spontaneous fusion with migratory leukocytes. The hybrids acquire genetic and phenotypic characteristics from both maternal cells (129).

Studies suggest that fusion between TAMs and cancer cells may be an underlying cause of tumor progression. The fusion process generates hybrids that acquire genetic and phenotypic characteristics from both maternal cells and exhibit a metastatic potential (130, 131). Pawelek et al, in several case reports, found clinical evidence indicating cell fusion. In allogeneic bone marrow transplants, alleles from both donor and recipient were found in primary tumor cells in patients who subsequently developed renal cell carcinomas (132). In two other separate cases with malignant melanoma metastasis (one with lymph node and a second with brain metastases) and previously received allogeneic bone marrow transplant because of hematologic malignancy, the same research group showed that alleles from the donor and recipient (patient) were found in metastatic tumor cells, indicating that cell fusion was likely involved in metastasis (133, 134).

Macrophage traits in tumor cells, such as CD163 expression, reported in breast and colorectal cancers, are associated with early tumor recurrence and lower patient survival (135-137). Based on the cell fusion theory, it has been suggested that macrophage phenotype in cancer cells might be caused by fusion between tumor-associated macrophages (TAM) and cancer cells (129, 138, 139).

Biomarkers

The cell cycle is a coordinated series of steps that regulate cellular division. It is divided into interphase (G1, S, and G2) and mitotic (M) phase. Cancers result from uncontrolled cell division. The cell cycle is controlled by regulatory proteins; when the genes coding for these proteins are mutated, the proteins may not function causing uncontrolled cell cycle and division (140).

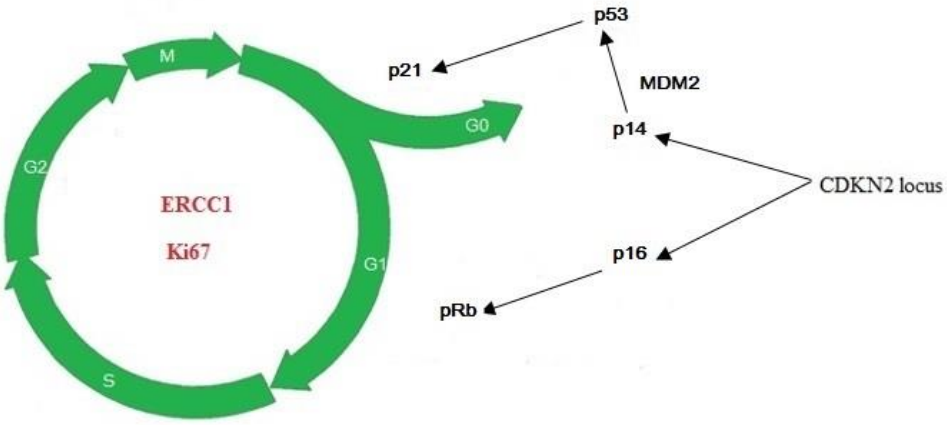

Figure 3. **The biomarkers studied in relation to the cell cycle**

p53 is a tumor suppressor protein and plays important roles in the regulation of the cell cycle, DNA repair, and apoptosis. There are discrepancies between studies about the prognostic value of p53. Previous studies show that nuclear accumulation is predictive of the outcome in patients treated with RC (141-143), whereas another prospective random studies attribute no prognostic value to p53 status in a series of muscle invasive lesions (144). The choice of anti-

body used, interpretation criteria, and specimen handling might affect the results in these studies (109, 115).

pRb is a cell cycle regulator and considered to be a tumor suppressor gene and nuclear protein. Recent evidence suggests that the prognostic value of pRb may be lower than that of other cell cycle regulators in UBC. The prognostic value of pRb remains to be validated (142, 143, 145-147).

p21 is a cyclin-dependent kinase inhibitor that regulates the cell cycle progression at G1. Activation of p21 is through p53, but p53 independent activation is also reported. Several studies showed that p21 was an independent predictor of both recurrence and CSS in UBC (142, 143, 148).

p16 is a tumor suppressor protein transcribed from an alternate reading frame of the CDKN2 locus located in the short arm of chromosome 9, and is involved in the G1 phase cell-cycle regulation. It is a cyclin-dependent kinase inhibitor. Loss or mutation of p16 gene would then result in loss of control over pRb phosphorylation, causing cell cycle progression (149-152).

$p14^{ARF}$ is also a tumor suppressor protein transcribed from an alternate reading frame of the cyclin-dependent kinase Inhibitor 2A (CDKN2A) locus located in the short arm of chromosome 9. It restrains cell growth by abrogating MDM2 inhibition of p53 activity, and thereby facilitating p53-mediated cell-cycle arrest and apoptosis. Deletion or mutation of $p14^{ARF}$ would result in increased MDM2 levels and increased p53 degradation (153-157)

Excision repair cross complementing 1 (ERCC1) is a component of the nucleotide excision repair (NER) pathway, which is a major repair mechanism of DNA damage. ERCC1 expression is associated with prolonged survival in patients with bladder cancer receiving platinum-based neoadjuvant chemotherapy (158, 159).

Ki67 is a nuclear protein produced by proliferating cells and is present during all active phases of the cell cycle (G_1, S, G_2, and mitosis), but is absent from resting cells (G_0). Ki-67 is therefore used as an indicator of cell proliferation in many studies. Its overexpression has been shown to be independently associated with tumor recurrence and CSS in UBC and several other types of tumors (160-164).

THE LN DETECTION PROBLEM

Locally advanced bladder cancer is a devastating tumor with a high rate of local recurrence, as well as regional lymph-node and distant metastasis in a large number of patients with little tendency toward improved outcome in spite of continuous efforts during the past decades (85, 165). In particular, LN metastasis has an important influence on outcome and no effective methods for preoperative diagnosis or prediction of such LN metastasis has been found during recent decades. Refined investigation methods need to be identified, and an understanding of the tumor metastatic mechanism in the tumor microenvironment level needs to be developed.

Current clinical staging methods remain limited. Preoperative radiological staging (CT and MRI) of LN metastasis does not reflect the true picture of the actual staging of LNs as they depend on the size criteria of metastasis. The detection of LN micro-metastases or even small and medium-sized macro-metastases is the most challenging issue in cancer staging. The level of LN dissection during cystectomy has its own drawbacks relating to extent of surgery, inadequate harvesting and prolonged surgical exposure. Even in meticulous and ambitious attempts to perform wide and extensive LN surgery, a single viable node with a micrometastatic deposit might remain in the patient after surgical closure. This may lead to extended metastatic disease and the ultimate propagation of the already established systemic disease. Therefore, preoperative or perioperative detection of LN metastases is crucial for optimum surgical treatment of these patients (37, 48).

