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Association of immunohistochemical markers of tumor subtype with response to neoadjuvant chemotherapy and survival in patients with muscle-invasive bladder cancer

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Purpose: A readily accessible biomarker to identify which patients with bladder cancer are more likely to respond to neoadjuvant chemotherapy (NAC) could help clinicians avoid unnecessary chemotherapy and prevent its subsequent complications in some patients. The primary objective of this study was to investigate the association of immunohistochemical markers of tumor subtype with response to NAC and survival of patients with muscle-invasive bladder cancer (MIBC).

Materials and Methods: MIBC patients treated with NAC were retrospectively included. The tissue microarrays were assembled from transurethral resection of bladder tumor (TURBT) specimens and immunohistochemistry (IHC) was performed. The association of independent variables, including IHC markers, and clinical covariates with clinical complete response to NAC and with overall survival was assessed by using logistic regression and Cox proportional hazard regression analysis, respectively. Kaplan-Meier curves were plotted for different IHC-based tumor subtypes.

Results: Data from 140 MIBC patients treated with NAC were retrospectively reviewed. A total of 63 patients with available TURBT specimens were eligible to be included in the analysis. Our results showed that the IHC signature of KRT5/6(+)/KRT20(-), as a combined marker of basal subtype, was the only covariate significantly associated with complete response to NAC (p=0.037). Moreover, we found no statistically significant differences in overall survival between different IHC-based subtypes (p=0.721).

Conclusions: The IHC expression of KRT5/6 and KRT20, as a readily accessible combined marker, may help us to identify the patients most likely to benefit from chemotherapy. The clinical utility of this marker needs to be established in larger prospective studies.

Keywords: Biomarker; Bladder cancer; Chemotherapy; Immunohistochemistry

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INTRODUCTION

Muscle-invasive bladder cancer (MIBC) is one of the leading causes of genitourinary cancer-related mortality [1]. Neoadjuvant chemotherapy (NAC) is recommended for patients with MIBC, but less than half of patients experience a substantial response to the chemotherapy [2,3]. Non-responding patients are unlikely to derive clinical benefit, are subject to considerable toxicity, and experience a delay in receiving subsequent treatment [4,5]. Thus, there is a high unmet need for clinically applicable biomarkers to guide personalized decision-making.

Recently, molecular subtypes of bladder cancer have attracted substantial interest [6-8]. In a whole-transcriptome study by Seiler et al. [9], basal bladder tumors showed the most improvement in survival with NAC compared with surgery alone. Those authors suggested that patients with the basal subtype should be prioritized for NAC [9]. Similar results were shown by other investigators [10,11].

Although potential applications of gene-expression-based molecular subtyping have been proposed to identify chemosensitive patients, the application of whole-transcriptome profiling for all patients in clinical practice may be an expensive and time-consuming procedure. Because immunohistochemistry (IHC) is an inexpensive, readily accessible, and reliable technique in practice, subtyping of bladder tumors using a limited number of IHC markers could be considered as an attractive surrogate for use in the clinical setting. Although a strong overlap has been shown between subtypes in some studies, a small panel of IHC markers, including KRT5/6 and KRT14 for basal tumors and GATA3 and KRT20 for luminal tumors, have shown promising differential expression patterns [11-13].

Given the existing evidence, we hypothesized that assessing the expression pattern of a panel of IHC markers of tumor subtype in transurethral resection of bladder tumor (TURBT) specimens could be effective for identifying patients who are likely benefit from chemotherapy. Thus, we aimed to investigate the association of IHC markers of tumor subtype with response to NAC and the survival outcome of patients with MIBC.

MATERIALS AND METHODS

1. Study design and patients

To perform this retrospective cohort study, we reviewed data from 140 consecutive patients with MIBC treated with platinum-based NAC in two tertiary referral hospitals (Labbafinejad Hospital and Shohada-e-Tajrish Medical Center) between 2009 and 2019. After excluding 72 patients for whom formalin-fixed paraffin-embedded samples were not available and 5 patients for whom data were missing on the status of treatment response, a total of 63 patients were included in a complete-case analysis. Patients were staged by using TURBT and computed tomography (CT) of the chest, abdomen, and pelvis. All patients were administered gemcitabine (1,000 mg/m^2, intravenously, 30 min) on days 1 and 8, every 21 days. Following gemcitabine administration, patients with a creatinine clearance >60 mL/min received cisplatin on days 1 and 2 every 21 days (70 mg/m^2, 60 min), whereas those with a creatinine clearance <60 mL/min received carboplatin (area under the curve [AUC]=5, intravenously over 30 min) on day 1 every 21 days. Carboplatin doses were adjusted for renal function per the label by using the Cockcroft-Gault formula. Patient characteristics and treatment outcomes were obtained by retrospective review. All tissue samples were re-reviewed by an expert uro-pathologist. Ethical approval for the study was obtained by the Institutional Review Board of the Shahid Beheshti University of Medical Sciences (approval number: IR.SBMU.UNRC.REC.1398.15). The requirement for informed consent was waived owing to the retrospective approach. All procedures performed in the study involving human samples were in accordance with the 1964 Helsinki Declaration and its later amendments.

