RESEARCH ARTICLE

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Identification of Molecular Subgroups of Muscle-Invasive Urothelial Bladder Cancer and Their Impact on Treatment Outcome

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Abstract

Purpose: Neoadjuvant chemotherapy (NACT) improves muscle-invasive bladder cancer (MIBC) survival. However, its efficacy is limited to a group of patients. This study explored CK5/6 and GATA3 for molecular subtyping and their prediction to response in patients with MIBC. **Methods:** This is a prospective study that includes 50 patients with TCC bladder. All Patients received 4 cycles neoadjuvant gemcitabine/ cisplatin then guided to further treatment according to the response to NACT. Responders (CR & PR) went for CCRTH whereas non-responders (SD & PD) went surgery if resectable or second line chemotherapy if non-resectable. The baseline TUR pathology specimens were examined for histopathological feature and CK5/6 and GATA3 and divided into 4 molecular subgroups.**Results:** The patients were divided into four molecular subgroups: luminal (n=12/26.7%), basal (n=8/17.8%), double-positive (n=21/26.7%), and double-negative (n=4/8.9%). There was no clinicopathological difference seen among the 4 molecular subgroups. The PFS was higher in patients with GATA3 positive (24 months) than GATA3 negative (17 months). Yet, it did not reach a statistically significant value (P = 0.1605). On the other hand, PFS was not affected by either CK5/6 status or different molecular subgroups. The OS was better in the luminal subgroup than the basal (20.8 months versus 16.16 months respectively, While the double positive showed the highest OS of 26 months(P=0.0352). **Conclusion:** GATA3 and CK5/6 IHC can classify MIBCs into four subtypes. These subtypes predicted treatment outcomes, however, were not correlated with the response to NACT. GATA3-positive tumors, luminal and double-positive subtypes tend to have higher OS and PFS. CK5/6 positivity did not impact the treatment outcome.

Keywords: Muscle invasive bladder cancer- molecular subtyping- neoadjuvant chemotherapy

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Introduction

Bladder cancer is considered the 9th most common cancer worldwide, and the second most common genitourinary malignancy [1] .Transitional Cell Carcinoma (TCC), or urothelial carcinoma (UC), is the most prevalent histological subtype among bladder malignancies, accounting for 90% of cases [1]. In Egypt, a significant change in the histopathological types of bladder cancer has been noticed. The relative frequency of UC increased from 22% in 1980 to 73% of bladder cancer diagnosed in 2005. However, bladder cancer remains the second most common cancer among Egyptian males [2].

Muscle-invasive bladder cancer (MIBC) represents about 20% of newly diagnosed cases [3]. It is more aggressive and has an inferior prognosis compared to the non-invasive disease, with a 5-year survival rate of 60% [4]. Treatment for MIBC involves neoadjuvant chemotherapy (NACT), which has been shown to enhance survival outcomes [5]. Two large meta-analyses found that platinum-based combination chemotherapy significantly improved overall survival, with a 5-8% absolute benefit at five years and a 13% reduction in mortality risk [6, 7].

However, the efficacy of NACT is limited to a group of patients, with a worthy proportion experiencing disease recurrence, and accurately forecasting responsiveness continues to be a significant obstacle [8]. Given the intricate nature of this situation, clinical management should identify prognostic and predictive factors that underlie treatment response. Currently, there are no available predictors for the response to NACT [9]. Nevertheless, several studies have investigated the potential impact of molecular subtyping on the response to NACT [10]. Molecularly, UC appears as a heterogeneous disease with numerous subtypes, characterized by a significant tumor mutational burden and genomic instability [11].

RNA and/or immunohistochemistry-based expression profiling of UC unveiled several molecular subtypes and

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classification schemes linked to prognosis or response to various therapies, including NACT [12]. Various classifications assigned distinct names to distinct subtypes; however, substantial overlap exists among them. The existence of two principal categories, luminal and basal, encompassing supplementary subtypes is a basic feature of these diverse classification systems [13]. To enhance the consistency of terminology utilized in RNA-based classification, the Bladder Cancer Molecular Taxonomy Group spearheaded a global endeavor to establish a consensus molecular classification of MIBC [14].

Therefore, oncologists need to investigate novel prognostic biomarkers and treatment response indicators for MIBC to realize individual precision medicine [9]. This study aimed to explore CK5/6 and GATA3 as surrogate markers for molecular subtyping and their impact on the response to neoadjuvant chemotherapy and survival in patients with MIBC.

Materials and Methods

This prospective study included 50 patients newly diagnosed with UC between December 2020 and June 2022 at Kasr Al Ainy Center of Oncology and Nuclear Medicine (NEMROCK).

The inclusion criteria were patients aged > 18 with ECOG performance status 0-2 with nonmetastatic UC indicated for NACT (stage cT2-T4a, N0-1). Patients with creatinine clearance < 60 ml/min., NHYA class > 2, or abnormal audiometry, and those refusing NACT were excluded from the study.

Before treatment, all patients underwent cystoscopy for biopsy and maximum TURB to confirm T2-T4a MIBC. They also did CT of the chest, abdomen, and pelvis, bone scan, echocardiography, audiometry and laboratory investigations (CBC, kidney and liver function tests, Ca, K, Na, and creatinine clearance). All patients received four cycles of NACT (gemcitabine 1000 mg/m2 on days 1 and 8, plus cisplatin 75 mg/m2 divided on days 1 and 2). Patients were monitored for chemotherapy toxicity according to CTCAE version 5. The response to this GC NACT protocol is assessed according to RECIST 1.1 criteria. After NACT, the patients were assessed by CT and cystoscopy and guided to further treatment according to the response to NACT. Patients in complete or partial remission were referred to definitive concurrent chemoradiotherapy. Patients with stable or progressive disease were left to the physician's choice, whether surgery if resectable or second-line chemotherapy if irresectable.

