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ARTICLE



Prediction of neo-adjuvant chemotherapy response in bladder cancer: the impact of clinical parameters and routine biomarkers

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ABSTRACT

Purpose: To investigate the role of clinical parameters and immunohistochemical (IHC) biomarkers in their feasibility to predict the effect of neo-adjuvant chemotherapy (NAC) in patients with muscle-invasive urothelial bladder cancer (MIBC).

Materials and methods: The first 76 consecutive patients with MIBC treated with NAC and radical cystectomy in two University hospitals in Finland between 2008 and 2013 were chosen for this study. After excluding patients with non-urothelial cancer, less than two cycles of chemotherapy, no tissue material for IHC analysis or non-muscle-invasive bladder cancer in re-review, 59 patients were included in the final analysis. A tissue microarray block was constructed from the transurethral resection samples and IHC stainings of Ki-67, p53, Her-2 and EGFR were made. The correlations between histological features in transurethral resection samples and immune-histochemical stainings were calculated. The associations of clinicopathological parameters and IHC stainings with NAC response were evaluated. Factors affecting survival were estimated.

Results: The complete response rate after NAC was 44%. A higher number of chemotherapy cycles was associated with better response to neo-adjuvant chemotherapy. No response to neo-adjuvant chemotherapy and female gender was associated with decreased cancer-specific survival. The IHC stainings used failed to show an association with neo-adjuvant chemotherapy response and overall or cancer specific survival.

Conclusions: Patients who do not respond to neo-adjuvant chemotherapy do significantly worse than responders. This study could not find clinical tools to distinguish responders from non-responders. Further studies preferably with larger cohorts addressing this issue are warranted to improve the selection of patients for neo-adjuvant chemotherapy.

Abbreviations: MIBC: Muscle invasive bladder cancer; NAC: Neo-adjuvant chemotherapy; RC: Radical cystectomy; TURBT: Transurethral resection of bladder tumor; OS: Overall survival; CSS: Cancer specific survival; TMA: Tissue microarray; GC: Gemcitabine Cisplatin; MVAC: Methotrexate Vinblastine Doxorubicin (Adriamycin) Cisplatin

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Muscle-invasive bladder cancer; neo-adjuvant chemotherapy; cystectomy; immunohistochemistry

Introduction

Muscle invasive bladder cancer (MIBC) is a disease with poor prognosis. Among patients with MIBC treated with radical cystectomy (RC) and pelvic lymphadenectomy alone, the 5-year overall survival (OS) is between 25 and 77%, decreasing with more advanced stage [1,2]. Platinum-based combination neo-adjuvant chemotherapy (NAC) prior to RC improves the absolute survival of patients at 5 years by 5–7% as compared to those receiving cystectomy alone [3,4]. The rationale to use NAC is to eradicate micro-metastasis and to downstage the primary tumor. Down-staging to non-muscle invasive disease and especially complete pathological response (pT0) is

associated with improved overall survival (OS) [5]. However, in the case of chemo-resistant tumors, NAC predisposes patients to possible adverse effects and increases the risk of cancer progression prior to surgery.

To date, there are no clinically validated methods to interrogate the chemo-sensitivity of MIBC prior to treatment. It has been shown that a low neutrophil-to-lymphocyte ratio is associated with better cancer-specific survival (CSS) and OS in patients receiving NAC prior to radical cystectomy [6]. In addition, certain genetic alterations (e.g., ERBB2 missense mutations and p53 status) are associated with improved NAC response [7].

In our study we describe the first 76 consecutive patients that were treated in Helsinki and Turku University

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Hospitals with NAC prior to open RC with bilateral pelvic lymphadenectomy.

Our objective was to investigate whether any pretreatment clinical variables and/or well established immune-histochemical (IHC) markers, namely p53, Ki-67, ERBB2 (Her-2) and EGFR, were associated with NAC response, OS or CSS. These markers were chosen as they are widely used in clinical practice of cancer diagnosis and treatment.

Materials and methods

Patients

Altogether the first 76 consecutive patients who underwent NAC prior to open RC for MIBC were included retrospectively in the study. Patients were treated in Helsinki University Hospital (2010–2013) and Turku University Hospital (2008–2013). After excluding patients with non-urothelial cancer (four small cell carcinomas, one squamous cell carcinoma), two patients with less than two cycles of NAC, four patients without muscle invasive carcinoma in the re-evaluation of the urologists (T.M. and P.T.) and six patients with insufficient tissue material for tissue microarray (TMA), there were $n=59$ patients eligible for the final analysis of both clinical data and IHC stainings (Figure 1). The study has been approved by the ethics committee of Helsinki University Hospital (169/13/03/02/2013).

