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Clinical-Bladder cancer

Molecular subtypes applied to a population-based modern cystectomy series do not predict cancer-specific survival

Petter Kollberg, M.D.[a,b,*], Gunilla Chebil, M.D.[c], Pontus Eriksson, Ph.D.[d], Gottfrid Sjödahl, Ph.D.[b], Fredrik Liedberg, M.D., Ph.D.[a,b]

[a] *Department of Urology, Skåne University Hospital, Malmö, Sweden*
[b] *Department of Translational Medicine, Lund University, Malmö, Sweden*
[c] *Unilabs Pathology Department, Helsingborg, Sweden*
[d] *Department of Clinical Sciences, Division of Oncology and Pathology, Lund University, Lund, Sweden*

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Abstract

Objectives: To investigate the preoperative prognostic value of molecular subtypes in relation to clinical information, histopathological findings, and molecular markers for patients with bladder cancer treated with radical cystectomy.

Patients and methods: After standard preoperative staging, a population-based cohort of 519 patients underwent radical cystectomy between 2006 and 2011. Following pathological review of all transurethral resection of bladder tumor specimens, tissue microarrays were constructed, and RNA was extracted from formalin-fixed tissue blocks. Immunohistochemistry (IHC) was performed using markers suggested to be relevant for prognosis (ZEB2, CCND1, CD3, CD68, CDH3, HER3, KRT14, CDKN2A(p16), TP63, FGFR3, EPCAM, GATA3, FOXA1, ERBB2, and EGFR). IHC- and gene-expression-based molecular classification was also conducted. Univariate and multivariate Cox proportional hazards regression were used for survival analyses.

Results: Clinical T3 stage (Hazard Ratio [HR] 1.6, Confidence Interval [CI] 1.1−2.3), hydronephrosis (HR 1.7, CI 1.2−2.3), lymphovascular invasion (LVI) (HR 2.6, CI 1.9−3.6), extensive necrosis (HR 1.6, CI 1.1−2.5), and CD68/CD3-ratio >1 (HR 1.3, CI 1.1−1.5) in the transurethral resection of bladder tumor specimen was associated with worse cancer-specific survival (CSS) and progression-free survival (data not shown). In multivariate analysis, higher clinical T stage (HR 1.3, CI 1.1−1.7; $P = 0.007$) and presence of LVI (HR 2.4, CI 1.7−3.5; $P = 1.8 \times 10^{-6}$) were associated with worse CSS, whereas only LVI was associated with progression-free survival. Molecular subtypes (assessed by Lund taxonomy and the Consensus molecular subtypes of muscle-invasive bladder cancer) and published single IHC markers were not associated with survival.

Conclusions: In the present large population-based cystectomy series, LVI and clinical stage were independently associated with CSS. However, molecular subtypes determined by global gene expression showed no such association with CSS according to either the Consensus molecular subtypes of muscle-invasive bladder cancer or Lund taxonomy.

Keywords: Bladder cancer; Radical cystectomy; Molecular subtypes; Survival

1. Introduction

Despite improved outcomes in most forms of cancer, no improvement in survival has been noted for bladder cancer [1]. Indeed, for invasive bladder cancer treated with radical cystectomy (RC), long-term recurrence-free survival has remained at the same level for decades [2,3]. Stage and grade provide prognostic information, although 2 different systems are used to grade urothelial cancer: WHO 2004/2016 and WHO 1973 [4,5]. Most patients treated with RC

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*Corresponding author. Tel.: +46730365002.
E-mail address: petter.kollberg@skane.se (P. Kollberg).

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are high grade/G3. Furthermore, preoperative classification of clinical tumor stage in this disease leads to understaging in more than 40% of patients [6]. Other clinical and histopathological parameters that offer prognostic information prior to RC are hydronephrosis and lymphovascular invasion (LVI) [7,8]. In transurethral resection of bladder tumor (TURB) specimens, it has also been noted that variant histology [9], presence of necrosis [10], and host immune response measured as ratio of CD68+ to CD3+ tumor-infiltrating immune cells [11] are independently associated with survival in the setting of RC. However, thus far, none of these suggested biomarkers have been validated, and hence they currently have no impact on management of bladder cancer prior to RC. Also, it has been proposed that many single immunohistochemistry (IHC) markers might help improve outcome prediction, although to date no individual markers of this type have been recognized or are in routine clinical use [12].

In recent years, gene-expression-based molecular classification of bladder cancer has increased our understanding of bladder cancer diversity [13–17]. The molecular classification systems published are largely in agreement but identify different numbers of molecular classes [18]. The two most detailed classification systems (the Lund taxonomy (which classifies also non–muscle-invasive bladder cancer) and Consensus molecular subtypes of muscle-invasive bladder cancer) each identify subtypes that show prognostic value [19,20], although the clinical importance and prognostic value these systems in consecutive patients has not yet been determined. Therefore, we investigated the prognostic value of molecular subtypes in the setting of RC for invasive disease in a large population-based cystectomy series after adjusting for preoperative clinical variables and histological features in TURB specimens.

2. Patients and methods

This population-based study initially included 519 consecutive RC patients at four hospitals in the Southern Healthcare Region in Sweden 2006-2011. Ninety-four of these subjects were excluded due to insufficient formalin-fixed paraffin-embedded (FFPE) tissue for tissue microarray (TMA) construction. An additional 28 patients were excluded because surgery was done for clinically non-invasive disease or cystectomy performed with palliative intent only. Thus 397 patients were analyzed regarding survival, clinical and pathological variables, and IHC data (Fig. 1). For 111 of those patients, tissue was lacking after TMA construction or RNA extractions gave a low yield, and hence gene expression data were available for 286 of the 397 cases. Three cases were excluded because the subtype was infiltrated or could not be determined for both mRNA and IHC classification, which left a total of 283 cases with molecular subtype classification (Fig. 1).