Radiotherapy

Patients started their sessions within 6-8 weeks of the end of NACT. Those with partial remission (PR) had maximum safe TURB before starting radiotherapy (RTH). The patients were followed up weekly and assessed clinically for adverse effects. Patients who develop side effects during RTH sessions are graded according to the RTOG and given the appropriate supportive measures [15]. For concurrent chemotherapy, patients received cisplatin 75 mg/m^2 on days 1 and 21, preceded by CBC, kidney, and liver function tests.

Surgery protocol

Patients with progressive or stationary disease went for radical cystectomy. Then, they were assessed for the need for adjuvant radiotherapy based on the surgical pathology report. The indications of adjuvant radiotherapy were pT3 or T4 and node-positive disease.

Second line chemotherapy

Patients with inoperable, progressive, or stationary disease were candidates for second-line chemotherapy. They received three cycles of paclitaxel and were assessed with chest, abdomen, and pelvis CT with contrast afterward. If the patient is responding, three more cycles are administered, and if not, the patient is for best supportive care.

The patients were followed up every three months with chest, abdomen, and pelvis CT +/- cystoscopy for two years. At the end of the follow-up, overall survival (OS) and progression-free survival (PFS) were calculated. OS is calculated from the date of starting treatment to the date of death or last follow-up. PFS was calculated from the date of starting treatment to the date of progression/ death or last follow-up.

Pathology

The baseline TURB pathology specimens were examined for histopathological features, CK5/6 and GATA3. Serial sections of 4 microns thick were prepared from each block; one was mounted on a glass slide and stained by Hematoxylin and Eosin (H&E) for histological evaluation. The other two were mounted on charged slides for immunohistochemical staining. The cases were divided into four groups depending on immunoreactivity for GATA3 and CK5/6 [16]: luminal (GATA3 +ve, CK5/6 −ve), basal (GATA3-ve, CK5/6 +ve), double +ve, and double -ve.

Immunohistochemical Staining

Immunostaining for GATA3 (Mouse monoclonal (L50-823), primary antibody) and Cytokeratin 5/6 (Mouse monoclonal (D5/16B4), primary antibody) was performed using a fully auto¬mated immune-histochemical system (Ventana-Ultra machine). Assessment of immunostaining was done using an Olympus light microscope (CX-34). A cut-off value of 20% positive nuclear staining (GATA3) and cytoplasmic staining (CK5/6) of tumor cells was applied as recommended by Ravanini et al. [17]. Expression in more than 20% of tumor cells was considered positive.

Sample size

Sample size was calculated using MedCalc® Statistical Software version 20.215 (MedCalc Software Ltd, Ostend, Belgium), the minimum required sample size for a two armed pilot study was calculated using the equation developed by Viechtbauer et al., to detect an event of death of progression of 10% among study population with confidence interval 95%, sample size was estimated as N per group= 22.

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Statistical Analysis

Statistical analysis was performed using MedCalc® Statistical Software version 22.003 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2023). The normality of distribution of continuous variables was tested using Shapiro-Wilk test. Mean and standard deviation or median and range were used to describe continuous variables. Pearson's Chi-square test (Fisher's exact test) was used to evaluate the association between categorical variables. Kruskal-Wallis test was used to test the difference in an abnormally distributed continuous variable between more than two groups. Kaplan-Meier method was used to perform survival analysis and plot survival curves, and the Log-rank test was used to compare survival curves. A value of p-value <0.05 was considered significant.

Results

Flow chart showing number of participants at each stage of the study (Flow chart 1). luminal (GATA3 +ve, CK5/6 −ve), basal (GATA3-ve, CK5/6 +ve), double +ve, and double -ve (Flow Chart 1).

Fifty patients with MIBC were enrolled, and five were excluded due to pathology problems (2 had scanty samples, one had technical processing issues, and two had lost their blocks). The mean age of the remaining 45 patients was 59.2±5.0 years. The baseline characteristics are shown in Table 1.

Nine patients had stage II disease (T2N0), and 36 patients had stage IIIA; T2N1 (n=2), T3N0 (n=24), T3N1 (n=5), T4N0 (n=4), T4N1 (n=1). The majority of the patients had grade 2 disease (63%). Grade 2 pleomorphism was seen in 23 patients (51.1%) and grade 3 in 15 patients (33.3%). CIS was positive in only nine patients (20.0%), whereas LVI was positive in 29 patients (64.4%), and PNI was positive in 26 patients (57.7%). Bilharzial ova were seen in 8 patients (17.7%). Lymphocyte infiltration was seen in 22 patients (48.9%). TCC with squamoid differentiation was found in 15 patients (33.3%).

Molecular markers

Of the 45 patients, 29 were CK5/6 positive (64.4%), and 33 were GATA3 positive (73.3%). Accordingly, the patients were divided into four molecular subgroups: luminal, basal, double positive, and double negative. The basal group constituted 17.8% (n=8), the luminal 26.7% (n=12), the double positive 46.7% (n=21) and the double negative 8.9% (n=4). There was no significant difference between the four molecular subgroups in age, smoking, pathological characteristics and response to NACT (Tables 2-3).