Diagnosis, treatment and follow-up

The diagnosis of MIBC was made by performing Transurethral resection of bladder tumor, TURBT). RC in males included the removal of urinary bladder, prostate and seminal vesicles. In females the uterus, ovaries, fallopian tubes and the anterior part of the vaginal wall was removed together with the bladder. Bilateral pelvic lymphadenectomy

was performed according to institution standards. Included with every patient's lymphadenectomy were at least areas of obturator fossae, external iliac vessels medial to genitofemoral nerve and internal iliac vessels up to iliac crossing.

Complete pathological response to treatment was defined as no detection of cancer in the cystectomy specimen (T0) and no detection of metastasis in the lymph nodes (N0). Partial response was defined as detection of non-muscle invasive cancer in the bladder (Ta, T1 or Tis) and no lymph node metastasis. Patients having muscle invasive (T2) or locally advanced (T3–T4) cancer or lymph node metastasis were defined as non-responders.

After cystectomy, follow-up was according to institutional guidelines, most commonly at 3 months postoperatively, semiannually for 2 years and annually thereafter. FU visits included physical examination, laboratory tests and cross-sectional imaging.

Construction of tissue microarrays (TMA)

Two urologists (TM, PT) reviewed the TURBT and cystectomy slides and selected corresponding areas in the TURBT slides for tissue microarray (TMA) construction. TMA was constructed by punching three 1.0 mm cores from each of the selected TURBT block presenting BC.

Immunohistochemistry and marker scoring

For the detection of protein expression, TMA slides were first pretreated for antigen retrieval in Ventana Ultra CC1 for 64 min at 98 °C (Ki-67, p53 and c-erbB2) or Target retrieval solution (Agilent DAKO, Glostrup, Denmark) for 24 min at 98 °C (EGFR). The antibody clones, dilutions and incubation times are shown in Appendix Table A. The detection kits with chromogens were Ventana Ultraview DAB Detection kit (Ki-67, p53, c-erbB2) and Dako REAL EnVision Detection

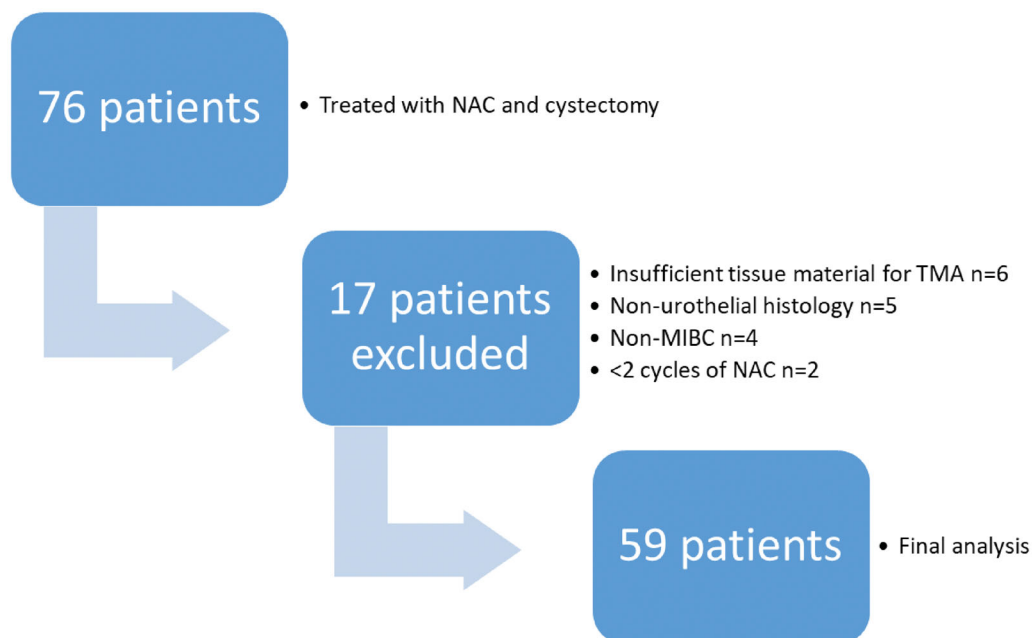


Figure 1. Inclusion of patients for analysis.