The study of tumor biology and microenvironment might improve our understanding of the role of the immune system in the process of progression and metastasis. One of the theories of TAM's role in cancer is the fusion between macrophage and cancer cells that lead to the generation of new hybrids with cancer and macrophage characteristics that might result in metastasis or the death of that hybrid. This phenomenon and the role of TAM in MI UBC have been subjected to few clinical studies previously. During the past 20 years many studies on p53 immunostaining and outcome have been published. However, little is known about p53 and LN metastases in UBC (166, 167). Few studies have been published on the tumor suppression proteins p53 in relation to proliferative protein Ki67, or DNA repair protein ERCC1 or the upstream control of p53 by $p14^{ARF}$. Further studies are needed in this field (161, 168, 169).

AIMS OF THE THESIS

Paper I

To evaluate PET/CT and conventional CT-scans for detecting pelvic LN metastasis in pre-operative N-staging of UBC patients before cystectomy.

Paper II

The purposes of this study were to examine

- The value of sentinel lymph node technique in detecting pelvic LN metastasis.
- The anatomical distribution of pelvic LN metastasis in relation to tumor location in bladder.
- The concept of LNMD as clinical variable in assessment of UBC.
- The results in relation to other clinical and pathological variables and outcome.

Paper III

Based on cell fusion theory and immunological importance of MI, the aim of this study was to

- Investigate the presence of macrophage traits in cancer cells and its clinical significance in UBC.
- Evaluate macrophage phenotype in relation to MI and cancer cell proliferation.
- Relate the results to LN metastasis, other clinical and pathological variables and outcomes.

Paper IV

To study the association of biomarkers p53, p21, pRb, p16, $p14^{ARF}$ as well as Ki67 and ERCC1 (DNA reparation protein) expression in UBC and their clinical significance in relation to LNs metastasis, other clinical and pathological variables and outcome.

PATIENTS AND METHODS

The patient cohort

We started in 2005 with a prospective study of all patients with UBC operated with RC and PLND. There were 172 patients at the end of the study in 2011, of whom 122 had pathological tumor stages of T1-4. Patients with pathological T0, Ta, and CIS (50) were excluded from the study due to the low incidence of LN metastasis in these groups. The SN method was used to investigate 103 of the patients. The PET/CT study, which started in 2010, included 28 patients from this cohort and 26 new patients.

The number of patients included in Study III was 103 because whole sections from available tumor tissue (blocks) for the other 19 patients (out of 122) showed no invasive tumor. Similarly, in Study 4 there were 10 patients had no evaluable tumor material in the TMA cores, leaving 112 patients for analysis of the biomarkers (Figure 4)

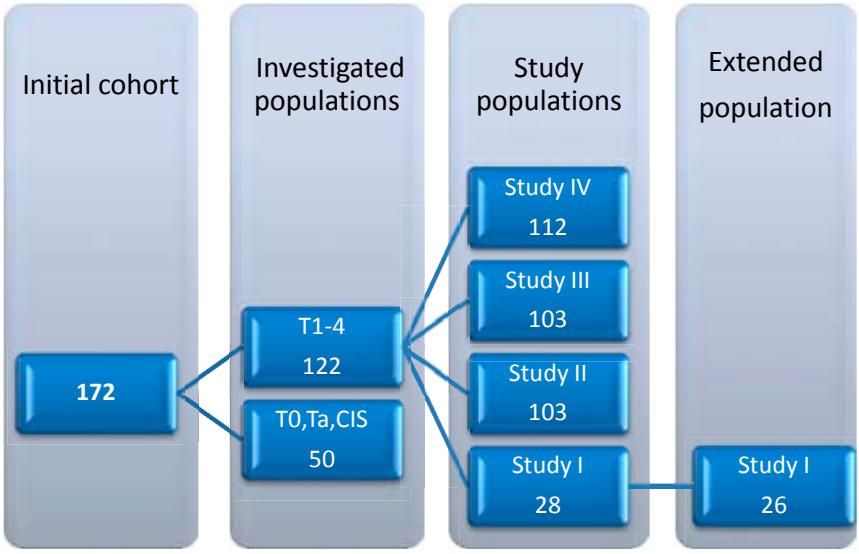

Figure 4. **The study cohort operated with cystectomy and PLND 2005-2011, inclusive and the patients included in the different studies**.

Table 3. Patients and tumor characteristics in the investigated population

Variables	N (%)
Gender	
Men	94 (77)
Women	28 (23)
cT stage organ confined	
Yes	76 (62)
No	46 (38)
pT stage organ confined	
Yes	34 (28)
No	88 (72)
pN	
N0	72 (59)
N+	50 (41)
Tumor type of tumor	
Urothelial	109 (89)
Squamous cell	13 (11)

Paper I

At the Department of Urology, University Hospital Linköping, Sweden, a total of 67 patients with urinary bladder cancer were scheduled for radical cystectomy with pelvic LN dissection between 2010 and 2012. All these patients underwent PET/CT and conventional CT of the thorax and abdomen as part of pre-cystectomy evaluation. Twelve of the patients did not treated with cystectomy for the following reasons: 4 had distant metastasis (M1; positive in both conventional CT and PET/CT); 2 were medically unfit for surgery; 6 preferred radiation therapy. One of the remaining 55 patients did undergo radical cystectomy but had no LN dissection and was therefore excluded from further analysis. The study was approved by the Regional Ethics Committee (Reference number M42-08) with written consent from the patients included. All operations were performed with curative intent. Patients with preoperatively known distant metastases (M1), previous radiotherapy of the pelvis and/or previous pelvic surgery were excluded from the study.

PET/CT was done according to a standard protocol. The patients fasted at least 4 h before injection of the FDG. Blood glucose was monitored immediately before the injection, and a level lower than 8 mmol/L was required to perform the investigation. The dose of FDG was 4

MBq/kg body weight. Images were acquired 60 min after the injection, and the patient drank 1 L of fluid or contrast medium during the 60-min interval between injection and imaging. The examination was done using a Siemens Biograph 40 PET/CT scanner with the patient in a dorsal recumbent position with the arms above the head. Full-dose CT with IV contrast was performed first, followed by PET (1.5–mm and 5–mm slices and a resolution of 4.2 mm). CT was performed using a thickness of 1.5 mm and a gap of 1.5 mm at 100 kV whereas mAS were regulated automatically according to the volume of the patient. The PET/CT images were evaluated by two radiologists (Kappa value 0.85). All PET images were evaluated by determining the maximum standardized uptake value (SUV), using a SUV-max cut-off of 2.5. LNs were considered positive by conventional CT if they had a diameter of ≥1 cm independently of whether large or small axes were considered, and positive by PET/CT if they exhibited higher levels of activity than the SUV max cut-off level regardless of their size.