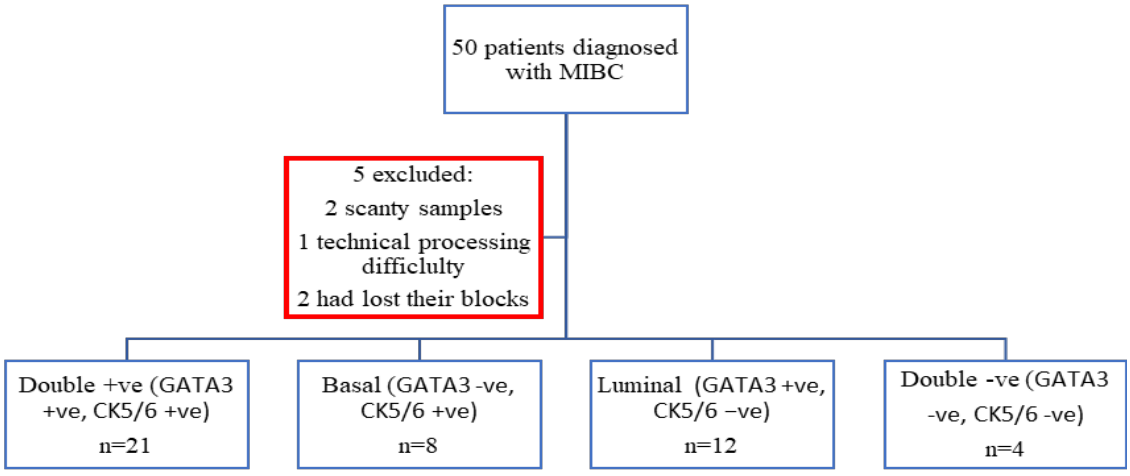
NACT

All patients underwent max TURB before NACT. All patients received four cycles of NACT Gemcitabine/ Cisplatin except for one patient who received only two cycles due to recurrent UTI, causing a delay in the NACT schedule. After NACT, the CT and cystoscopic assessment

Table 1. Baseline Characteristics of the Patients and Disease

		value
Age (years)		59.2±5.0
Sex (male/female)		37/8
Smoking		31 (68.8%)
Performance status (I/II)		40/5
Diabetes mellitus		9 (20.0%)
Hypertension		6 (13.3%)
American Joint Committee on Cancer Stage (II/III)		36/9
Grade (2/3)		29/16
Degree of Pleomorphism (1/2/3)		7/23/2015
CIS		9 (20.0%)
Lymphovascular invasion		29 (64.4%)
Perineural invasion		26 (57.8%)
Degree of Lymphocyte infiltration	No	23 (51.1%)
	1	14 (31.1%)
	2	7 (15.6%)
	3	1 (2.2%)
Differential histology	Squamoid	15 (33.3%)
	Glandular	1 (2.2%)
	Glandular & Squamoid	2 (4.4%)

Data are presented as mean±SD or number (%)



Flow Chart 1. The Number of Participants at each Stage of the Study

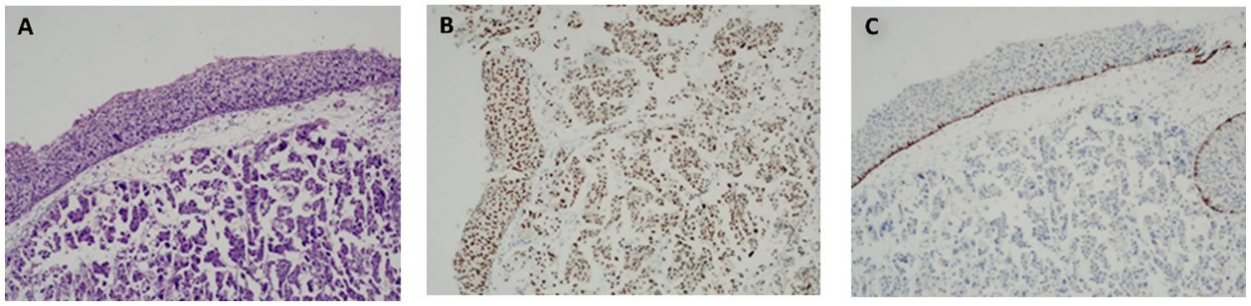


Figure 1. A. Invasive urothelial carcinoma (H&E 100X), B. GATA3 positive nuclear expression in tumor cells with positive control in surface urothelium (IHC 100X), C. Ck5/6 Negative expression in tumor cells with positive control in basal cell layer of surface urothelium, a case of TCC GATA3 positive and CK5/6 negative

Table 2. Characteristics of Different Molecular Subgroups

		Double +ve n=21	Basal n=8	Luminal n=12	Double -ve n=4	p-value
Age (mean ±SD)		58.4±5.7	60.6±5.7	59.2±4.4	60.5±5.7	0.211
Sex	Males	19	4	11	3	*
	Females	2	4	1	1	
Stage	Stage II	6	1	1	1	*
	Stage III	15	7	11	3	
T stage	T2	6	2	2	1	*
	T3	13	6	7	3	
	T4	2	0	3	0	
N stage	N0	19	5	10	3	*
	N1	2	3	2	1	
Differential histology	No	11	1	11	4	*
	Yes	10	7	1	0	
CIS	Negative	19	7	6	4	*
	Positive	2	1	6	0	
LVI	Negative	6	3	4	3	0.424
	Positive	15	5	8	1	
PNI	Negative	7	3	6	3	0.434
	Positive	14	5	6	1	
Smoking	Non-smoker	6	4	3	1	0.713
	Smoker	15	4	9	3	

showed that 21 patients were responders (46.6%) while 24 were non-responders (53.3%). Complete response was seen in 16 (35.6%), partial response in 5 (11.1%), whereas 10 patients (22.2%) showed progressive disease.

Table 3 shows that smoking negatively affected response to NACT (p=0.025). Also, ten out of 12 patients with T4 and/or N1 disease did not respond to NACT (p=0.015). On the other hand, molecular subgroups did not affect response to NACT (p=0.344).