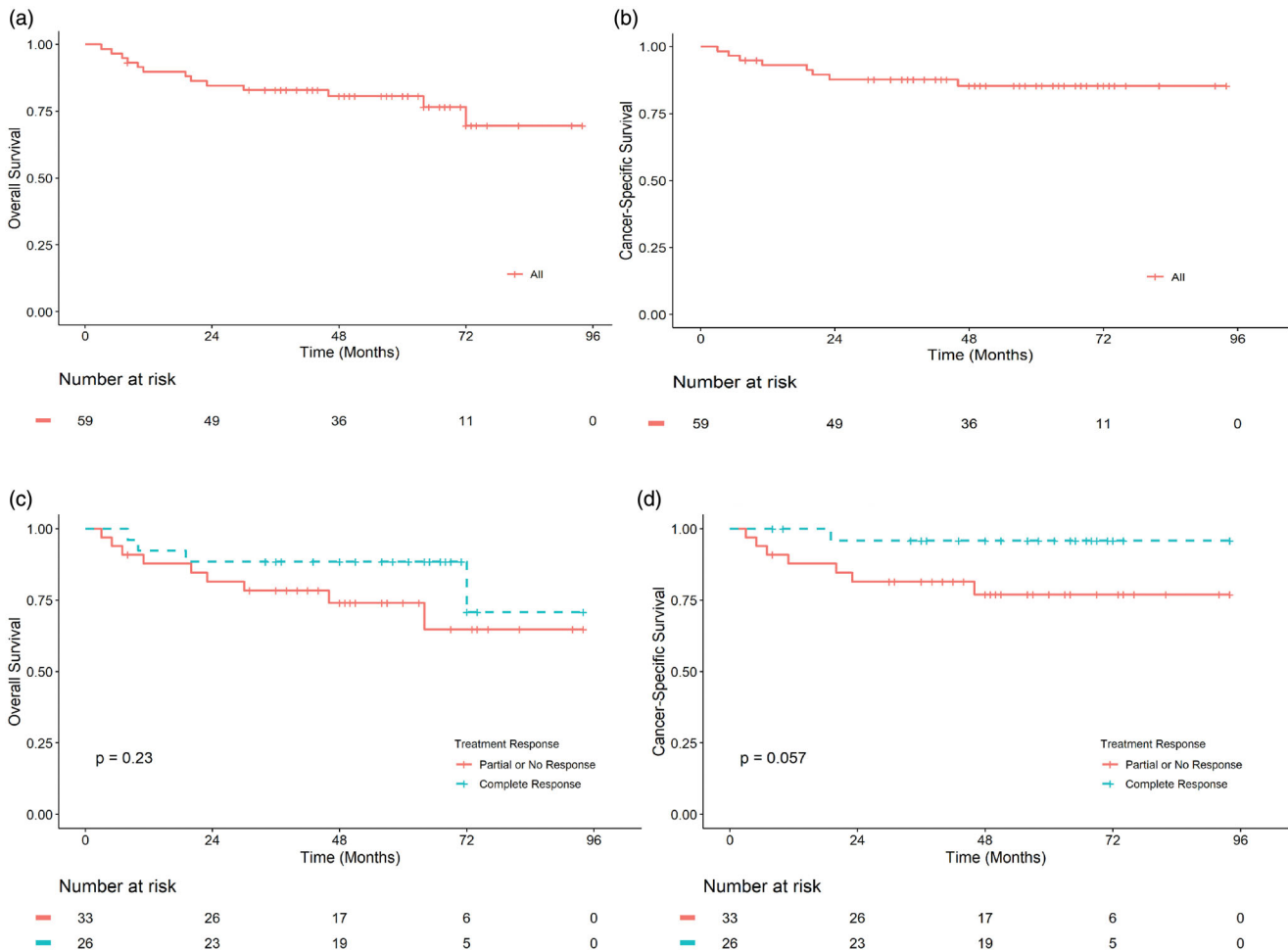


Figure 2. OS (a and c) and CSS (b and d) in months from TURBT of urothelial MIBC patients treated with NAC and radical cystectomy in Helsinki and Turku University Hospitals between 2008 and 2013 ($n = 59$). The whole patient cohort (a and b), according to treatment response (c and d). TURBT, Transurethral resection of bladder tumour; MIBC, Muscle invasive bladder cancer; NAC, Neo-adjuvant chemotherapy; Complete response, pT0N0; Partial response, < pT2N0; No response, \geq pT2 and/or N+.