Radical cystectomy was performed according to a standardized LN dissection and handling of the specimen described in Study II. Each harvested LN was sectioned in the middle along the larger axis, and a single 4-µm section was mounted on a slide and stained with hematoxylin eosin before analysis. Sections from the tumor at cystectomy and from all LNs as well as the new tumor sections were re-evaluated by an experienced uropathologist (HO) who was blinded for the clinical data of the study. Primary tumors were graded according to WHO 1999 and WHO 2004 systems as well as the TNM 2010 classification system and examined for the presence of lympho-vascular invasion without vessel specific immunohistochemical staining. Results of PET/CT, conventional CT, and histopathological examination were compared.

Statistical analysis: Using the histopathological examination as gold standard, each PET/CT and conventional CT examination was classified as true positive or false positive, and true negative or false negative. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated. Otherwise, differences between groups were calculated using the chi square test with $P < 0.05$ as statistically significant value.

Paper II

All 103 patients who were planned for treatment with open cystectomy and lymphadenectomy as treatment for locally advanced UBC and with pathological category pT1–pT4 were included. The study was performed during 2005–2011 at the Department of Urology University Hospital Linkoping, Sweden, and was approved by the Regional Ethics Committee (Reference number; Lu 01–48 and Li 03– 268) with written consent from the patients included. All operations were performed with curative intent. Patients with preoperatively known distant metastases (M1), previous radiotherapy of the pelvis and/or previous pelvic surgery were excluded from the study.

After anesthesia, cystoscopy was performed, tumor location in the bladder was recorded and 1 mL of 99mTc-nanocolloid (70 MBq/mL) and 1 mL of Patent Blue (Sigma-Aldrich, Stockholm, Sweden) were injected at four sites around the tumor margin in the detrusor muscle just before cystectomy. For further location of tumor, the following regions within the bladder were considered: the apex, right side, left side, posterior side, anterior wall, and trigone.

Radical cystectomy and pelvic lymphadenectomy were performed in all patients. Lymphadenectomy was performed systematically with defined anatomical landmarks before RC. The cranial limit of PLND was at the level of the ureteric crossing of the common iliac vein immediately superior to the confluence of the external and internal iliac veins but in 55 patients, lymph node dissection was extended to the aortic bifurcation at the decision of the operating surgeon. The caudal extension of PLND was at the level of Cooper's ligament, with the genitofemoral nerve as the lateral boundary. On both sides of the pelvis, lymphatic and connective tissue were removed separately from the following four anatomical stations: obturator, external iliac, internal iliac, and common iliac. A handheld gamma probe (Neoprobe 2000; Neoprobe Corp) was used to detect SNs in tissue removed from each anatomical region, outside the body at a distance from the radioactive bladder. Tissue samples expressing radioactivity were defined as SN stations and were sent separately for pathological evaluation. All specimens were processed according to the standard procedure at the Department of Pathology, Linköping University Hospital. LNs were prepared using standard protocol with one section of 5-µm in the middle of the node stained with H&E. All the LNs were similarly sectioned at two additional levels situated at 0.5 mm and 1.0 mm, respectively, from the initial section.

The purpose of re-sectioning all the LNs was to detect additional metastases that could not be found in the initial routine pathological examination.

Sections from the tumor at cystectomy, from all LNs as well as the new tumor sections were re-evaluated by an experienced uropathologist (HO) who was blinded for the clinical data of the study. Primary tumors were graded according to WHO 1999 systems as well as the TNM 2010 classification system and examined for the presence of lympho-vascular invasion without vessel specific immunohistochemical staining.

Statistical analysis: The concordance between SN pathological evaluation and definitive pathological status of LNs in the same anatomical site was calculated by the diagnostic tests of sensitivity, specificity and positive and negative predictive values. All other differences between groups were calculated using the chi-squared test and logistic regression analysis. Univariate Cox proportional hazards analysis and Kaplan– Meier analysis with the log-rank test were used for CSS analysis with $P < 0.05$ as statistically significant. To evaluate the optimum cut-off value for LNMD, we used receiver-operating characteristic (ROC) analysis. Sensitivity and 1-minus specificity data on cancer-specific survival as outcomes were used, and area under curve (AUC), in this case 0.74, was calculated with 95% confidence interval (CI) for LNMD means as dichotomous variable.

Paper III

All patients with pathological stage pT1-4 treated with cystectomy 2005 – 2011 were included in the study, which was approved by the Regional Ethics Committee (Reference number; Lu 01-48 and Li 03-268). All operations were performed with curative intent. Patients with preoperatively known distant metastases (M1), previous radiotherapy of the pelvis and/or previous pelvic surgery were excluded from the study. RC, PLND, processing of tumor specimen and histopathological evaluation of primary tumors and LN were performed according to the methods described in Paper II.

Whole sections of 5 μm were cut from formalin-fixed paraffin-embedded tissue blocks, deparaffinized in xylene and hydrated in a series of graded alcohols (100%, 95%, and 70%). The sections were stained for CD163 and Ki67 using mouse anti-human monoclonal CD163 anti-

body (Clone 10D6, Abcam, USA) and monoclonal antibody (Mib-1, Dako, Denmark), respectively. Positive Ki-67 expression was defined by nuclear staining in cancer cells. The Ki-67 expression in tumor areas showing the highest density of stained tumor cells was determined by an initial scan at low magnification (x200). The number of positively stained nuclei was counted in 200 tumor cells at high magnification (×400) and was recorded as a percentage of the total number of positive cells.

The positive immunoreactivity of CD163 was defined as a granular cytoplasmic or a cytoplasmic and membrane staining pattern. TAMs and cancer cells were distinguished based on morphological criteria. Cells expressing CD163 with small and regular nuclei were recognized as M2 macrophages. To avoid overestimation of the number of TAMs, which was a possibility due to extended cytoplasmic ramifications, we counted only cells with a visible nucleus. The infiltration of TAMs in tumor stroma was evaluated over a whole section and was classified in three grades: no/low, moderate, or high (Figure 5). Cancer cells were enlarged and atypical, with pleomorphic hypertrophic and darker nuclei, and also showed a decreased cytoplasmic to nuclear ratio. The proportion of cancer cells staining for CD163 in a tumor section was estimated semi-quantitatively. A tumor was regarded as positive if it contained any CD163-positive cancer cells and was considered negative if it lacked such cells. Immunostaining was evaluated by three of the co-authors (FA, IS and HO) independently and all were blinded for clinical information about the patients at the time of the examination of the slides. Inter-observer agreement, calculated as Cohen kappa index, was $\kappa = 0.56$. The cases with discordant results were discussed between all investigators to reach consensus.

Statistical analysis: Pearson's chi-square test was applied to evaluate the relationship between CD163 expression and MI grade in relation to clinical data and tumor characteristics. Comparison of clinical data, MI, and proportion of cancer cells expressing Ki-67 and CD163 was achieved by one-way analysis of variance (ANOVA) together with a post-hoc Bonferroni's test. Kaplan-Meier analysis with the log-rank test was used for CSS rate analysis with $P < 0.05$ considered statistically significant.