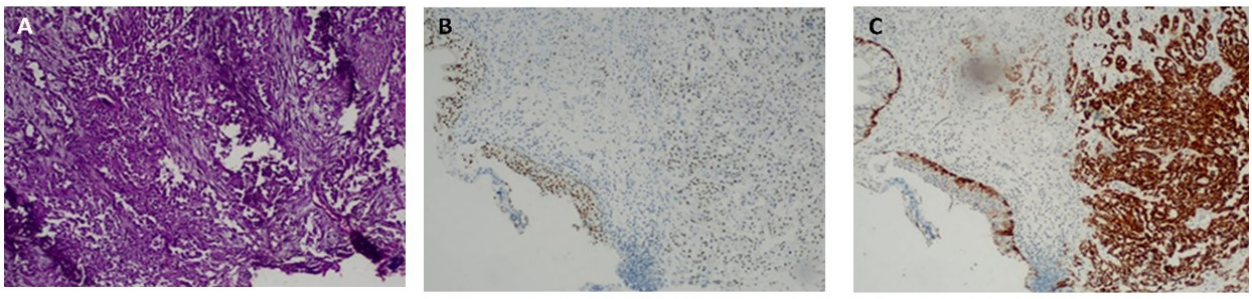


Figure 2. A. Invasive high-grade urothelial carcinoma (H&E 100X), B. GATA3 moderate positive expression in tumor cells with positive control (IHC 100X), C. CK5/6 strong positive expression in tumor cells with positive control, double positive case

Table 3. Factors Affecting Response to NAC

		Response to NACT		p-value
		Yes n=21	No n=24	
GATA3	Positive	17 (51.5%)	16 (48.5%)	0.28
	Negative	4 (33.3%)	8 (66.7%)	
CK5/6	Positive	12 (41.4%)	17 (58.6%)	0.338
	Negative	9 (56.3%)	7 (43.8%)	
Molecular Subtypes	Double +ve	10 (47.6%)	11 (52.4%)	0.525
	Basal	2 (25.0%)	6 (75.0%)	
	Luminal	7 (58.3%)	5 (41.7%)	
	Double -ve	2 (50.0%)	2 (50.0%)	
Smoking	No	10 (71.4%)	4 (28.6%)	0.025
	Yes	11 (35.5%)	20 (64.5%)	
T-stage	2	6 (54.5%)	5 (45.5%)	0.905
	3	13 (44.8%)	16 (55.2%)	
	4	2 (40.0%)	3 (60.0%)	
N-Stage	0	20 (54.1%)	17 (45.9%)	0.051
	1	1 (12.5%)	7 (87.5%)	
Stage categories	T4 +/- N1	2 (16.7%)	10 (83.3%)	0.015
	Other stages	19 (57.6%)	14 (42.4%)	
Grade	2	11 (37.9%)	18 (62.1%)	0.118
	3	10 (62.5%)	6 (37.5%)	
Extent of pleomorphism	1	6 (85.7%)	1 (14.3%)	0.01
	2	6 (26.1%)	17 (73.9%)	
	3	9 (60.0%)	6 (40.0%)	
CIS	Negative	17 (47.2%)	19 (52.8%)	1
	Positive	4 (44.4%)	5 (55.6%)	
LVI	Negative	8 (50.0%)	8 (50.0%)	0.739
	Positive	13 (44.8%)	16 (55.2%)	
PNI	Negative	10 (52.6%)	9 (47.4%)	0.493
	Positive	11 (42.3%)	15 (57.7%)	
Differential histology	No	13 (48.1%)	14 (51.9%)	0.807
	Yes	8 (44.4%)	10 (55.6%)	

Data are presented as the number (%)

Toxicity of NACT

Anemia was reported in 6 patients, neutropenia in 4, and thrombocytopenia in 3. Moreover, grade 1 vomiting was seen in 1 patient. Anemia, thrombocytopenia, and neutropenia were seen mainly in smokers. Yet, bilharziasis did not affect the toxicity of NACT. (Table 4)

Treatment arms

All the responders received CCRTH. Of the non-responders, 20 patients went for radical cystectomy and PLND, and four patients received second-line CTH (paclitaxel) due to advanced stage.

CCRTH arm

Most radiotherapy adverse events were tolerable. Grade 1 early GU toxicity was reported by 11 patients (52.4%), grade 2 by 7 (33.3%), and grade 3 by 3 (14.3%). Early GIT toxicity was reported by 14 patients, 11 (52.4%) of grade 1 and 3 (14.3%) of grade 2. No grade 4 early or late toxicity was reported. The 3-month assessment

Table 4. Toxicity to NACT

		G1	G2	G3	G4
Hematological	Anemia	1	2	3	0
	Thrombocytopenia	0	1	0	2
	neutropenia	0	2	2	0
GIT	Vomiting	1	0	0	0

Data are presented as the number of patients.

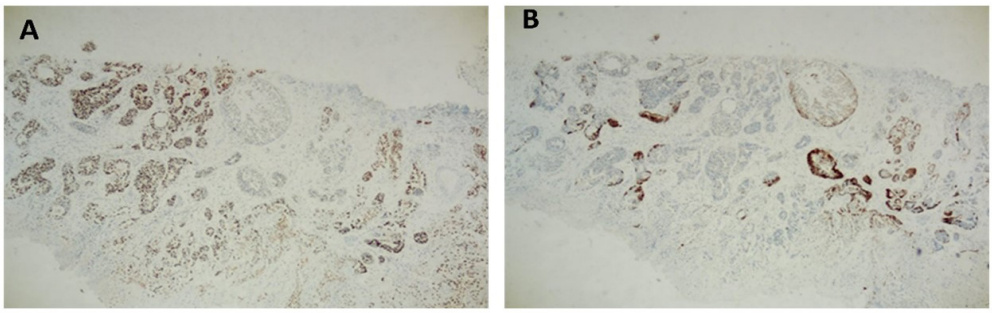


Figure 3. A. GATA3 positive expression in tumor cells (IHC 40X). B. Ck5/6 positive expression in tumor cells (IHC 40X), double positive case

showed that 2/21 patients had residual disease and were sent for salvage cystectomy. The toxicity to CCRTH was not affected either by smoking or bilharziasis.