System (EGFR), and the reactions were conducted in Ventana Benchmark ULTRA or LabVision Autostainer, respectively.

For the scoring of p53 IHC, TMA cores were considered positive if $>10\%$ of all cells were stained positive, and negative if $\leq 10\%$ of all cells were stained positive as described by Shariat et al. [8]. Ki-67 scores were calculated by counting the number of positively stained cells out of 500 cancer cells. The received values were then dichotomized by mean value (62.8). Her-2 and EGFR scoring was done by comparing no staining with any staining.

The highest count of positively stained cells in any of the three punches was used in the statistical analysis.

Statistical analysis

All of the statistical analyses were performed with SPSS v.22 (IBM, Chicago, IL). The correlations between histological features in TURBT samples and IHC stainings were calculated by Pearson Chi-square or Fisher's exact test where less than five units were available. The associations of clinicopathological parameters and IHC marker status with NAC response were evaluated by binary logistic regression. Univariate Cox

proportional hazards regression analysis was used to estimate the factors affecting OS and CSS.

Results

The demographics of patient cohort are described in Table 1. Most of the patients were male (83%) and the median age was 65 years. Both Helsinki and Turku treated an equal number of patients. Almost three out of four (73%) of the patients had a high grade (WHO grade 3) urothelial carcinoma in the initial diagnosis made with TURBT, but only 27% had lympho-vascular invasion (LVI). Ten patients (17%) had primarily concurrent carcinoma *in situ* (data not shown). Gemcitabine and cisplatin (GC) was the chosen chemotherapy regime for 95% of the patients, and half (49%) received two or three cycles and the remainder had four or more cycles. During the median follow-up time of 49 months, eight (14%) patients succumbed to metastatic disease while five patients (8%) died due to other causes.

The positively stained cancer cells in the TMAs by individual IHC markers are shown in Table 2. Almost half (47%) of the patients had over 10% staining for p53. Her2 staining was positive in 72% of the patients (any staining intensity),

with intermediate staining intensity being the most prevalent (38%). EGFR staining, on the other hand, was positive in only 24% of the patients.

Table 1. Urothelial MIBC patients treated with NAC and radical cystectomy in Helsinki and Turku University Hospitals between 2008 and 2013 ($n = 59$).

Age	Years, mean/median (range)	64/65 (47–76)
Gender	Male, n (%)	49 (83)
Smoking status known ($n = 53$)	active or stopped	45 (76)
Center	Turku University Hospital	29 (49)
	Helsinki University Hospital	30 (51)
Grade (WHO 1973) (TURBT)	1	1 (2)
	2	15 (25)
	3	43 (73)
LVI (TURBT)	Yes	16 (27)
	No	43 (73)
pT-category (Cystectomy)	pT0	26 (44)
	pTa-T1, Tis	10 (17)
	pT2	11 (19)
	pT3	10 (17)
	pT4	2 (3)
pN-category (Cystectomy)	pN0	52 (88)
	pN+	6 (10)
	data missing	1 (2)
Response to NAC	Complete Response (T0N0)	26 (44)
	Partial Response (Ta-T1, Tis, N0)	10 (17)
	No Response (\geq T2 and/or N+)	23 (39)
NAC regimen	Cisplatin-Gemcitabine	56 (95)
	Carboplatin-Gemcitabine	3 (5)
Cycles of NAC	2	7 (12)
	3	22 (37)
	4	29 (49)
	6	1 (2)
Follow-up	months, mean/median (range)	49/49 (3–93)
Patient status	Alive, no evidence of disease	46 (78)
	Dead do to MIBC	8 (14)
	Dead do to other reason	5 (8)
MIBC	Muscle invasive bladder cancer	
NAC	Neo-adjuvant chemotherapy	
TURBT	Trans-urethral resection of bladder tumour	
LVI	Lympho-vascular invasion	

Table 2. Immuno-histochemical markers selected to stain the cancer samples from TURBT sections and their distribution according to expression.

Marker		n (%)
p53 ($n = 59$)	\leq 10% expression	31 (53)
	$>$ 10% expression	28 (47)
Ki-67 ($n = 59$)	Low expression	29 (49)
	High expression	30 (51)
Her-2 ($n = 58$)	No expression	16 (28)
	Any expression	42 (72)
EGFR ($n = 59$)	No expression	45 (76)
	Any expression	14 (24)

Ki-67 was dichotomized by the mean value (62.8).