After standard preoperative staging by computed tomography (CT) of the abdomen and chest, patients underwent RC including extended lymph node dissection, with the exact proximal limit set according to the preference of the surgeon. The preoperative and perioperative/postoperative patient characteristics are given in Tables 1a and 1b,

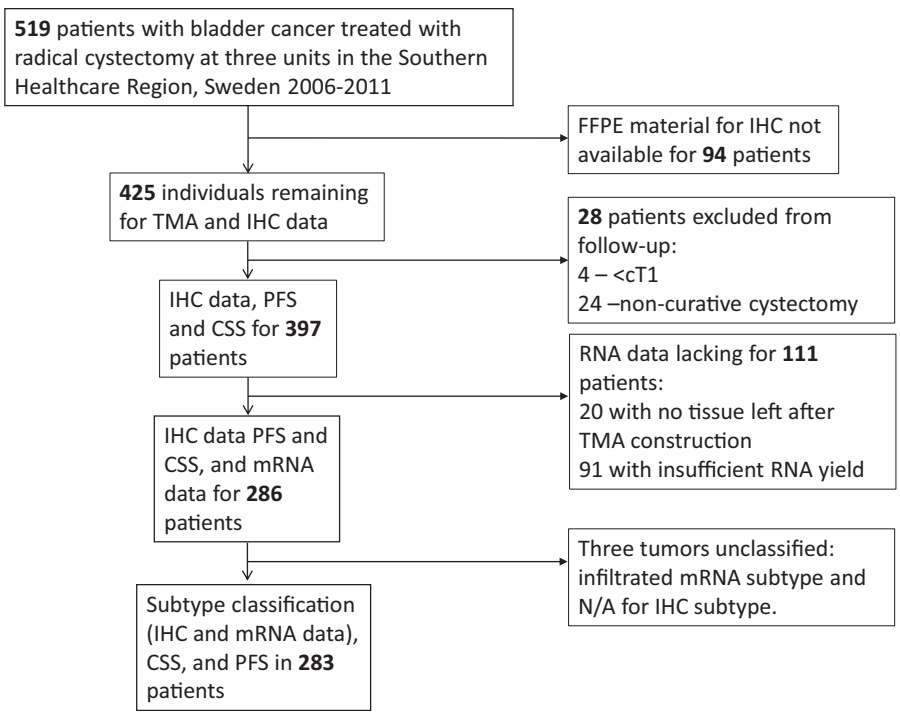

Fig. 1. Description of the cohort and how the tumor specimens were retrieved. CSS = cancer-specific survival; IHC = immunohistochemistry; PFS = progression-free survival; TMA = tissue microarray.

Table 1a
Preoperative characteristics of the 397 patients

Men	313	(79%)
Women	84	(21%)
WHO 1999[a] grade based on TURB specimen		
WHO II	39	(10%)
WHO III	343	(86%)
N/A	15	(3.8%)
Clinical T-stage		
T1	66	(17%)
T2	172	(43%)
T3	116	(29%)
>T3	34	(9%)
Tx	9	(2%)
Clinical N-stage		
N0	370	(93%)
N+	18	(5%)
Nx	9	(2%)
Hydronephrosis		
Yes	105	(26%)
No	292	(74%)
Cis in TURB specimen		
Yes	107	(26%)
No	288	(73%)
N/A	2	(0,5%)
LVI in TURB specimen		
Yes	124	(31%)
No	266	(67%)
N/A	7	(2%)
Necrosis in TURB specimen		
None	178	(45%)
Limited	145	(37%)
Extensive	71	(18%)
N/A	3	(1%)
Variant histology		
Yes	37	(9%)
No	360	(91%)
Preoperative chemotherapy		
Neoadjuvant (3 courses)	17	(4%)
Induction chemotherapy	20	(5%)
None	360	(91%)

[a] WHO 1999 grades II and III are equivalent to high grade in WHO 2004/2016.

respectively. An experienced uro-pathologist (GC) re-evaluated the histological sections from the TURB specimens with regard to stage, grade, presence of carcinoma in situ, variant histology including squamous cell differentiation (defined as presence of keratin or desmosomes), apoptosis, presence of necrosis, and LVI. Tumor cell necrosis was defined as the presence of at least 5 necrotic cells clustered together in a group, and apoptosis was defined as fewer than 5 apoptotic cells. The extent of tumor necrosis was semiquantitatively assessed at low magnification and recorded as none, limited (≤10% of the tumor area), or extensive (>10% of the tumor area) based on the histological evaluation of all available tumor blocks. Presence of necrosis on gross examination was not included in the assessment [21]. LVI was defined as tumor cells within or invading a vessel, but no immunohistochemical marker was used to identify the endothelium.