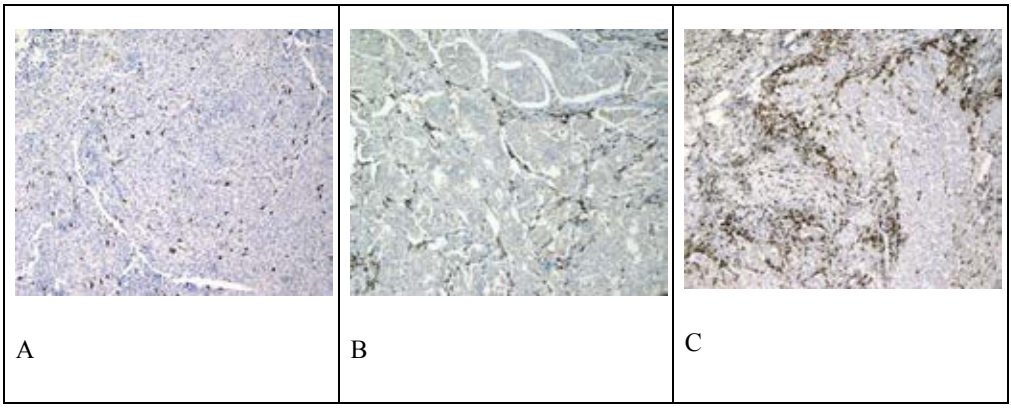

Figure 5. Infiltration of M2 tumor-associated macrophages in muscle-invasive bladder cancer graded as no/low (A), moderate (B), or high (C) density.

Paper IV

Patients with stage pT1-4 UBC who were treated with RC between 2005 and 2011, inclusive at the Department of Urology, University Hospital, Linköping, Sweden, were included prospectively. The study was approved by the Regional Ethics Committee (Ref No; Lu 01-48 and Li 03-268). All operations were performed with curative intent. Patients with preoperatively identified distant metastases and previous radiotherapy of the pelvis were excluded from the study. RC, PLND, handling of the tumor specimen and histopathological evaluation of the primary tumors and LN were performed according to the methods described in Paper II.

The paraffin blocks of primary tumors were chosen carefully with respect to the presence of invasive UBC. A tissue microarray (TMA) was constructed using three random tissue cores (diam. 0.6 mm) that were taken from tumor regions in the paraffin-embedded primary UBC block and subsequently inserted in a recipient paraffin block in an array pattern. Serial 5-µm sections were obtained from formalin-fixed paraffin-embedded TMA tumor specimens. The sections were deparaffinized in xylene and then rehydrated, pretreated with Tris-EDTA buffer

(pH 9) or citrate (only for pRb), and stained in an automated immunostainer (DAKO TechMate-TM Horizon, DAKO Denmark A/S). All the antibodies used in this study are listed in Table 4. Positive and negative controls were employed throughout. All antibodies were initially individually optimized with respect to the best pretreatment method and dilution following the standard procedure at the department and according to the manufacturers' recommendations.

All slides were evaluated at 400x magnification by an experienced uropathologist (HO) who was blinded for clinical data. Only nuclear staining was considered for $p14^{ARF}$, pRb, Ki67 and p21. Nuclei and cytoplasm were considered for p53 and p16. For ERCC1, nuclear staining only was also used, but in this case both the number of stained tumor cells and the intensity of staining (1 = weak, 2 = intermediate, 3 = strong) were evaluated and calculated as H score, which represents the proportion of stained tumor cells multiplied by the intensity of staining. Tumors with an H score >1% were considered ERCC1 positive (159). For further statistical analysis, all biomarkers were assigned to one of two categories: normal (wild type) or abnormal (altered). Cut-offs for p53, 21, p14ARF, and Ki67 were chosen as the median values for stained tumor cells, whereas p16 and pRb were considered to be altered if there was no staining or >50% of tumor cells were stained (170). The cut-off values are summarized in (Table 1). For each patient, the core with the highest value was considered to be representative for that individual. This strategy was applied, because there is always a risk that a hot spot in a tumor will be missed in a TMA core, as studied by others (171-173).

Statistical analysis: Statistical analyses were performed using SPSS statistics software, version 23 (IBM Corporation, USA). Fisher's exact test and the chi square test were conducted to evaluate the association between p53, p21, pRb, p16, $p14^{ARF}$, Ki67, ERCC1, pathologic stage, LN status, and LVI. The Kaplan-Meier method was applied to calculate survival , and differences were calculated using the log-rank test. Statistical significance was set at $P < 0.05$. All reported P-values are two-sided.

Table 4. Antibodies used for immunohistochemistry

Antibody	Clones	Source	Dilution	Abnormal	Positive
p14^{ARF}	Polyclonal (canine)	SPRING Bioscience	1:25	>0%	Nuclei
p53	DO-7	DAKO, Denmark	1:100	≥10%	Nuclei and cyto-plasm
pRb	G3-245	BD Pharmingen	1:100	0% or >50%	Nuclei
p21	SX118	DAKO, Denmark	1:50	<8%	Nuclei
p16	6H12	Novocastra	1:20	0% or >50%	Nuclei and cyto-plasm
Ki67	Mib-1	DAKO, Denmark	1:50	>46%	Nuclei
ERCC1	8F1	NeoMarkers	1:200	>0.01 median H score	Nuclei

RESULTS

Paper I

Of 54 patients evaluated in this study, 47 were men and 7 were women. The mean age was 68 years (range 46–85 years). Considering all 54, histopathological examination showed no LN metastasis (N0) in 37 patients (69%) but revealed 1 or more positive LNs in 17 patients (31%). Sixteen (94%) of those 17 patients had pT3-pT4 disease. FDG uptake was detected in 12 patients (22 %), and 7 of those observations were confirmed by pathology. Conventional CT alone showed enlarged LNs in 11 patients (20%), which were confirmed by pathology in 7 cases. Equivalent findings were obtained by both PET/CT and conventional CT in 43 (80%) of the 54 patients. The following was observed in the remaining 11 patients (20%): 9 were positive by PET/CT and negative by conventional CT, and only 1 of those findings was confirmed by pathology; 2 were positive by conventional CT and negative by PET/CT, and neither case was confirmed by pathology. There were 1,518 LNs (mean 28 nodes per patient) excised from 347 sites in the stipulated anatomical regions, and metastases were confirmed in 99 LNs (7% of all LNs). PET/CT was negative in 41 sites with metastases, which was confirmed by pathology, whereas conventional CT was negative in 48 sites with metastases. PET/CT had 41% sensitivity, 86% specificity, 58% PPV, and 76% NPV, whereas the corresponding figures for conventional CT were 41%, 89%, 64%, and 77%.