2. Outcome assessment

Clinical complete response (CR) to NAC and overall survival (OS) were selected as the study endpoints. Clinical CR was defined as no residual bladder cancer in post-NAC cystoscopy and CT scan, as assessed almost 1 month after the end of treatment. OS was defined as the interval between the start of treatment and patient death or the last follow-up. Patients were followed by review of medical records or by telephone contact. The assessors of outcome were blinded to the study covariates. A panel of IHC markers and patient characteristics were considered as the covariates.

3. Tissue microarray building

Tissue microarrays (TMAs) were assembled as previously described [14]. All hematoxylin and eosin (H&E)-stained slides were reviewed by a pathologist with subspecialty expertise in urologic pathology, who identified well-preserved areas rich in tumor cells. The corresponding areas were marked on paraffin blocks and three parallel tissue cores were obtained per tumor to account for intratumoral heterogeneity. Then, the cores were assembled in recipient paraffin blocks using a tissue arrayer (Galileo TMA CK3500 Tissue Micro arrayer;

ISETMA Software, Integrated System Engineering, Milan, Italy). Tissue arrays were constructed by placing cores of 1-mm diameter in recipient paraffin blocks. Finally, consecutive sections (with a thickness of 3 μm) were cut from each TMA block, mounted on microscope slides, and immunohistochemically assayed. Four noncancerous bladder tissues were also included in the TMA blocks as a control group.

4. Immunohistochemistry technique and antibodies

IHC was performed on the TMA slides with a standard technique as previously defined with some modifications [14,15]. Briefly, tissue slices were deparaffinized at 55°C for 10 minutes, cleared in xylene, and then rehydrated by incubating the slices in solutions with decreasing alcohol content. Antigen retrieval was conducted by boiling the samples in tris-EDTA buffer (pH 9.0) for 34 minutes in a standard microwave. The endogenous peroxidase was blocked with 3% H_2O_2 for 10 minutes. Samples were immunostained at 4°C in blocking solution with primary antibodies. After washing with phosphate buffered saline (3 times/5 min), the sections were incubated with appropriate secondary antibody (Detection kit; MAD-000237QK; Master Diagnostica, Granada, Spain) for 45 minutes. Then, the TMA slides were visualized with 3,3'-diaminobenzidine substrate as chromogen for 10 minutes at room temperature. The sections were counterstained with hematoxylin, dehydrated in alcohol, cleared with xylene, and mounted for examination. All IHC analyses were performed by researchers who were blinded to clinical data. The stained slides were reviewed by the pathologist, using a semi-quantitative scoring system in a coded manner, who was blinded to clinical data. Samples were scored on both intensity and percentage of positive tumor cells. Intensity was scored as 0 (absent), 1 (weak), 2 (moderate), or 3 (strong). All cases with an intensity score ≥2 and tumor positivity ≥20% were considered positive, as previously defined [12].

The following primary antibodies were selected for use in this study, based on the IHC markers introduced by Dadhania et al. [12]: KRT5/6 (MAD-000651QD) and KRT14 (MAD-005103QD) as basal markers; KRT20 (MAD-005105QD) and GATA3 (MAD-000632QD) as luminal markers; and desmin (MAD-001011QD) as a p53-like marker. To determine two additional rare phenotypes, i.e., mesenchymal-like and small-cell/neuroendocrine-like [16], antibodies against vimentin (MAD-000326QD) and CDH1 (MAD-000761QD) were also used. All antibodies were from Master Diagnostica.

5. Statistical analysis

The associations between CR and independent variables were evaluated by using logistic regression analysis. Interaction terms between basal and luminal markers were assessed as potential combined IHC markers. Patient survival was estimated by using the Kaplan–Meier method. A log-rank test was used to compare OS between the patient groups. A Cox proportional hazard regression analysis was used to identify the significant determinant factors for OS. A bootstrapping technique was applied by using 1,000 random data sets generated from the original data. Median follow-up time was calculated by using a reverse Kaplan–Meier method. All statistical tests were two-sided and p-values ≤0.05 were considered statistically significant. The statistical analyses were performed by using IBM SPSS, version 23 (IBM Corp., Armonk, NY, USA).