Table 1b
Perioperative and postoperative patient, tumor (pathological tumor stage in the cystectomy specimen), and treatment characteristics in the study cohort

Cystectomy centre		
Hospital 1	$n = 140$	(35%)
Hospital 2	$n = 137$	(35%)
Hospital 3	$n = 34$	(9%)
Hospital 4	$n = 86$	(22%)
Cystectomy indication		
Primary MIBC	$n = 275$	(69%)
Primary NMIBC	$n = 32$	(8%)
Progression from NMIBC or treatment-refractory NMIBC	$n = 88$	(22%)
Salvage cystectomy after curative radiation	$n = 2$	(0.5%)
Extent of lymphadenectomy		
None	$n = 14$	(4%)
Limited	$n = 19$	(5%)
To the ureteric crossing	$n = 205$	(52%)
To the aortic bifurcation	$n = 159$	(40%)
Nerve-sparing cystectomy		
yes	$n = 23$	(6%)
no	n = 369	(93%)
N/A	$n = 5$	(1%)
Urinary diversion		
Ileal conduit	$n = 293$	(74%)
Lundiana pouch	$n = 49$	(12%)
Orthotopic neobladder	$n = 54$	(14%)
Other	$n = 1$	(0%)
Cystectomy type		
Open radical cystectomy	$n = 384$	(97%)
Robotic-assisted cystectomy	$n = 13$	(3%)
pT stage based on cystectomy specimen		
pT0	$n = 43$	(11%)
pTa	$n = 15$	(4%)
pTcis	$n = 31$	(8%)
pT1	$n = 42$	(11%)
pT2a	$n = 23$	(6%)
pT2b	$n = 46$	(12%)
pT3a	$n = 79$	(20%)
pT3b	$n = 72$	(18%)
pT4a	$n = 40$	(10%)
pT4b	$n = 6$	(2%)
pN stage based on cystectomy specimen		
pN0	$n = 282$	(71%)
pN1	$n = 36$	(9%)
pN2	$n = 50$	(13%)
pN3	$n = 16$	(4%)
pNx	$n = 13$	(3%)
Positive margin		
No	$n = 371$	(93%)
Yes	$n = 18$	(5%)
suspicious	$n = 7$	(2%)
N/A	$n = 1$	(0%)
Number of lymph nodes		
Median	29	
IQR	20–40	
Mean	30	
Adjuvant chemotherapy		
Yes	$n = 34$	(9%)
No	$n = 363$	(91%)

MIBC = muscle-invasive bladder cancer; NMIBC = non–muscle-invasive bladder cancer.

For each tumor, 2 representative 1.0-mm cores were obtained from FFPE tissue blocks, and TMA blocks were constructed. RNA extraction was performed on 10-μm sections from macro-dissected tissue areas located close to the TMA cores (see Supplementary material and methods). Following TMA construction, remaining tumor-rich areas with the same features as described above were identified and marked on the H and E slides. Marked areas were isolated by cutting into the paraffin block with an awl, and sections (4−10 × 10 μm) were obtained. Fewer sections were taken if the hollowed out area was large, and conversely, more sections were taken if the area was small. The isolated area in each section was carefully collected and used as starting material for RNA extraction.

Tissue flakes were deparaffinized in xylene and rehydrated by serial incubation with ethanol/xylene mixture and pure ethanol. Rehydrated tissue was dried and subjected to proteinase K digestion at 56˚C overnight (Roche HighPure RNA extraction kit for FFPE tissue). The lysate was split for RNA and DNA extraction, and nucleic acids were extracted by on-column DNaseI digestion according to the standard protocol supplied with the Roche HighPure kit. Final RNA samples were eluted in 25 μl of elution buffer, and concentrations were determined using Nanodrop before storage at −80˚C.

Molecular classification based on global RNA profiling was performed according to the Lund taxonomy and the Consensus molecular classification of muscle-invasive bladder cancer, and is further described in Supplementary material and methods. The full gene expression data are publicly available at Gene Expression Omnibus under accession number GSE83586.

We used several antibodies that have been reported to be of prognostic value after RC when assessed in the TURB specimen (Supplementary material and methods): ZEB2, CCND1, CD3, CD68, CDH3, HER3, KRT14, CDKN2A (p16), TP63, FGFR3, EPCAM, GATA3, FOXA1, ERBB2, and EGFR.

2.1. Statistics

Oncological outcomes were calculated as time from cystectomy to recurrence (progression-free survival [PFS]) and time to death from bladder cancer (cancer-specific survival [CSS]). Disease recurrence was defined as radiological or clinical tumor recurrence occurring locally in the cystectomy bed or regional lymph nodes, or as distant metastasis. Survival was visualized using Kaplan-Meier curves. Median follow-up time was 69 months (interquartile range 46−92 months). At the end of follow-up, 192 patients were alive, 150 had succumbed to bladder cancer, and 55 had died of other causes. We applied univariate Cox regression to analyze associations between CSS/PFS and clinical variables, histopathological factors, individual immunohistochemical markers suggested to predict survival, and molecular subtypes according to the Lund taxonomy and Consensus molecular subtypes of MIBC-system. A multivariate model was created using significant prognostic factors from the univariate analyses. P values <0.05 were considered significant. All statistical calculations were performed with R version 3.1.1.

3. Results

Characteristics of the patients, tumors, and treatment are outlined in Tables 1a and 1b. The median age in this population-based cohort was 68.6 years (interquartile range 61.1−74.5), and 84 patients (21%) were females. Of the assessed preoperative clinical and histopathological factors, hydronephrosis occurred in 105 individuals (26%), LVI in 124 (31%), and variant histology in 37 (9%). Seventy-one patients (15%) received perioperative chemotherapy, either as neoadjuvant/induction treatment (Table 1a) or in the adjuvant setting (Table 1b). The majority of the patients (92%) underwent extended pelvic lymphadenectomy.