Paper II

Of the 103 patients included in the study, 80 (77%) were male. The median age of the patients was 69 years. Seventy one patients (69%) had non-organ-confined tumors (T3 or T4 tumor stage) and all patients had negative surgical margins. A total of 3,253 LNs were sectioned at three levels and examined for the presence of metastases. The mean number of harvested LNs was 31 (range 7–68) per patient. LN metastasis was found in 41 (40%) of the patients, of whom 11 (11%) had single LN metastasis (N1 stage) and 30 (29%) had multiple LN metastases (N2 stage). Seventy-five percent of patients had tumors which involved more than one region in the urinary bladder. SN was detected in 83 (80%) patients, 20 (25%) of whom had pathological SNs. Metastasis occurred in single SNs in 17 patients (85%). One patient had four pathological SNs, and the remaining 2 patients had two pathological SNs each. In 3 N1 patients, the only positive LN was also SN.

The sensitivity and specificity for detecting metastatic disease by SNB varied between LN stations, with averages of 67% and 90%, respectively. The negative and positive predictive value ranges were 63–100% and 0–99%, respectively. In patients with detectable nonpathological SNs (n = 70), 13 (19%) had LN metastasis in other pelvic LN specimens (Table 5).

Looking only at the side of the patients (right or left) with respect to LNs involved instead of particular stations, sensitivity and specificity for the right side were 77% and 100%, respectively, and for the left side they were 72% and 100%, respectively.

Table 5. The specificity and sensitivity of sentinel node biopsy for detecting lymph node metastasis in invasive urinary bladder carcinoma

Pelvic region	Sensitivity, % (95% CI)	Specificity, % (95% CI)	PPV, % (95% CI)	NPV, % (95% CI)
Obturator				
Right	100 (2.5–100)	93.6 (78.58–99.2)	33.3 (0.84–90.57)	100 (88–100)
Left	66.67 (22.28–95.67)	95.3 (81.0–99.9)	80 (28.36–99.49)	92.86 (76.5–99.12)
Internal iliac				
Right	75 (19.41–99.37)	86.67 (59.54–98.34)	60 (14.66–94.73)	92.86 (66.13–99.82)
Left	0 (00–97.5)	87.5 (61.65–98.45)	0 (00–84.2)	93.3 (68.1–99.83)
External iliac				
Right	100 (2.5–100)	91.4 (76.94–98.2)	25 (0.63–80.6)	100 (89.1–100)
Left	50 (11.8–88.2)	81.8 (59.7–94.8)	42.86 (9.9–81.59)	85.7 (63.66–96.95)
	LN location, stratified by side of pelvis			
Right side	77 (46.19–94.96)	100 (91.96–94.96)	100 (69.2–100)	93.6 (82.46–98.66)
Left side	72 (46.52–90.31)	100 (91.4–100)	100 (75.3–100)	89 (76.4–96.4)

LN, lymph node; NPV, negative predictive value; PPV, positive predictive value.

The mean LNMD in all patients was 8%. In relation to CSS, the highest AUC was identified for an LNMD value of 8% (AUC 0.74; 95% CI 0.67–0.87). Based on these data, an LNMD

value of 8% was used as a cut-off in further statistical analysis. Patients with LNMD ≥8% had a mean survival time of 22 months compared with patients with LNMD <8%, who had a mean survival time of 53 months. Lymph node metastatic density ≥8% was associated with advanced N-stage tumor recurrence and poor survival. Patients with N0 stage (n = 62, (60%)), LNMD <8% (n = 14, (14%)) and LNMD ≥8% (n = 27, (26%)) had 5-years CSS rates of 71%, 43% and 19%, respectively (P < 0.001) (Figure 6).

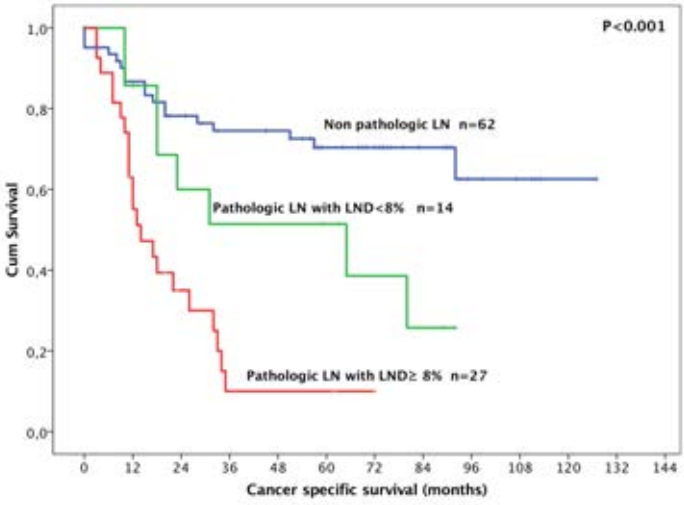

Figure 6. CSS curves in relation to a metastatic lymph node density (LNMD) rate of 8% estimated according to Kaplan-Meier. Patients with LNMD >8% had shorter disease-free survival.

Patients with organ-confined (n = 32, (31%)) and non-organ-confined tumors (n = 71, (69%)) had cancer-specific survival rates of 81% and 41%, respectively, after 5 years (P < 0.001). Patients with LVI (n = 67, (65%)) and patients without LVI (n = 36, (35%)) had cancer-specific survival of 78% and 40%, respectively, (P < 0.001). Non-organ confined tumors and LVI were significantly associated with pathological LN.

Paper III

The study included 103 UBC patients with a median age of 69 years (range 51–87 years), the majority whom (78%) were males. Eighty patients (78%) had non-organ-confined tumors (stage T3 or T4). Forty-three (42%) of the 103 patients had LN metastasis, which was detected in a single LN (N1) in 12 (12%) of the subjects and in multiple LNs (> N1) in 31 (30%). Almost all the patients (99%) had advanced tumors exhibiting moderate (9%) to poor (91%) differentiation. Whole-tumor sections samples from all 103 patients were available for immuno-histochemical staining. On average, 78% of tumor cells expressed Ki-67. Tumors from 80 (78%) of the patients showed CD163 expression in some of the cancer cells. The average proportion of cancer cells expressing CD163 was 34%. There was no correlation between CD163 expression and clinical variables such as age, gender, tumor stage, and lympho-vascular invasion or outcome. We classified the patients into two categories as follows: a strong MI group including patients with high and moderate MI, and weak MI group comprising patients with low or no MI.

LN metastases were more frequent in patients with weak intratumoral MI (58%) than in those with strong intratumoral MI (36%), although not at a significant level ($P = 0.06$). Gender, age, differentiation grade, lympho-vascular invasion, and pathological stage were not associated with MI. MI was associated, although not at a significant level, with CSS in UBC (Figure 7a).