Surgery arm

As for the patients in the surgery arm, stage I was seen in 1 patient, stage II in one, and 18 had stage III disease. One patient received two cycles of adjuvant CTH, while

Table 5. Progression-Free Survival and Its Relation to Prognostic Factors

Factor		n	Events	Cumulative survival at 18 months (%)	Median survival (months)	p-value
Whole Group		45	16	66.0	21.9 (18.5-25.3)	
GATA3	Positive	33	10	71.8	*	0.186
	Negative	12	6	50.0	14.2 (18.5-25.3)	
CK5/6	Positive	29	10	68.2	*	0.707
	Negative	16	6	62.1	21.9 (18.5-25.3)	
Management	CCRT	21	5	83.1	21.9 (18.6-25.2)	0.019
	Surgery	20	8	44.8	14.2 (13.4-15.1)	
	2nd CTH	4	3	50.0	10.6 (0.0-22.3)	
Management	CCRT	21	5	83.1	21.9 (18.6-25.2)	0.078
	Surgery	20	8	44.8	14.2 (13.4-15.1)	
Smoker	Yes	31	11	56.5	19.4 (9.7-29.1)	0.493
	No	14	5	78.6	21.9 (18.6-25.2)	
T.stage	2	11	4	57.3	*	0.207
	3	29	9	71.2	*	
	4	5	3	60.0	*	
N.Stage	0	37	10	76.8	*	<0.001
	1	8	6	0.0	11.8 (9.3-14.3)	
Grade	2	29	12	63.1	20.2 (16.7-23.7)	0.794
	3	16	4	74.5		
CIS	Positive	9	4	30.5	14.7 (13.3-16.0)	0.286
	Negative	36	12	72.2	21.9 (18.4-25.3)	
LVI	Positive	29	9	64.5	*	0.377
	Negative	16	7	67.7	20.2 (13.6-26.9)	
PNI	Positive	26	7	67.7	*	0.121
	Negative	19	9	62.0	19.4 (13.6-25.2)	
Types	Double +ve	21	5	78.8	*	0.16
	Basal	8	5	37.5	14.2 (13.3-15.1)	
	Luminal	12	5	56.3	*	
	Double -ve	4	1	75.0	*	
Types	Double +ve	21	5	78.8	*	0.087
	Others	24	11	52.9	20.2 (12.8-27.7)	
T4	T4 +/- N1	12	8	25.0	12.3 (10.6-14.0)	< 0.001
	Others	33	8	77.7	*	

*, Median survival cannot be calculated

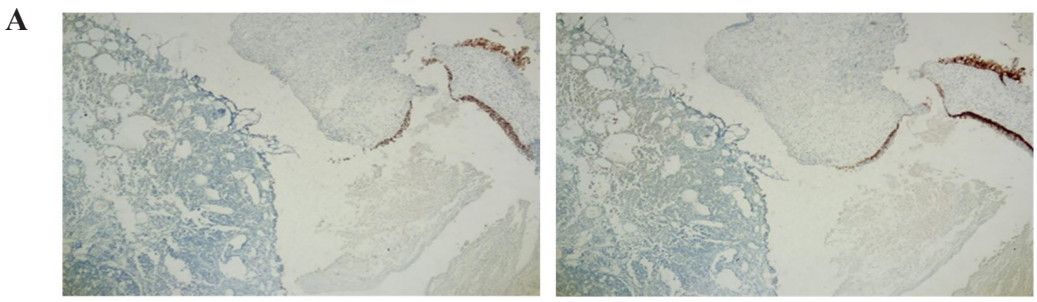


Figure 4. A. GATA3 negative expression in tumor cells with positive control in surface urothelium (IHC 40X); B. CK5/6 negative expression in tumor cells with positive control in basal cell layer of surface urothelium (IHC 40 X), double negative case

8 received adjuvant RTH.

Progression-free survival (Table 5)

The median PFS of the whole group was 21.9 months (95%CI: 18.5-25.3). The cumulative PFS was 66%. PFS was affected by the type of definitive management after NACT. CCRTH had the best PFS compared to surgery and 2nd line CTH (p=0.019). If CCRTH was compared

Table 6. Overall Survival and Its Relation to Prognostic Factors

Factor		n	Events	Cumulative survival at 18 months (%)	Median survival (months)	p-value
Whole group		45	13	70.2	*	
GATA3	Positive	33	7	78	*	0.052
	Negative	12	6	49.4	14.2 (7.1-21.3)	
CK5/6	Positive	29	8	74.7	*	0.572
	Negative	16	5	61.7	*	
Management	CCRT	21	3	88.2	*	0.003
	Surgery	20	7	46.7	*	
	2nd CTH	4	3	50	5.9 (0.2-23.2)	
Management	CCRT	21	3	88.2	*	0.021
	Surgery	20	7	46.7	*	
Smoker	Yes	31	10	60.8	*	0.245
	No	14	3	85.7	*	
T.stage	2	11	3	67.3	*	0.113
	3	29	7	73.8	*	
	4	5	3	60	*	
N.Stage	0	37	8	79.8	*	<0.001
	1	8	5	0	13.0 (12.1-13.8)	
Grade	2	29	10	66.8	*	0.697
	3	16	3	80.8	*	
CIS	Positive	9	4	30.5	14.7 (13.3-16.0)	0.095
	Negative	36	9	77.5	*	
LVI	Positive	29	7	66.9	*	0.454
	Negative	16	6	74	21.8 (20.8-22.9)	
PNI	Positive	26	5	70.2	*	0.122
	Negative	19	8	67.4	21.8 (12.7-31.0)	
Types	Double +ve	21	3	88.7	*	0.061
	Basal	8	5	37.5	14.2 (13.3-15.1)	
	Luminal	12	4	56.3	*	
	Double -ve	4	1	75	*	
Types	Double +ve	21	3	88.7	*	0.026
	Others	24	10	52.2	21.9 (12.4-31.3)	
T4	T4 +/- N1	12	7	27.8	13.8 (12.6-15.0)	< 0.001
	Others	33	6	81		