Worse outcome was observed for patients with higher tumor stage, hydronephrosis, LVI, necrosis, or high ratio between tumor-associated macrophages (TAM-CD68 positive) and tumor-infiltrating lymphocytes (TIL-CD3 positive; Fig. 2). Thus, clinical stage T3 was associated with poorer CSS (HR 1.62, 95% CI 1.12−2.34; P = 0.01) compared to ≤T2 disease. Also presence of hydronephrosis (HR 1.65, 95% CI 1.18−2.32; P = 0.003), LVI (HR 2.62, 95% CI 1.89−3.62; $P = 1.7 \times 10^{-9}$), necrosis (extensive vs. none or limited) (HR 1.64, 95% CI 1.07−2.50; P = 0.02), and a high TAM/TIL ratio (HR 1.28, 95% CI 1.01−1.54; P = 0.008) was associated with increased risk of bladder cancer death (Table 2). Similar associations were noted for PFS (Supplementary Table 1).

Survival (CSS/PFS) was not affected by abnormal expression of single biomarkers, with the exception of high FOXA1 expression, which was associated with improved CSS (HR 0.86, 95% CI 0.74−0.99; P = 0.04) but not PFS or Over-all Survival (Supplementary Table 2 and 4). Similarly, molecular subtypes based on global gene expression according to the Lund taxonomy and Consensus molecular Subtypes of MIBC-system were not associated with survival (Table 2, Fig. 3).

In a multivariate model for CSS, clinical T stage and presence of LVI were independently associated with poorer survival. Presence of LVI was also independently associated with shorter PFS (HR 2.05, CI 1.45−2.91; $P = 4.7 \times 10^{-05}$), whereas tumor stage was not.

4. Discussion

This study covered a large population-based cystectomy series that included molecular subtypes and prognosis in relation to available clinical and histopathological prognostic factors. We found that the 2 global gene-expression-based classifiers we evaluated (the Lund taxonomy and the Consensus molecular Subtypes of MIBC-system) had no

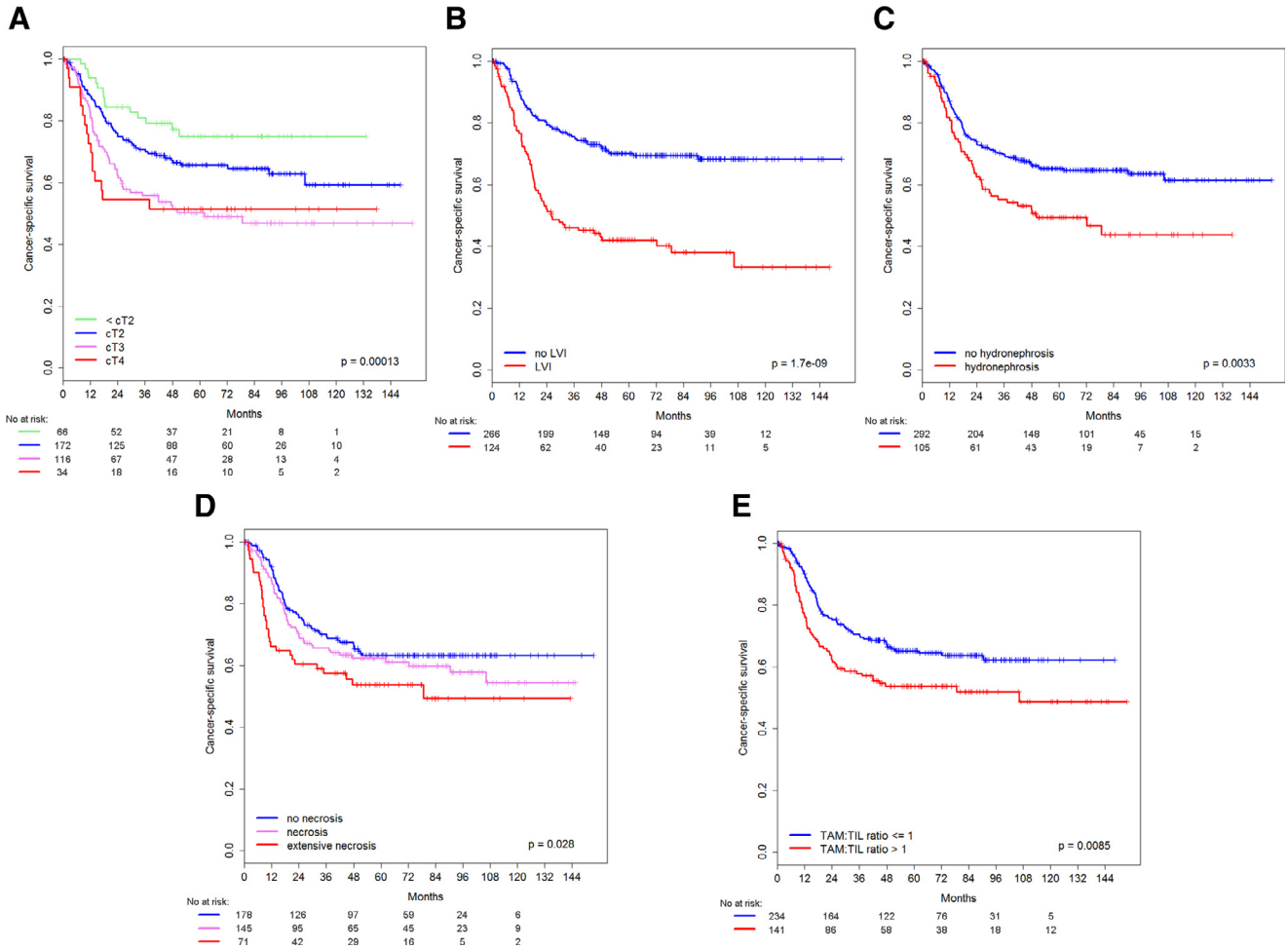

Fig. 2. Cancer-specific survival stratified by clinical stage (A), LVI (B), hydronephrosis (C), necrosis (D), and TAM/TIL-ratio (E).

prognostic ability. LVI and clinical stage were the only preoperative prognostic factors that were independently associated with CSS.