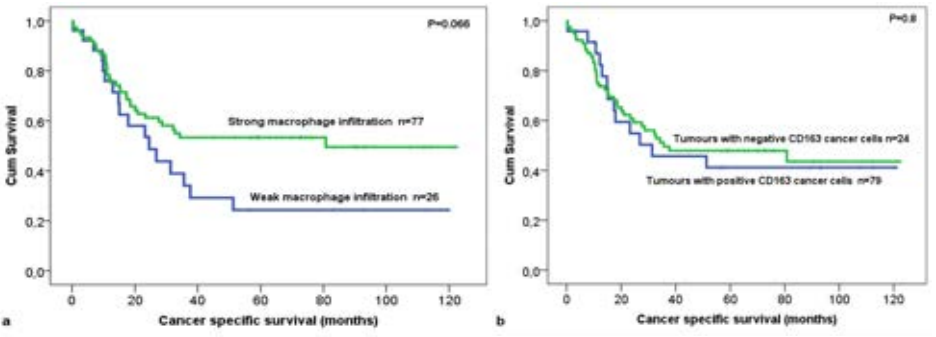

Figure 7. **CSS in locally advanced bladder cancer with respect to CD163 immunostaining for strong and weak MI (a) and CD163 expression by tumor cells (b).**

The mean proportion of cancer cells expressing CD163 in tumors with low MI was 18%, which was significantly lower than the rates observed in tumors with moderate (42%) or high (42%) MI (P = 0.01 vs P = 0.015). The mean CSS was 65 months in patients with CD163-positive tumors and 66 months in subjects with CD163-negative tumors, but the indicated difference in CSS was not statistically significant (P = 0.7) (Figure 7b).

Paper IV

The study comprised 112 patients whom 86 (77%) were male. The median age of all patients was 69 years (range 51–87 years). Eighty three patients (74%) had non organ-confined tumors (>T2). LN metastasis was found in 47 (42%) of all patients, of whom 32 (29%) had multiple LN metastases (>N1). Lympho-vascular invasion was seen in 76 (69%) of the cases. Chemotherapy was given to 34 patients, 27 adjuvant, 5 neoadjuvant whereas 2 patients had both neoadjuvant and adjuvant treatment. Immunostaining showed altered biomarker expression as follows; p53-45 patients (49%), p21 -51 patients (50%), pRb – 51 patients (49%), p16- 92 patients (91%), $p14^{ARF}$ – 57 patients (52%), Ki67 – 46 patients (47%) and ERCC1 – 50 patients (47%). Logistic regression analysis found no association between studied biomarker

expression and T category, N category or LVI (data not shown). $p14^{ARF}$ was significantly associated with ERCC1 and high T stage. There was no association between improved CSS and p53 altered compared with p53 non-altered in a chi square analysis (P =0.08) or in a Kaplan-Meier analysis with log rank test (P = 0.223). For altered $p14^{ARF}$, ERCC1 or p21 CSS was lower in case of low p53 and improved in case of high p53, but not at a significant levels. Thus it seems that altered $p14^{ARF}$, ERCC1 and p21 implies a low survival, but patients with altered p53 might be protected from cancer death.

DISCUSSION

In P**aper I,** we compared PET/CT and conventional CT regarding the rate of detection of positive LNs in UBC patients before RC. Both these methods showed low levels of sensitivity and specificity for detecting pelvic LN metastases and our data suggest that, FDG PET/CT, provided no improvement in detection and localization of regional LN metastases in urinary bladder cancer compared with conventional CT. In contrast, Drieskens et al, Liu et al, and Kibel et al, and Goodfellow et al (174-177), noted sensitivity rates ranging from 60 to 77%§ when using protocols for PET/ CT imaging and analyses similar to those employed in our study. We also found that sensitivity decreased from 41% on a patient level to 25% on a regional LN level, which agrees with Drieskens et al, who also noted a drop in sensitivity from 60% to 50% on the regional level (35).There is no obvious explanation for this discrepancy. However, most of the studies mentioned were rather small, so just a few additional patients with positive PET/CT results might have had a marked impact on the rate of sensitivity. Another plausible reason for differences in the LN detection rate might be related to the extent of LN dissection. However, our findings are consistent with the results reported in previous studies (15, 178, 179). In a meta-analysis, Zhang et al, demonstrated that FDG PET/CT had a pooled sensitivity of 82% and a pooled specificity of 92% PPV 6.80; NPV 0.27. The results indicate that FDG-PET/CT have relatively high sensitivity and specificity for the diagnosis of urinary bladder cancer (180). The author recommended further prospective randomized, controlled studies with larger case numbers to confirm the results. A major concern in meta-analysis studies is publication bias because studies reporting positive or significant results tend to be published, whereas those whose results are non-significant or negative are often rejected (181). Mayoral et al, demonstrated that PET/CT has low sensitivity in lymph node staging of cervical and endometrial cancer due to the lack of detection of micro-metastasis (182). In another meta-analysis, Lu et al showed that there is no solid evidence to support the routine clinical application of PET/CT in the pre-therapeutic evaluation of lymph node status in patients with colorectal cancer (183). In a review article Kiss et al, demonstrated that despite the multitude of new imaging techniques available today, a meticulous histologic workup of lymph nodes retrieved by an extended pelvic lymph node dissection still has the highest accuracy for detection of lymph node metastases in bladder or prostate cancer (18).

Possible explanations for the discrepancy between different studies are that most of the studies were rather small, therefore just a few additional patients with positive PET/CT results might have had a marked impact on the rate of sensitivity. Other possible reasons for differences in the LN detection rate might be related to the extent of LN dissection, techniques for pathological detection of LN in the specimen, sectioning of LN and staining procedures (180). In EAU guidelines there is no consensus about the use of PET/CT in staging of MIBC and further trials are required before a recommendation can be made (3). The important lesson from our study is not to rely on PET/CT or conventional CT in LN staging of muscle-invasive UBC due to low sensitivity and specificity in identifying metastasis.

Paper II, SNB is a feasible method to detect SN in UBC. We demonstrated 80% detection rate. The mean sensitivity and specificity for detecting LN metastasis in each pelvic lymphatic station were 67% and 90%, respectively. These findings indicate that as UBC metastasis to pelvic LNs is difficult to predict accurately using the SNB technique, it has low clinical value for the detection of pathological pelvic LN. Liss et al demonstrated in a meta-analysis that the ideal candidates for SNB are patients with T1 or T2 tumors on resection with clinically negative CT scans prior to cystectomy as the results of investigation in patients with T3 tumors and enlarged LN are difficult to interpret. Under optimal conditions, the technique may provide the surgeon with improved ability to guide lymph node dissection decisions and reduce patient morbidity (184). This meta- analysis was based on 7 previous studies with small numbers of patients. Our results are consistent with the results obtained by Liedberg et al, who reported an 87% SN detection rate and a 19% false-negative SN rate (69). Based on previous experience, Munbauhal et al reviewed the inability to identify a constant SN or packet of SLNs for prostate cancer (185). In other systemic review, van der Zaag et al, showed that the overall sensitivity rate of SN mapping in patients with colorectal cancer was 70% (186). These low sensitivity rates in detecting pelvic LN metastasis with SNB might be explained by the anatomical variation of lymphatic drainage from the bladder to the pelvic LNs and rerouting of afferent lymphatic drainage as a consequence of obstructed pathological SNs. As a result, there would be no radiotracer uptake in these LNs. This phenomenon is described in several other tumor types and may help explain the low sensitivity rate in the present study (67, 187). Technical factors such as the volume of injection might influence the detection rate as shown in previous studies (69, 188). Another finding from Paper II was that the additional

sectioning of LNs identified micro-metastases in 1/103 patients (<1%). Our result is in agreement with other published results in this field indicating that the additional sectioning of LNs do not result in a better detection rate of additional LN metastasis and furthermore is both costly and time- consuming (189-191). A detailed guideline on how to handle the LN specimens at the pathological department is needed.