Table 3. Correlation between marker status with both WHO Grade and Lympho-vascular invasion (LVI) from TURBT sections.

Marker		Grade (WHO 1973)			LVI		
		1-Feb	3	p -value	No	Yes	p -value
p53 ($n = 59$)	\leq 10% expression	14 (45)	17 (56)	0.001*	26 (84)	5 (16)	0.077
	$>$ 10% expression	2 (7)	26 (93)		17 (61)	11 (39)	
Ki-67 ($n = 59$)	Low expression	11 (38)	18 (62)	0.084	21 (72)	8 (28)	1.000
	High expression	5 (17)	25 (83)		22 (73)	8 (27)	
Her-2 ($n = 58$)	No expression	3 (19)	13 (81)	0.514	12 (75)	4 (25)	1.000
	Any expression	13 (31)	29 (69)		31 (74)	11 (26)	
EGFR ($n = 59$)	No expression	14 (31)	31 (69)	0.310	34 (76)	11 (24)	0.485
	Any expression	2 (14)	12 (86)		9 (64)	5 (36)	

Pearson Chi-square test was used to calculate the correlation between marker status and Grade or Lympho-vascular invasion.

Fisher's exact test was used if less than five units were available.

Ki-67 was dichotomized by the mean value (62.8).

*Significant p -value.

The correlation between IHC status and cancer grade or LVI are shown in Table 3. Higher cancer grade (WHO 3) was correlated with 10% or less expression of p53 ($p = 0.001$). Staining results from other biomarkers were not correlated with grade or LVI.

The associations of clinical variables and marker status with chemotherapy response are described in Table 4. Initially patients with complete response were compared to partial and non-responders. Then the comparison was made by grouping complete responders together with partial responders and comparing them to non-responders. A higher number of chemotherapy cycles was associated statistically significantly with the complete and partial response group ($p = 0.014$; OR = 4.044, 95% CI = 1.321–12.377). Other statistically significant associations were not observed.

Presented in Table 5 are the factors affecting OS and CSS in a univariate Cox proportional hazard regression model. Female gender was associated significantly with decreased CSS ($p = 0.011$). There was also a trend toward association with OS, but this was not statistically significant ($p = 0.072$). MIBC (\geq T2) found in cystectomy was also associated with poorer CSS compared to \leq T1 ($p = 0.035$). As expected, positive lymph nodes (N+) were associated both with poorer CSS ($p = 0.011$) and OS ($p = 0.004$). None of the investigated biomarkers were associated with CSS or OS.

Discussion

We demonstrate here that NAC induced down-staging of non-MIBC or a complete response is associated with improved CSS. We also observed that a higher number of chemotherapy cycles was associated with better response to NAC treatment. However, none of the tested immune-histochemical markers were associated with NAC response or survival.

The tumor protein 53 (p53), the so-called gatekeeper of the cell cycle has been demonstrated to correlate with bladder cancer grade and advanced stage when overexpressed (presumably due to a mutation) in cancer cells [9]. Only a few studies have examined the role of p53 and its value to predict NAC response or survival in MIBC patients after cystectomy. In a prospective study of Plimack et al. [10] with accelerated Methotrexate, Vinblastine, Doxorubicin (=Adriamycin) and Cisplatin (MVAC) as neo-adjuvant

Table 4. Binary logistic regression analysis evaluating associations of variables with chemotherapy response in cystectomy specimen.

	Complete response (vs partial and No response)		Complete and partial response (vs No response)	
	OR	95 % CI	OR	95 % CI
Age	1.029	0.958–1.105	1.027	0.955–1.103
Gender				
Male	Reference		Reference	
Female	0.818	0.25–3.268	0.354	0.088–1.429
Smoking (data available $n = 53$)				
Active/Stopped	Reference		Reference	
Never	2.281	0.485–10.732	1.111	0.236–5.238
WHO Grade (TURBT)				
1 or 2	Reference		Reference	
3	2.100	0.623–7.074	0.918	0.281–2.994
LVI (TURBT)				
Yes	Reference		Reference	
No	1.018	0.320–3.236	0.918	0.281–2.994
Cycles of chemotherapy				
2 or 3	Reference		Reference	
4 or 6	2.171	0.760–6.200	4.044	1.321–12.377
p53				
$\leq 10\%$	Reference		Reference	
$> 10\%$	1.200	0.428–3.361	0.733	0.257–2.095
Ki-67				
Low	Reference		Reference	
High	1.636	0.580–4.615	0.514	0.177–1.491
Her-2 ($n = 58$)				
No expression	Reference		Reference	
Any expression	3.300	0.914–11.910	2.000	0.620–6.452
EGFR				
No expression	Reference		Reference	
Any expression	1.368	0.411–4.558	1.827	0.497–6.715