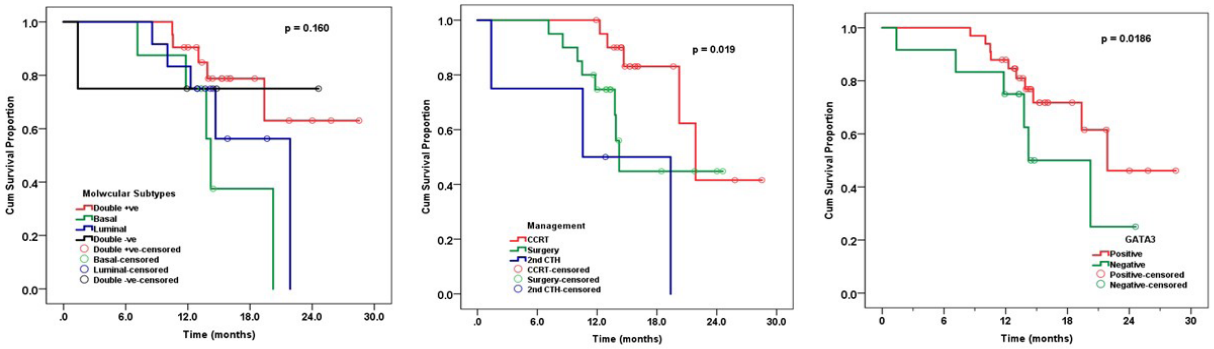


Figure 5. Progression-Free Survival in Relation to GATA3 Expression, Management Modality, Stage, and Molecular Subtypes

Table 7. Multivaiate Cox Hazard Regression Model for Predictor of Death

	P value	HR	95.0% CI
T	0.687	1.281	0.385-4.59
N	0.013	7.068	1.518-32.903
CCRTH (reference)	0.137		
Second line chemo	0.054	6.49	0.965-43.629
Surgery	0.133	3.025	0.714-12.821

Table 8. Multivaiate Cox Hazard Regression Model for Predictor of Disease Progression

	P value	HR	95.0% CI
T	0.916	0.945	0.327-2.727
N	0.003	8.108	2.04-3229
CCRTH (reference)	0.277		
Second line chemo	0.112	3.909	0.727-21.008
Surgery	0.342	1.794	0.537-6

to surgery only, it had better PFS (83.1% vs. 44.8%, respectively) without statistical significance differences between groups (p= 0.078). PFS was significantly higher in patients with N-stage 0 compared to N-stage 1 (p< 0.001). Patients with T4 and/or N1 disease had significantly lower cumulative PSF than others (25.0% vs. 77.7%, p<0.001). PFS was not affected by smoking, grade, CIS, LVI, or PNI.

GATA3 positive cases had slightly – but not significantly – higher PFS than negative cases (p=0.186). There was no significant impact of CK5/6 positivity on PFS (p=0.707). Double +ve cases had a higher PFS than other molecular subtypes, but the difference was not statistically significant (p=0.087). However, the difference in PFS between the four molecular subtypes was not significant (p=0.160).

Multivariable cox regression model showed that nodal status was the only independent predictor for relapse with p value 0.003 and HR 8.1 (95% CI 2.04-32.229), while T stage and treatment groups showed no statistically significant increase hazard of progression after adjustment for nodal status with p values 0.916, and 0.277 (Table 7).

Overall survival (Table 6)

The cumulative OS of the whole group at 18 months was 70.2%. The OS was higher in patients with GATA3 positive (78%) than GATA3 negative (49.4%), without statistically significant difference with a p-value of 0.052. On the other hand, the OS was not affected by the CK5/6 status (p=0.572). CCRTH had the higher OS compared to surgery and 2nd line CTH (p=0.003). pairwise comparison of CCRTH versus surgery showed a statistically significant longer OS with p value 0.021.

OS was significantly higher in patients with N-stage 0 compared to N-stage 1 (p< 0.001). Patients with T4 and/or N1 disease had significantly lower cumulative OS than others (27.8% vs. 81%, p<0.001). The OS was better in the luminal subgroup than the basal (56.3% vs. 37.5, respectively), while the double positive showed the

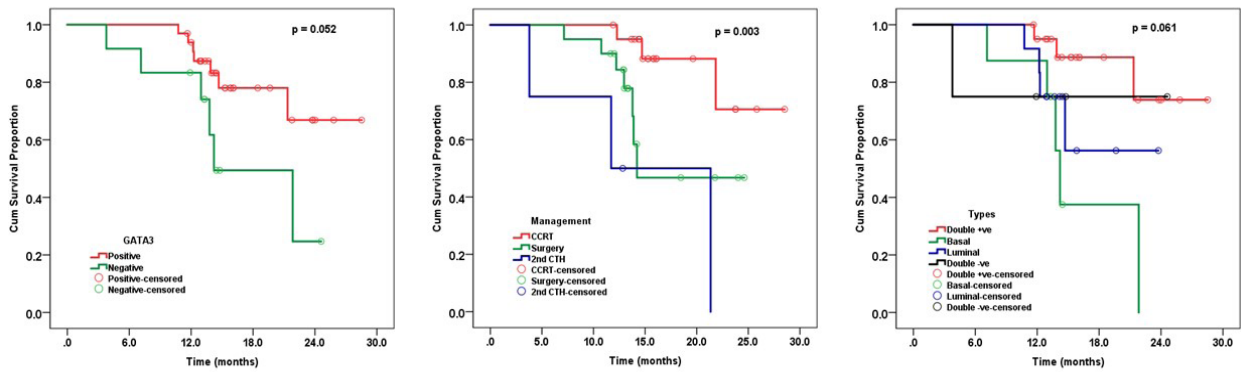


Figure 6. Overall Survival in Relation to GATA3 Expression, Management Modality, and Molecular Subtypes

highest OS of 88.7, yet the difference was not statistically significant (p=0.061). OS was not affected by smoking, grade, CIS, LVI, or PNI.