Molecular subtype classification of bladder cancer is currently attracting increased attention, and several systems have been suggested [22]. Furthermore, it has been claimed that molecular subtypes are not only of prognostic value in invasive bladder cancer [23,24], but also provide predictive information regarding response to neoadjuvant chemotherapy [24,25]. In the present study, 2 different classification systems indicated that none of the subtypes were of prognostic value, which disagrees with previous reports [23,24]. There may be plausible explanations for the conflicting findings. Our investigation was population based and comprised of patients that were consecutively recruited over a period of 6 years, which represents a different setting compared to previously published reports [23,24]. The patients in our study underwent similar preoperative investigations that were in line with current guidelines, and they were selected for cystectomy and the surgery was performed in a standardized manner. For example, only 3% of our patients did not have a lymphadenectomy compared to 10% in the TCGA cohort [23]. Surgery was performed by a limited number of surgeons and with curative intent. Palliative cystectomies without curative intent were excluded from the present survival analyses. These circumstances might differ from those applied in a previous study that considered the prognostic value of molecular subtypes in patients sampled in 36 units and also in 10 patients with M1 disease [23]. As an endpoint, we used CSS rather than overall survival, which may also have influenced the outcome of the survival analyses. Furthermore, the 2 studies reporting independent prognostic value of molecular subtypes refrained from including relevant clinical information such as presence of LVI and hydronephrosis in the statistical analyses [23,24], which may have affected the outcome of those evaluations.

On the other hand, in the present series, use of exclusion criteria such as perioperative findings of disseminated disease or palliative cystectomy was to some extent subjective and may have generated a selection bias regarding the remaining patients, even if only 24 of 425 (6%) were excluded for such reasons. In short, it can be questioned whether those patients should have been excluded from the survival analysis. The aim of our investigation was to study all patients who underwent RC with curative intent and had no known spread of disease prior to the surgery.

Table 2
Univariate (above) and multivariate (below) Cox regression survival analysis of cancer-specific survival using clinical, histopathological, and individual IHC markers

Variables (univariate analysis)	Number	HR	Lower 95% CI	Upper 95% CI	Log-rank p	Z-dist p
Age (year included)	397	1.02	0.99	1.04	0.15	
Sex (female)	397	1.11	0.76	1.64	0.59	
TURB Grade (G3)	382	1.50	0.83	2.71	0.18	
TURB squamous (yes vs. no)	393	1.15	0.80	1.70	0.45	
TURB cis. (yes vs. no)	395	0.70	0.47	1.02	0.064	
TURB LVI (yes vs. no)	390	2.62	1.89	3.62	1.67E−09	
TURB necrosis (no necrosis ref.)	394				0.028	
Necrosis		1.18	0.82	1.70		0.37
Extensive necrosis		1.64	1.07	2.50		0.022
TURB apoptosis (yes vs. no)	389	1.24	0.81	1.91	0.32	
Variant histology (yes vs. no)	397	1.24	0.74	2.09	0.41	
Squam/Gland. Diff (yes vs. no)	397	0.81	0.47	1.38	0.43	
Clinical tumour stage (cT2 ref.)	388				1.3E−04	
<cT2		0.63	0.35	1.10		0.10
cT3		1.62	1.12	2.33		0.01
cT4		1.70	0.97	2.94		0.062
Clinical node status (N+ vs. N0)	388	1.57	0.82	2.98	0.17	
Hydronephrosis (yes vs. no)	397	1.65	1.18	2.32	3.3E−03	
Neoadjuvant MVAC (yes vs. no)	397	1.41	0.85	2.33	0.18	
Molecular subtypes						
LundTax 2017 (Basal/Squamous-like ref.)	283				0.46	
Urothelial-like		0.76	0.47	1.24		0.27
Genomically unstable		0.78	0.44	1.36		0.38
Mesenchymal-like		1.18	0.51	2.75		0.70
Small-cell/Neuroendocrine-like		0.53	0.23	1.23		0.14
TCGA subtype (Basal/Squamous ref.)	286				0.69	
Luminal-papillary		0.76	0.50	1.17		0.21
Luminal		0.69	0.36	1.32		0.27
Luminal-infiltrated		0.81	0.43	1.51		0.50
Neuronal		0.71	0.28	1.80		0.47
Consensus subtypes (Basal/Squamous ref.)	286				0.37	
Luminal-Papillary		0.64	0.38	1.08		0.095
Luminal-NS		0.83	0.46	1.50		0.55
Luminal-unstable		0.63	0.35	1.11		0.11
Stroma-rich		0.72	0.41	1.27		0.26
Neuroendocrine-like		0.42	0.13	1.36		0.15
IHC markers						
Immune cell ratio TAM (CD68):TIL (CD3)	375	1.28	1.07	1.54	0.0079	
ZEB2	367	1.28	0.96	1.71	0.092	
CCND1	394	1.16	0.97	1.38	0.11	
CD3	395	0.93	0.82	1.07	0.31	
CD68	397	1.07	0.92	1.24	0.40	
CDH3	390	0.99	0.84	1.18	0.95	
HER3	390	0.98	0.83	1.16	0.80	
KRT14	392	0.98	0.80	1.20	0.81	
p16	389	0.84	0.69	1.02	0.071	
TP63	392	0.99	0.94	1.04	0.62	
FGFR3	374	0.87	0.72	1.05	0.15	
EPCAM	381	0.97	0.78	1.19	0.74	
GATA3	389	0.96	0.82	1.12	0.62	
FOXA1	376	0.86	0.74	0.99	0.041	
ERBB2	389	1.01	0.85	1.21	0.87	
EGFR	372	1.08	0.89	1.32	0.43	
Variables (multivariate analysis)	**HR**	**Lower 95% CI**	**Upper 95% CI**	**Z-dist p**		
TURB LVI (yes vs. no)	2.41	1.68	3.45	1.75E−06		
Clinical tumour stage (< T2, T2, T3, T4)	1.36	1.09	1.71	6.7E−03		
Hydronephrosis (yes vs. no)	1.31	0.90	1.91	0.16		
Necrosis (none, limited, extensive)	0.92	0.71	1.19	0.53		
TAM:TIL ratio	1.19	0.94	1.49	0.14		
FOXA1 IHC	0.88	0.75	1.04	0.13		