Complete response pT0N0; Partial response $< pT2N0$; No response pT2 and/or N+. Ki-67 was dichotomized according to mean value (62.8).

treatment, p53 status, determined by DNA sequencing, failed to select patients with complete response in cystectomy. On the other hand, p53 protein overexpression (suggestive of *TP53* mutation) was shown to have an independent predictive role for survival in a multivariate analysis by Sarkis et al. [11] after MVAC as NAC and cystectomy. They used a cut-off of $\geq 20\%$ in positively stained cells determining altered p53 staining in IHC. In contrast, Maluf et al. [12] demonstrated that altered p53 status was associated with improved long-term survival ($p = 0.05$), but not with pathologic down-staging after NAC in their retrospective study. The patient cohort studied was relatively small ($n = 59$) and the cut-off of $\geq 20\%$ was similarly used. It is worth mentioning the prospective randomized study of Stadler et al. [13], where 272 T1/T2 bladder cancer patients with p53 overexpression (cut-off $\geq 10\%$) were randomly assigned for MVAC adjuvant treatment or surveillance after cystectomy. The authors found no difference between the groups in the recurrence-rates or survival. Based on these results, to date p53 cannot be regarded as an (established) predictive marker for treatment response or for survival in NAC treated MIBC.

Previously, a high percentage of Ki-67 (a proliferation marker) positive cells has been shown to correlate with high stage and poor differentiation in bladder cancer [14]. In one study a high Ki-67 percentage was associated with higher stage, higher grade, lympho-vascular invasion and metastasis to lymph nodes. Among patients ($n = 91$) not receiving neo-adjuvant or adjuvant chemotherapy, a high Ki-67 percentage was also an independent predictor of both disease recurrence and cancer-specific mortality [15]. According to the literature only one published paper

addresses the association of Ki-67 expression in NAC treated MIBC patients. In the sub-study of the SWOG8710 trial where patients were randomized prospectively to NAC and cystectomy versus cystectomy alone in locally advanced bladder cancer, a high Ki-67 percentage was related to better progression-free survival ($p = 0.063$) [16]. The median survival of patients with higher Ki-67 expression was 73 months, compared to 38 months for those with lower expression, but such difference failed to reach statistical significance ($p = 0.25$). Although the evidence is quite weak, it suggests that a high percentage of Ki-67 in NAC treated patients leads to better outcome after cystectomy, while cystectomy only patients do worse.

Human Epidermal Growth Factor Receptor-2 (Her-2 also erbB2) is an oncogenic growth factor receptor and overexpression of EGFR has been reported in 12–53% of bladder cancers and more frequently in MIBC [17–19]. Studies have shown that, in MIBC Her-2, overexpression is associated with worse CSS [20,21]. Groenendijk et al. [7] showed that MIBC with Her-2 missense mutations characterize a subgroup of MIBC patients with excellent response to NAC. A total of $n = 9$ of 38 complete responders had Her-2 missense mutations, while none of the 33 non-responders had any ($p = 0.003$). Her-2 overexpression was identified in both responders and non-responders, and there was no association with response to NAC ($p = 0.52$). Using immunohistochemistry, Kramer et al. [22] demonstrated that overexpression of EGFR correlates with higher grade and stage and strong EGFR expression was associated with poorer survival in univariate, but not multivariate analysis. To our knowledge, this is the first study report of EGFR expression and NAC

Table 5. Univariate Cox regression analysis (OS and CSS) evaluating association of variables for survival.