Multivariable cox regression model showed that nodal status was the only independent predictor for mortality with p value 0.013 and HR 7.1 (95% CI 1.518-32.903), while T stage and treatment groups showed no statistically significant increase hazard of progression after adjustment for nodal status with p values 0.687, and 0.137 (Table 8), (Figure 1-6)

Discussion

This prospective study demonstrated that the immunohistochemical biomarkers GATA3 and CK5/6 can be used to classify cases of MIBC into distinct molecular subgroups. These subgroups did not show a difference in the response to NACT. However, the treatment outcome was correlated with GATA3 expression. GATA3-positive tumors showed a trend towards better OS than those with GATA3-negative tumors (p=0.052). OS was also better in the luminal and double-positive subtypes. The double-positive subgroup had better PFS than other molecular subgroups with a trend toward statistical significance (p=0.087). On the other hand, there was no significant impact of CK5/6 positivity on OS (p=0.572) or PFS (p=0.707). Survival was primarily affected by treatment modality and N-staging.

We used GATA3 and CK5/6 based on the findings of a meta-analysis of the genome expression profiles involving 937 samples of MIBC [13]. The authors reported over 90% accuracy in classifying bladder tumors into basal and luminal categories using the expression levels of these two markers. By comparing the IHC staining patterns of GATA3 and Ck5/6 with the outcomes of mRNA-based classification, Guo et al. [18] demonstrated that in the majority of cases, it is possible to distinguish between the luminal and basal molecular subtypes reliably.

Of the 45 patients, GATA3 expression was detected in 33 (73.3%). Previous studies reported widely variable rates of GATA3 positivity in UC from below 5% to 100% [19]. In several studies, GATA3 was the most frequently expressed marker in MIBC cases [20–24]. On the contrary, CK5/6 was the most frequent marker in the present study (64.4%). Other studies reported proportions varying between 10% and 45% [16, 25–29]. Low expression was demonstrated in many studies, e.g., Hashmi et al. found positive CK5/6 expression in 19.7% of their series of 127 cases [30]. In two studies from Sweden, basal/squamous tumors were the least common type detected in approximately 10% of cases [31, 32]. However, relatively higher CK5/6 expression was reported in two studies from Egypt; one reported positive expression in 48.3% of 60 cases [33], and the other found 66% CK5/6 in cases of MIBC [34]. Geographic variation may be a factor accounting for the difference between the current study and the two Egyptian studies. We did not observe a relationship between CK5/6 expression and history of bilharziasis or detection of bilharzia ova. The high expression might be attributed to the relatively advanced stage in the current study as 80% of the patients had stage IIIA disease. As most basal-type cases involve invasive tumors, a significant proportion is anticipated to comprise T2 and T3 tumors [26].

Numerous studies have shown that GATA3 inhibits the migration, invasion and epithelial-mesenchymal transition of cancer cells. It was observed that high-grade MIBC was accompanied by GATA3 loss [35]. GATA3 over-expression can predict a favorable prognosis, lower grade and earlier tumor stage. High expression of GATA3 in papillary tumors accounted partially for the favorable prognosis of these tumors compared to nonpapillary tumors [36].

CK5/6 is a basal cytokeratin typically found in squamous cell carcinoma and squamous epithelium. Up to 50% of advanced UC may exhibit divergent differentiation (including squamous component) associated with a poor prognosis for the disease [37]. IHC markers, such as CK5/6, were utilized by Gaisa et al. [38] to identify squamous differentiation in a significant proportion of UC lacking morphologic evidence of differentiation. Langer et al. [37] assessed the prognostic significance of keratin subtyping in UC and uncovered the prognostic implications of several cytokeratin staining techniques such as CK5/6. It was shown that a higher proportion of patients with basal/squamous-like tumors, characterized by low GATA3 expression, exhibited a favorable response to platinum-based NACT [39].

In the present study, molecular subtypes did not affect the response to GC NACT. Also, GATA3 and CK5/6 expressions were not associated with the response to NACT. Besides, the four molecular subgroups were not associated with the patient's age, smoking or pathological characteristics. We noticed that 75% of patients with basal/squamous-like tumors and 40% of luminal tumors were non-responsive to NACT with no significant intergroup difference (p=0.197). Our results are similar to Kamoun et al. [14] where the response to NACT was not significantly different between various molecular subgroups.

The response to NACT in the present study was assessed using CT and cystoscopy. The response rate was 46.6%, including those with CR and PR, while ten patients (22.2%) showed progressive disease. Many studies assessed the response to GC in the NACT setting. Dracham et al. [40] tested the response rate and toxicity to three cycles of GC NACT regimen in MIBC. They reported 22.5% and 65% CR and PR rates, respectively. The CR rate was lower than that of the present study (35.6%), while the PR was much higher (11%). This difference could be attributed to different inclusion criteria. We enrolled patients with stage T2-T4a, N1, and M0, while all patients in Dracham's study were node-negative. The pathological CR has varied from 26% to 42% in different studies [41–44]. In a similar group of 128 patients with T2-T4aN1M0 disease, Sung et al. reported a 41% CR rate [45]. A low rate of CR of 10.2% was recorded in another study, where 75% of the participants had T3-4 disease [46]. Noteworthy is that this study tested the effect of NACT without prior maximal TURB.

We found that T4 and/or N1 disease was associated with low response to NACT (p=0.015). Besides, smoking negatively affected response to NACT (p=0.025).

N-Stage 1 per se is associated with NACT (p=0.051). On the other hand, molecular subgroups did not affect response to NACT (p=0.344). Currently, molecular subtypes lack consistent association with CR to NACT, and the available experience is insufficient to warrant their routine application as response predictors [47].

In agreement with our study, Boeri et al. [48] found smokers to be more likely non-responders (p=0.007). Contrarily, Kim et al. [49] found no such association. Nicotine was found to stimulate Stat3 (signal transducer and activator of transcription), a transcriptional factor involved in cellular proliferation and apoptosis. This consequently leads to cisplatin chemoresistance in human BC lines [50]. Smoking status was significantly associated with recurrence after RC [51].