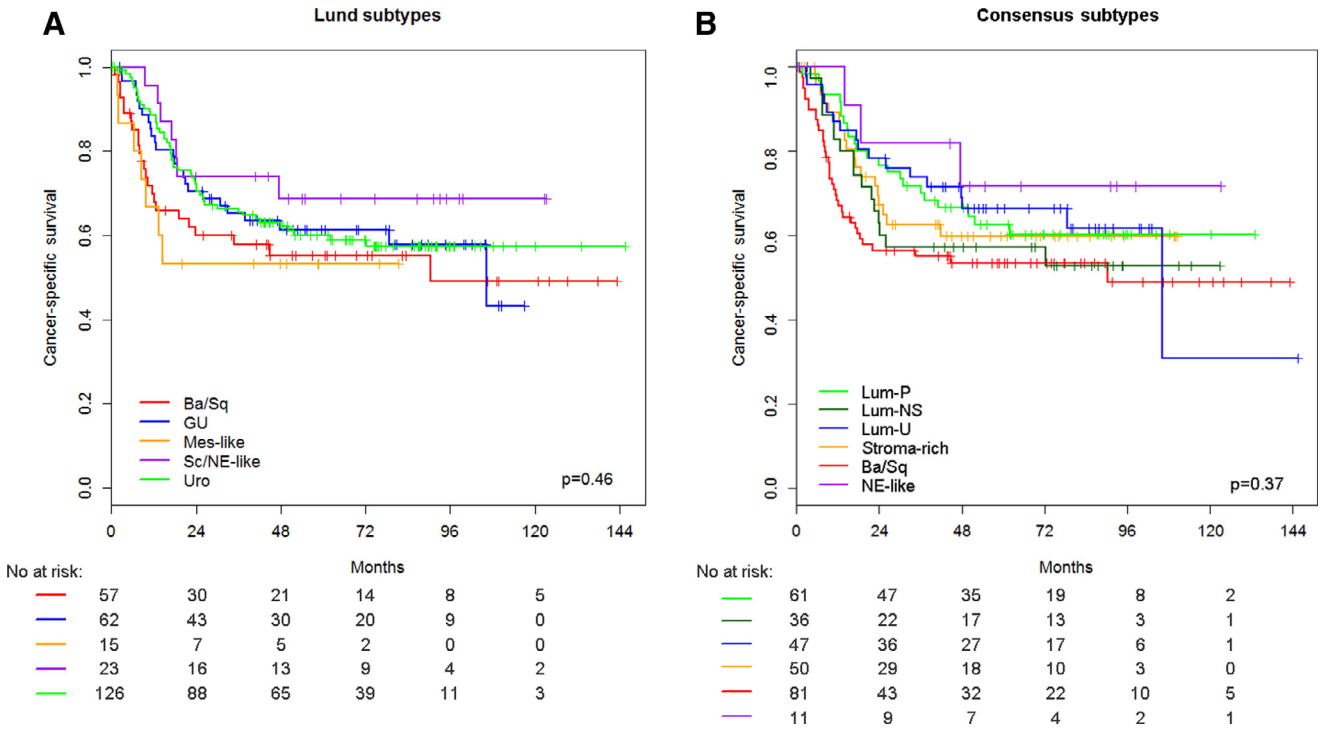

Fig. 3. Cancer-specific survival stratified by Lund Taxonomy molecular subtypes (A) and Consensus molecular subtypes of MIBC-system (B).

Furthermore, only information for clinical decision-making that was available before cystectomy (ie, TURB specimen data) was used to evaluate the outcomes. Today, FDG-PET-CT investigations and improved CT investigations are widely performed before RC [26], and hence unexpected intraoperative detection of advanced tumor status is now less common than at the time the patients in the current study underwent surgery. Among the 24 patients excluded for having undergone noncurative cystectomy prior to our survival analysis, it was technically possible to classify 21 according to subtype. In these 21 subjects, basal/squamous-like and urothelial-like each represented a proportion of approximately 33%, whereas these subtypes occurred at proportions of 20% and 45%, respectively, in the remaining cohort. Thus, according to both Seiler and co-workers and the TCGA [23,24], there was an enrichment of the basal/squamous-like subtype with worse outcome among the specified 21 patients excluded from our study. It is possible that the exclusion of these cases improved the survival outcome for the cohort that remained for survival analysis. Other limitations of the present investigation are associated with the selection mechanisms that were introduced in the cohort due to lack of tissue for RNA extraction and insufficient yield of RNA extraction in 95 individuals (Fig. 1), thus such lack of tissue and RNA also contributed to a reduction of the final cohort to 283/519 (55%) individuals. An additional drawback that decreases the generalizability of our findings is that only a minority of patients subjected to RC for muscle-invasive bladder cancer in Sweden did receive neoadjuvant chemotherapy during the investigated time-period.