Furthermore, Paper II shows that LNMD and LVI have a significant prognostic impact in UBC which is in agreement with previous studies. However, the cut off value in our study was 8% for LNMD which is lower than the cut off value in other studies (97).

Paper III, Tumor associated macrophages (TAM) are an important subset of non-neoplastic cells in tumor stroma and in several solid tumors may influence cancer growth and progression (123, 192). However, the prognostic impact of MI is still contradictory in several types of tumors, such as lung and colorectal cancers (193-197). In this paper we have found that increased MI in UBC is associated with decreased rates of metastasis and improved CSS, although not at a significant level. Maniecki et al (2012) reported, in a UBC material from transurethral resections, that high infiltration of CD163-expressing macrophages in tumor stroma was associated with advanced tumor stages (137). However, in a similar study conducted by Sjödahl et al, the infiltration of TAMs alone (i.e., CD163+ or CD68+ macrophages) was not related to disease-specific survival. Sjödahl and co-workers also demonstrated that a high ratio of CD68/CD3 infiltration had a negative impact on prognosis in those patients (198). Hanada et al used CD68 as a macrophage marker and found that patients with a high MI had shorter survival time than those with a low MI, and that MI was related to the microvascular count. In this study, no details about patient management and definition of survival end points were reported (199). The current findings in Paper III indicate that increased MI might have a protective effect in advanced UBC, which seems to be synergistic with the immune response against urinary bladder cancer by reducing LN metastasis and may eventually improve survival. Based on our own and previously reported observations, the clinical impact of MI in UBC is contradictory which may be due to differences between patient cohorts and sample material examined in these studies. Moreover, in Paper III macrophages are detected by the M2-specific marker CD163, which is more selective for TAMs than for other macrophage markers, such as CD68 (200-203).

Macrophage trait in cancer cells is reported in several types of tumors, such as breast and colorectal cancers and may be explained by cell fusion between TAM and cancer cells (204-206). Cell fusion is a natural biological process in normal development and tissue regeneration (207, 208). Fusion between cancer cells and macrophages results in hybrids that acquire genetic and phenotypic characteristics from both maternal cells (129, 139, 209). There is a growing body of in vitro and in vivo data indicating that this process also occurs in solid tumors and may play a significant role in tumor progression (133, 134, 138, 139, 210, 211). In Paper III, M2-macrophage trait evaluated by CD163 expression in cancer cells was not correlated to lymph node metastasis or survival time. These observations are opposed to the general understanding that fusion between myeloid cells and cancer will generate hybrids with increased metastatic ability (131, 133, 212). The expression of CD163 in UBC tumor cells was also reported by Maniecki et al but this expression was evaluated as staining intensity, and no data were given regarding the proportion of cancer cells expressing CD163. Using real-time PCR analysis, Maniecki and colleagues also found that CD163 expression was related to poor prognosis. In this study, the authors have not used micro-dissection and as the sample material (CD163 RNA) may originate from macrophage and cancer cells, the correlation between CD163 expression and survival cannot be associated with CD163 expression selectively in cancer cells. Sjödahl et al observed that tumor cells in urothelial carcinoma expressed CD163 in 6% and CD68 in 5% of cases. The authors did not report further analysis of how this expression was associated with clinical data.

The discrepancy of CD163 expression by cancer cells in UBC and other tumor types, such as breast colorectal and malignant melanoma, in relation to clinical outcomes is notable and may be explained by the biological differences between these tumors. In order to metastasize, tumor cells must acquire characteristics similar to those inherent in normal lymphoid cells such as proteolytic degradation or intra- and extravasation capabilities (213, 214). Furthermore, the development of secondary tumors involves tumor cells (seed) and normal cells in the different target organs (soil) (215). According to the “seed and soil theory”, metastatic cells exhibit tissue tropism, preferring to grow in certain organs and this phenomenon cannot be explained by circulatory or anatomic patterns alone. The pattern of affected organs is depending on the cancer type (216). UBC metastasizes mainly to the pelvic lymph nodes. In line with the “seed and soil theory” and homing mechanisms, the findings in Paper III suggests that macrophage

phenotype in cancer cells in advanced UBC may not contribute to selective lymphogenic metastasis.

Paper IV, Studying the cell cycle regulatory proteins and other biomarkers might be important in understanding the mechanisms of regulation and patterns of growth control in cancer cells. In the present study we found no association between p53 immunostaining and LN metastases, in accordance with other studies (143, 165). Our findings show that p53 accumulation might result in improved CSS, although not at a significant level. In particular, an improved survival is observed, although not at a significant level, if p53 accumulation is associated with altered $p14^{ARF}$, p21 or ERCC1 indicating possible interaction between these biomarkers and p53. Such interaction might imply stimulation of p53 production due to altered p21 and ERCC1 as well as stabilization of existing p53 by inactivation of MDM2 by $p14^{ARF}$ and gain of function of mutant p53 (157, 159). These results indicate that $p14^{ARF}$ might be an important factor in UBC micro-environment, possibly by interaction with p53 function and might be a potential prognostic biomarker in urinary bladder cancer. ERCC1 expression may also be an important factor for tumor control and serve as a prognostic marker for survival in bladder cancer patients in association with other biomarkers, in particular p53. Alteration of tumor suppressor proteins such as p53, p21, pRb, p16 is involved in the pathology of UBC, and together with the proliferating marker Ki-67 is suggested to have prognostic significance in patients treated with radical cystectomy for bladder cancer (217). Freier et al. studied cervical cancer and demonstrated better CSS rates in patients with tumors expressing mutated nuclear p53 protein. It was proposed that this unexpected finding might be explained by a better response to chemotherapy (218). The discrepancies between the findings of the cited studies might be related to differences regarding the choice of antibodies, interpretation of the data and stratification criteria, and specimen handling and technical procedures (105). Other plausible explanations for these differences are the pathological sample processing from surgical removal to immunohistochemistry staining, differences in cut-off levels, and sample size (168, 219-222). The use of TMA in immunohistochemical studies of invasive urinary bladder cancer might be questionable as intra-tumor heterogeneity might affect the results compared with whole section, which might be due to sampling only small areas of the tumor (173, 223).