	OS		CSS	
	HR	95% CI	HR	95% CI
Age	1.057	0.974–1.148	1.007	0.915–1.108
Gender				
Male	Reference	–	Reference	–
Female	2.978	0.907–9.781	6.019	1.497–24.200
Smoking (n = 53)				
Active/Stopped	Reference	–	Reference	–
Never	1.244	0.267–5.800	3.145	0.575–17.196
Grade (TURBT)				
1 or 2	Reference	–	Reference	–
3	2.044	0.450–9.280	2.044	0.450–9.280
LVI (TURBT)				
No	Reference	–	Reference	–
Yes	1.846	0.603–5.657	2.954	0.736–11.851
Chemocycles				
2 or 3	Reference	–	Reference	–
4 or 6	0.924	0.281–3.033	0.935	0.234–3.741
NAC				
Cis-Gem	Reference	–	Reference	–
Regimen				
Car-Gem	1.388	0.180–10.707	2.441	0.300–19.851
pT				
T0	Reference	–	Reference	–
Ta-T4	2.010	0.617–6.546	5.990	0.736–48.730
T0, Ta, T1, Tis	Reference	–	Reference	–
T2–T4	2.299	0.767–6.888	5.610	1.128–27.909
pN				
N0	Reference	–	Reference	–
N+	6.188	1.801–21.261	6.430	1.526–27.092
p53				
No expression	Reference	–	Reference	–
Any expression	1.238	0.414–3.701	3.375	0.681–16.723
Ki-67				
Low	Reference	–	Reference	–
High	0.781	0.262–2.326	0.546	0.130–2.287
Her-2				
No expression	Reference	–	Reference	–
Any expression	0.700	0.225–2.177	2.633	0.324–21.406
EGFR				
No expression	Reference	–	Reference	–
Any expression	0.509	0.112–2.323	0.459	0.056–3.728

Ki-67 was dichotomized according to the mean value (62.8).

response in MIBC and we could not see any predictive value with EGFR immunohistochemistry.

In our study 61% of MIBC patients were down-staged to either non-MIBC (17%) or pT0N0 (44%). This was associated with a significantly higher number of NAC cycles (2–3 versus 4–6, $p = 0.014$). Peyton et al. [23] described a complete response rate of 41% in patients treated with dose dense MVAC (on average 3.3 cycles) and 25% in patients treated with GC (on average 3.7 cycles), respectively. In an international retrospective study cohort consisting of 146 MIBC patients treated with GC, the complete response rate was 31% in patients who received four or more cycles of NAC [24]. Taken together, these results suggest that four or more cycles of GC NAC favor likelihood complete response.

In our study we observed that women had poorer survival than men in MIBC, a finding in line with previously reported results. For CSS this difference was statistically significant ($p = 0.011$). Similarly, a large Swedish study by Radkiewicz et al. [25] showed that MIBC in women leads to excess mortality when most confounding factors are taken into account.

Patients who did not have a response to NAC (\geq pT2) had an over 5-fold risk of dying from MIBC compared to

responders. There was, however, no significant difference in OS between the groups. The patients with negative pN-status had better CSS ($p = 0.011$) and OS ($p = 0.004$) compared to pN+ patients. Sonpavde et al. [26] observed similar results in their substudy of SWOG-S8710. Interestingly Bhindi et al. [27] showed in their retrospective registry study that patients with muscle invasive or advanced residual tumor after NAC had worse CSS and OS than the patients with similar postoperative residual tumor but no NAC prior to cystectomy. This suggests that today there are only a few treatment options for MIBC found to be chemoresistant after NAC and cystectomy, and the prognosis of such patients is particularly poor.

Promising results of NAC treatment in MIBC patients has been previously shown by molecular subtyping of tumors. For example, Seiler et al. [28] demonstrated that basal tumors responded well to NAC when OS was used as an endpoint.

The weaknesses of our study is the retrospective nature and the small patient cohort. Benefits include detailed real world clinical follow-up data from two academic institutes.

In conclusion, the detection of protein expression by IHC of Ki67, HER2, EGFR, and p53 (as detected by IHC) is unable to stratify patients for NAC treatment before cystectomy. Bladder cancer specific and genetic DNA level studies are needed to advise MIBC patients with cisplatin resistant cancers and urologists treating them to optimize the treatment.

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Appendix Table A. Antibodies used in this study and their preparation for staining

Antigen	Antibody clone and vendor	Antibody dilution	Primary antibody incubation time
Ki-67	MIB-1, DAKO	1:100	32 min
p53	DO-7, DAKO	1:500	24 min
c-erbB2 (HER-2)	4B5, Roche	1:166*	48 min
EGFR	31G7, Invitrogen	1:25	30 min

*Ready-to-use solution with an antibody concentration of approximately 6 µg/mL.