The present data show that the highest HR for cancer-specific death in multivariate analysis was associated with LVI HR (2.4, CI 1.7−3.5) has also been observed in 2 previous studies that assessed LVI in TURB specimens [8,27]. Our finding suggesting that LVI is even more important than clinical stage prior to RC as a prognostic factor strengthens the indication for neoadjuvant chemotherapy prior to cystectomy in patients with LVI in TURB samples. It has also been proposed that LVI status be included in the TNM classification of bladder cancer, as is done for testicular cancer [28], and this conclusion is supported by our data.

In the clinical situation prior to RC, the presence of hydronephrosis has been shown to have an independent prognostic value in some evaluations [29] but not others [30]. Our study did not identify any independent prognostic value of preoperative hydronephrosis, despite standardized conditions. A colinearity between other prognostic variables such as non−organ-confined disease and lymph node positivity has been suggested [31], which makes it more difficult to prove causality and limits the prognostic use of hydronephrosis based on our results and the divergent outcomes in the literature.

The field of host immune response as a prognostic feature is of great interest in many forms of cancer, especially considering that the advent of checkpoint inhibitors has now provided a systemic treatment option also in bladder cancer [32]. Many immunologic and inflammatory markers have been suggested to have prognostic implications, but as of yet none of them are in clinical use [33]. We previously described a poor prognosis for patients with a high ratio

between tumor-associated macrophages and tumor-infiltrating lymphocytes [11]. In the present series, this ratio was prognostic in univariate analysis, but not in multivariate analysis when adjusting for clinical and histopathological information. To assess the density of tumor-infiltrating immune cells, whole section IHC is likely preferable over TMAs, and we cannot exclude that the full section ratio of TAM/TIL density would give different results.

Presence of necrosis in the cystectomy specimen has previously been associated with CSS [10]. In our study, assessment of necrosis in the TURB specimen indicated no impact on survival after adjusting for the other variables that were associated with survival in univariate analyses (Table 2). Of all the single IHC markers we used, only FOXA1-expression was associated with improved survival in univariate analysis. However, in multivariate analysis, no independent prognostic value could be verified. This observation disagrees with the results of another recent study [34], although that investigation adjusted only for female gender, non−organ-confined disease, and node positivity, and it assessed FOXA1 in the cystectomy specimen rather than the preoperatively obtained TURB specimen.

Thus, the findings of the present population-based study suggest that molecular subtype classification of TURB specimens prior to RC is of no prognostic use. Nevertheless, there are reports indicating that molecular subtype classification may provide predictive information regarding both chemo sensitivity [24] and response to checkpoint inhibition [35]. Such use of molecular subtypes to predict treatment response is currently being investigated in multiple settings and is also being compared with other potential response predictors, such as defects in DNA repair genes and oncogenic mutations associated with actionable targets like FGFR3 [36]. To be a successful and validated tool for predicting treatment response in invasive bladder cancer, second-generation biomarkers of this type must capture both the complexity of the biology of the molecular subtypes in the disease and the tumor microenvironment, and to some extent also tumor heterogeneity [37].

Supplementary materials

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.urolonc.2019.04.010.