To date, there are no established predictors of response to NACT. Several predictive factors were investigated, including clinical, histological, pathological, and molecular predictors [52]. Many molecular predictors have been studied. Basal MIBCs have been demonstrated to be aggressive; however, their sensitivity to cisplatin-based CTH was shown [53], but not in the current investigation. Seiler et al. discovered that NACT is most beneficial for patients with basal tumors [54]. Luminal MIBC subtypes are typically responsive to NACT whereas CTH resistance is believed to be present in p53-like tumors [55]. According to the findings of Sjodahl et al. [56], genomically unstable tumors exhibited higher rates of CR (52%) in comparison to subtypes that were basal/squamous (21%) and urothelial-like (31%).

In the present study, the adopted combined modality therapy (CMT), i.e., maximal TURBT, NACT, and CCRT, was associated with significantly better OS and PFS than those managed by surgery or second-line CTH. As the modality was based on the response to NACT. This bladder-sparing therapy has witnessed a surge in recent years [57]. However, there has been no effective comparison of the oncological outcomes of CMT and RC in a prospective randomized trial. Many observational studies have compared their oncological outcomes; the 5-year OS and the salvage cystectomy rates have fluctuated between 36% and 74% and 10% and 30%, respectively [58–60]. A meta-analysis of the pooled data from six RTOG bladder-sparing studies revealed salvage cystectomy rates of 21% and 5-year OS of 57% [61]. Besides, in most studies, patients treated with CMT were usually older and had more co-morbidities when compared with those who underwent RC; hence, they may have a poorer prognosis [62].

In addition to the treatment modality, OS and PFS were negatively affected by the N-stage in the present study. It was noticed that patients with T4 and/or N1 disease had significantly lower OS and PSF than others. Patients with GATA3-positive tumors showed a trend towards better OS (78%) than those with GATA3-negative tumors (49.4%, p=0.052). PFS was slightly higher in GATA3 positive cases (p=0.186). OS was better in the luminal than the basal subgroup (56.3% vs. 37.5, respectively), while the double positive showed the highest OS of 88.7%, yet the difference was trending to statistical significance (p=0.061). However, the difference in PFS between the four molecular subtypes was insignificant (p=0.160). Compared to other molecular subtypes, the double-positive type had a better OS (p=0.026) and an apparently higher PFS (p=0.087). There was no significant impact of CK5/6 positivity on OS (p=0.572) or PFS (p=0.707).

Many studies reported better prognosis in patients with GATA3-positive and double-positive tumors, which agrees with the current research. Yet, some reported the association of good prognosis in CK5/6 tumors. In agreement with the present study, Jangir et al. reported the best outcome in patients with tumors expressing GATA3, irrespective of the expression of other markers. At the same time, CK5/6 was not associated with the outcome [16]. Also, Yuk et al. [63] reported findings concordant with the present study. They found that GATA3 expression > 10% was positively correlated with recurrence-free survival (p=0.032). Even in patients with GATA3 expression of only ≥ 1, there was a tendency for better OS and recurrence-free survival than those with < 1%.

Another study found that luminal subtype tumors were associated with a better PFS but similar OS compared to double-positive tumors [64]. Wang et al. reported in CTH naïve patients that recurrence-free survival was observed in association with low GATA3 staining on multivariate analyses (p = 0.002) [20]. In patients managed by RC, both GATA3 and CK5/6 were significantly associated with better OS (p= 0.004 and 0.02). The mixed subtype had the highest 5-year OS of 42.8%, while the double-negative subtype had the lowest (7.14%) [65]. Hodgson et al. found a trend towards worse disease-specific survival in patients with basal-subtype tumors (p=0.078).[25] In a recent study, the luminal subtype of MIBC had a significantly longer OS and DFS than the basal subtype based on an IHC panel of GATA3, CK5/6, and P53 [66]. Like the present study, CK5/6 expression did not affect the OS of 140 patients with MIBC treated with NACT [67]. Also, Koll et al. [68] found no association between CK5/6 or GATA3 outcomes of patients with MIBC.

The limited sample size and absence of gene expression profiling to validate concordance between molecular subtypes and IHC marker expression constituted the limitations of our study. Moreover, the discrepancy in the results may be attributed to the false positivity of CK5/6 due to the polyclonal nature of the antibodies used together with the low cut off of the positivity (>20%). However, the polyclonal antibody was a better option in our study as the paraffin blocks were initially fixed and processed in various pathology centers [69] Nevertheless, the risk of selection bias and clinicopathologic stage discordance cannot be disregarded. Furthermore, only two IHC-marker analyses were used to approximate basal and luminal molecular subtypes. These limitations are added to the numerous drawbacks of the IHC technique despite its affordability, speed, and accessibility, such as the absence of a universally accepted scoring system and discrepancies in the sensitivity and specificity of the antibodies.

In conclusion, this study supports the clinical utility of immunohistochemistry for predictive classification of MIBC patients. A combination of GATA3 and CK5/6 IHC can classify MIBCs into prognostically significant subtypes. These subtypes were not correlated with the

response to NACT but correlated with treatment outcomes. GATA3-positive tumors and luminal and double-positive subtypes tend to have better OS and PFS. CK5/6 positivity did not impact the treatment outcome. Neoadjuvant CTH may help select patients for bladder-preserving definitive treatment.

Author Contribution Statement

Hagar Hamdy: protocol writing, data collection, patient follow up, assessment of response. Ahmed Hamed: manuscript writing, data review and management, patient follow up, assessment of adverse events. Mohamad Emam: Reviewing the H&E slides and the immunohistochemical slides. Ahmed Assem: Baseline cystoscopy for all patients, follow up cystoscopy for the CCRTH arm and radical cystectomy for the patients in the surgery arm. Ahmad Hassan: patient follow up, assessment of adverse events. Emad Hamda: senior supervision.

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Scientific approval

Study protocol was submitted and approved by the local scientific committee of oncology department followed by approval from scientific committee of faculty of medicine, Cairo university.This study is for MD thesis.

Ethical considerations

The study protocol was approved by the Ethical Committee of NEMROCK. All patients provided written informed consent before enrollment in the study after fully explaining the study procedures and possible risks with ethical code.

Conflict of interest

None.

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