FUTURE PROSPECTIVE AND RESEARCH

It is obvious from the references mentioned in the introduction and the discussion of every study included in this thesis, that LN metastasis is a major factor in predicting prognosis of patients with locally advanced UBC. The challenge issue is to detect LN metastasis in normal size LNs. Unfortunately, there is no accurate detection method that can detect metastasis in normal size LNs and the available techniques introduced for this purpose during the past 20 years have low accuracy. As a result of the low sensitivity and specificity of the available techniques and the poor CSS associated with locally advanced UBC, PLND remains the standard method associated with cystectomy to eliminate as much as possible of LNs that might harbor metastasis. This is at the expense of increasing the operative time, the risk of major perioperative bleeding, and the risk of post-operative morbidity. Despite the appropriate PLND, the incidence of local recurrence, regional LN metastasis or distant metastases remain high.

Detection of LN metastasis

The focus of the first two studies in the present thesis was on pre-operative detection (radiological PET/CT) and peroperative detection (radio-nuclear SN detection) in relation to post-operative (pathological) LN metastasis status. Due to the small number of patients in common, Studies I (PET/CT) and II (SNB) were not sufficiently statistically conclusive to study the combination of the two techniques in detection of LN metastasis in UBC. It might be possible to study such an association in a larger study cohort. Employing novel tracers or the use of other imaging modality such as MR/PET might enhance detection of LN metastases (224). Most recently Beinat et al identified an 18F-labeled pyruvate kinase M2 (PKM2) specific radiotracer showing promising cell uptake results which warrants further evaluation for its ability to detect and monitor cancer noninvasively (225). Prostate-Specific Membrane Antigen (PSMA) PET imaging for prostate cancer staging is another newly tested tracer which needs further investigation in UBC (226). No UBC specific marker has been discovered yet.

New studies for lymphatic mapping and identification of SN perioperatively using indocyanine green (ICG) with near-infrared fluorescence imaging (NIR-ICG) as new tracer modality have been published (227-229). The use of tracer that only delineates lymphatic drainage

transforms the procedure into a radio-guided or fluorescence-guided dissection (185). The future of a true SN concept seems to depend on the identification of a cancer-specific targeted ligand which is bound to an intraoperatively detectable tracer

Tumor microenvironment and tumor cellular pathology

The focus of the last two studies in the present thesis was the association of tumor microenvironment and tumor cellular pathology with LNs metastasis and CSS. The results of Study III and IV might help in understanding the relationship between different nuclear biomarkers, TAM, macrophage trait and clinical outcome including LN metastasis. We used the TMA technique in Study IV and whole slide in Study III in Ki67. There were differences in immunohistochemistry results for the same tumors between the two techniques, with lower values when using the TMA technique. This might be due to tumor heterogeneity, which leads to lower estimation of the immunostaining in TMA. Therefore, further evaluation of the TMA technique in UBC immunostaining is required.

By using CD163, we evaluated M2 infiltration in tumor stroma in relation to the clinical outcome. Further studies using larger population groups with a wider spectrum of T stage and with evaluation of both M1 with M2 using different macrophage specific markers to calculate the ratio between them might elucidate the functional role of macrophages in UBC. The lymphocyte, dendritic cells and TREG infiltrations in the tumor stroma or tumor margin are other interesting fields in tumor microenvironment studies. Macrophage traits both in the primary tumor and in LN metastasis in a larger study cohort might help achieve a better understanding of the mechanisms involved in LN metastasis. Mutation of various tumor suppressor genes in association with the level of their tumor suppressor protein expression such as p53, p21, $p14^{ARF}$ as well as DNA repair gene ERCC1 both in the tumor and LN metastasis is another interesting field to study and further elucidate the underlying mechanisms between LN metastasis and tumor microenvironment. Larger cohorts are needed to study the interrelationship between various tumor biomarkers, MI, macrophage trait, LN metastasis and clinical outcome in UBC.

CONCLUSIONS

PET/CT: Provided no improvement in detection and localization of regional LN metastases in bladder cancer. Both PET/CT and conventional CT showed low sensitivity in detecting LN metastases, and sensitivity decreased with a more exact level of LN localization.

SNB: Is not a reliable technique for identifying LN metastasis. Nevertheless, LNMD at 8% cut-off level and LVI have a significant prognostic impact on CSS.

M2 macrophage: Positively related to age, CD163 expression in cancer cells. MI was also associated with lower rate of LN metastasis and improved CSS, although not at a significant level in MIBC.

Biological markers: In patients with altered markers for ERCC1, $p14^{ARF}$ or p21 survival is lower when p53 is not altered and improved if p53 is altered. This indicates a protective effect of the altered p53 in association with these biomarkers.

METHODOLOGICAL CONSIDERATIONS AND LIMITATIONS

Study 1: As this investigation comprised a small number of patients, no firm conclusions can be drawn from the results. Furthermore, it was our intention to explore the possibility of routine PET/CT for all patients undergoing cystectomy. Considering the substantial number of PET/CT examinations that were negative in our subjects, it might have been more appropriate to study patients who were at greater risk of having positive LNs. Our assessments relied on pathological examination of single sections of LNs, and it is possible that using step-sectioned nodes for comparison would have improved the sensitivity and specificity of PET/CT. Finally, the time from PET/CT to surgery in our study was too long in some cases, which might have influenced the results.

Study 2: The LN dissection was performed to the level of the ureter, crossing the common iliac vessels, as a standard procedure. This might have had an influence on both LN and SN detection. However, the number of LN per patient in our study was high, indicating a satisfactory PLND. The number of previous TURB might be associated with an altered lymphatic drainage and nodal metastases, possibly to different nodal sites. It was sometimes difficult to identify the exact anatomical location of the LNs dissected, especially in the proximity of the division of common iliac vessels in to external and internal iliac arteries. Therefore, we also used the entire left and right sides of the lymph node stations for analysis of the SNB with improved but not satisfactory results.

Study 3: The majority of patients had poorly-differentiated locally advanced UBC, so the observations reported here are not applicable to all stages and grades of UBC. Another limitation is that MI is examined by a single M2-macrophage specific marker (CD163). The detection of macrophages, both M1 (Ly6C, CD68 and MAC387) and M2 (CD206, ERG2 and c-Myc) with other specific markers may improve the understanding of MI and macrophage phenotype in UBC.

Study 4: Factors that might influence the results of IHC staining, such as the antibodies, dilution, fixation, and specimen handling that are applied might affect the results. The cut-off value for immunostaining of biomarkers is another important factor that might explain disparities between the findings obtained in previous studies. The biomarkers were studied on a small group of patients with locally advanced UBC. Furthermore, most markers could not be analyzed in all patients and particularly immunostaining for p53 and Ki 67 were lacking in more than 10% of the cases. The differences between the whole section and TMA results of immunohistochemistry should be taken into consideration as it might affect the results of immunostaining in UBC

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