References

[1] Jahnson S, Hosseini Aliabad A, Holmäng S, et al. Swedish National Registry of Urinary Bladder Cancer: no difference in relative survival over time despite more aggressive treatment. Scand J Urol 2016;50:14–20.
[2] Hautmann RE, de Petriconi RC, Pfeiffer C, et al. Radical cystectomy for urothelial carcinoma of the bladder without neoadjuvant or adjuvant therapy: long-term results in 1100 patients. Eur Urol 2012;61:1039–47.
[3] Stein JP, Skinner DG. Radical cystectomy for invasive bladder cancer: long-term results of a tandard procedure. World J Urol 2006;24:296–304.
[4] Moch H, Humphrey PA, Ulbright TM, et al. WHO classification of tumours of the urinary system and male genital organs. 4th ed. Lyon, France: WHO; 2016.
[5] Eds.. In: Mostofi FK, Sobin LH, Torloni H, eds. Histological typing of urinary bladder tumours, Geneva, Switzerland: World Health Organization; 1973.
[6] Svatek RS, Shariat SF, Novara G, et al. Discrepancy between clinical and pathological stage: external validation of the impact on prognosis in an international radical cystectomy cohort. BJU Int 2011;107:898–904.
[7] Eisenberg Manuel S, Boorjian Stephen A, Cheville John C, et al. The SPARC score: a multifactorial outcome prediction model for patients undergoing radical cystectomy for bladder cancer. J Urol 2013;190:2005–10.
[8] Abufaraj M, Shariat SF, Foerster B, et al. Accuracy and prognostic value of variant histology and lymphovascular invasion at transurethral resection of bladder. World J Urol 2018;36:231–40.
[9] Black PC, Brown GA, Dinney CP. The impact of variant histology on the outcome of bladder cancer treated with curative intent. Urol Oncol 2009;27:3–7.
[10] Hodgson A, Xu B, Satkunasivam R, et al. Tumor front inflammation and necrosis are independent prognostic predictors in high-grade urothelial carcinoma of the bladder. J Clin Pathol 2018;71:154–60.
[11] Sjödahl G, Lövgren K, Lauss M, et al. Infiltration of $CD3^+$ and $CD68^+$ cells in bladder cancer is subtype specific and affects the outcome of patients with muscle-invasive tumors. Urol Oncol 2014;32:791–7.
[12] Liedberg F, Anderson H, Chebil G, et al. Tissue microarray based analysis of prognostic markers in invasive bladder cancer: much effort to no avail? Urol Oncol. 2008;26:17–24.
[13] Lerner SP, McConkey DJ, Hoadley KA, et al. Bladder cancer molecular taxonomy: summary from a consensus meeting. Bladder Cancer 2016;2:37–47.
[14] Sjödahl G, Lauss M, Lovgren K, et al. A molecular taxonomy for urothelial carcinoma. Clin Cancer Res 2012;18:3377–86.
[15] Choi W, Czerniak B, Ochoa A, et al. Intrinsic basal and luminal subtypes of muscle-invasive bladder cancer. Nat Rev Urol 2014;11:400–10.
[16] Damrauer JS, Hoadley KA, Chism DD, et al. Intrinsic subtypes of high-grade bladder cancer reflect the hallmarks of breast cancer biology. Proc Natl Acad Sci USA 2014;111:3110–5.
[17] Cancer Genome Atlas Research Network. Comprehensive molecular characterization of urothelial bladder carcinoma. Nature 2014;507:315–22.
[18] Aine M, Eriksson P, Liedberg F, et al. On molecular classification of bladder cancer: out of one, many. Eur Urol. 2015;68:921–3.
[19] Marzouka NA, Eriksson P, Rovira C, et al. A validation and extended description of the Lund taxonomy for urothelial carcinoma using the TCGA cohort. Sci Rep 2018;8:3737.
[20] Kamoun A, de Reynies A, Allory Y, et al. The bladder cancer molecular taxonomy group. the consensus molecular classification of muscle-invasive bladder cancer. BioRxiv488460; doi: https://doi.org/10.1101/488460
[21] Langner C, Hutterer G, Chromecki T, et al. Patterns of invasion and histological growth as prognostic indicators in urothelial carcinoma of the upper urinary tract. Virchows Arch 2006;448:604–11.
[22] Black PC. Seeking the molecular truth in bladder cancer: biology=genome×$(\text{transcriptome})^2$. Eur Urol 2017;72:366–7.
[23] Robertson AG, Kim J, Al-Ahmadie H, et al. Comprehensive molecular characterization of muscle-invasive bladder cancer. Cell 2017;171:540–56.
[24] Seiler R, Ashab HAD, Erho N, et al. Impact of molecular subtypes in muscle-invasive bladder cancer on predicting response and survival after neoadjuvant chemotherapy. Eur Urol. 2017; 72: 544−54.
[25] Choi W, Porten S, Kim S, et al. Identification of distinct basal and luminal subtypes of muscle-invasive bladder cancer with different sensitivities to frontline chemotherapy. Cancer Cell 2014;25:152–65.

[26] Kollberg P, Almquist H, Bläckberg M, et al. [(18)F]Fluorodeoxyglucose - positron emission tomography/computed tomography improves staging in patients with high-risk muscle-invasive bladder cancer scheduled for radical cystectomy. Scand J Urol 2015;49:296–301.
[27] Kim HS, Kim M, Jeong CW, et al. Presence of lymphovascular invasion in urothelial bladder cancer specimens after transurethral resections correlates with risk of upstaging and survival: a systematic review and meta-analysis. Urol Oncol 2014;32:1191–9.
[28] Mathieu R, Lucca I, Rouprêt M, et al. The prognostic role of lymphovascular invasion in urothelial carcinoma of the bladder. Nat Rev Urol 2016;13:471–9.
[29] Bartsch GC, Kuefer R, Gschwend JE, et al. Hydronephrosis as a prognostic marker in bladder cancer in a cystectomy-only series. Eur Urol 2007;51:690–7.
[30] Thrasher JB, Frazier HA, Robertson JE, et al. Clinical variables which serve as predictors of cancer-specific survival among patients treated with radical cystectomy for transitional cell carcinoma of the bladder and prostate. Cancer 1994;73:1708–15.
[31] Stimson CJ, Cookson MS, Barocas DA, et al. Preoperative hydronephrosis predicts extravesical and node positive disease in patients undergoing cystectomy for bladder cancer. J Urol 2010;183:1732–7.
[32] Bellmunt J, de Wit R, Vaughn DJ, et al. KEYNOTE-045 investigators. pembrolizumab as second-line therapy for advanced urothelial carcinoma. N Engl J Med 2017;376:1015–26.
[33] Masson-Lecomte A, Rava M, Real FX, et al. Inflammatory biomarkers and bladder cancer prognosis: a systematic review. Eur Urol 2014;66:1078–91.
[34] Reddy OL, Cates JM, Gellert LL, et al. Loss of FOXA1 drives sexually dimorphic changes in urothelial differentiation and is an independent predictor of poor prognosis in bladder cancer. Am J Pathol 2015;185:1385–95.
[35] Mariathasan S, Turley SJ, Nickles D, et al. TGFβ attenuates tumour response to PD-L1 blockade by contributing to exclusion of T cells. Nature. 2018;554:544–8.
[36] Vlachostergios PJ, Faltas BM. The molecular limitations of biomarker research in bladder cancer. World J Urol 2018. https://doi.org/10.1007/s00345-018-2462-9: [Epub ahead of print] PubMed PMID: 30171455.
[37] Jakobsson L, Chebil G, Marzouka NA, et al. Low frequency of intratumor heterogeneity in bladder cancer tissue microarrays. Bladder Cancer 2018;4:327–37.