RESULTS

1. Patient characteristics

A total of 140 consecutive adult patients with MIBC treated with NAC between April 2009 and April 2019 were retrospectively evaluated for inclusion in the study. A total of 72 patients for whom formalin-fixed paraffin-embedded samples were not available and 5 patients for whom the re-

Table 1. Baseline characteristics of the patients

Characteristic	CR (n=20)	No CR (n=43)
Age (y)	68.4±9.5	68.6±10.5
Gender		
Woman	3 (15.0)	4 (9.3)
Man	17 (85.0)	39 (90.7)
T stage		
T2	14 (70.0)	26 (60.4)
T3	5 (25.0)	14 (32.6)
T4a	1 (5.0)	3 (7.0)
Tumor grade		
Low	0 (0.0)	2 (4.7)
High	20 (100.0)	41 (95.3)
Node status		
Negative	15 (75.5)	36 (83.7)
Positive	5 (25.0)	7 (16.3)
Chemotherapy regimen		
Gem/Cis	11 (55.0)	23 (53.5)
Gem/Carbo	9 (45.0)	20 (46.5)
Creatinine clearance (mL/min)	49.7±15.4	55.7±20.0
Previous BCG therapy		
No	18 (90.0)	32 (74.4)
Yes	2 (10.0)	11 (25.6)
Smoking status		
No	12 (60.0)	30 (69.8)
Yes	8 (40.0)	13 (30.2)

Values are presented as mean±standard deviation or number (%).
CR, complete response; Gem, gemcitabine; Cis, cisplatin; Carbo, carboplatin; BCG, bacille Calmette-Guérin.

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sponse status was not documented were excluded from the analysis. Thus, 63 patients were considered for the complete-case analysis and were followed until April 2020. Patient characteristics are summarized in Table 1. None of the clinical covariates differed significantly between patients with and without CR.

2. Expression of immunohistochemistry markers

Representative images of positive and negative IHC stains for selected basal (KRT5/6 and KRT14) and luminal (KRT20 and GATA3) markers in TMA samples are illustrated in Fig. 1.

Because KRT14 stains were positive in 10 only cases, and GATA3 stains were negative in only 8 cases, these two markers were not considered to represent an effective differentiation marker in our study. In addition to the interaction between KRT5/6 and KRT20, as the best combination, other potential combined markers identifying basal and luminal subtypes, and their relationships with CR are indicated in Supplementary Table 1. According to the best dual-marker signature, 15 (23.8%), 12 (19%), 11 (17.5%), and 25 (39.7%) patients were classified into the following subtypes: basal, as assessed by KRT5/6(+)/KRT20(-); luminal, as assessed by KRT20(+)/KRT5/6(-); double-negative, as assessed by KRT5/6(-)/KRT20(-); and double-positive, as assessed by KRT5/6(+)/KRT20(+), respectively. A heatmap depicting the expression of these markers on the basis of IHC-based subtypes is shown in Supplementary Table 2. No associations between the IHC-based subtypes and clinical variables were found. Vimentin was expressed in infiltrating mesenchymal cells of the patients and not by the tumor cells. As expected, almost all tumor cells were positive for CDH1 protein and negative for vimentin; therefore, CDH1 and vimentin were not analyzed further. Desmin was expressed in the stromal component of almost all tumor samples. In addition, 19% of the tumor cells were also positive for this stromal marker. The IHC stains for CDH1, vimentin, and desmin in representative TMAs are illustrated in Supplementary Fig. 1.

3. Tumor response

After chemotherapy, clinical CR was achieved in 20 (31.7%) patients. The associations of singular and combined IHC markers with CR are shown in Table 2. Our results indicated that the IHC signature of KRT5/6(+)/KRT20(-), as a combined marker of basal subtype, was the only covariate significantly associated with the CR to NAC (p=0.037). As shown in Table 2, 40.0% of patients with CR were classified in the basal group, compared with only 16.3% of patients without CR. The relationships between CR and additional potential combined markers identifying basal and luminal subtypes are indicated in Supplementary Table 1.

Notably, there was no significant association between CR and the chemotherapy regimens used (p=0.906). Among patients who received gemcitabine/cisplatin, 32.4% exhibited CR, compared to 31% in the gemcitabine/carboplatin group. Also, no significant relationships were found between other clinical variables and CR (Table 2).

4. Survival outcome

After a median follow-up of 41 months (range, 12–76 months), 30 patients died. Achievement of clinical CR after chemotherapy was significantly associated with better survival in our population (p=0.004). Kaplan–Meier survival curves for the basal, luminal, double-negative, and double-positive groups, on the basis of IHC expression of KRT5/6 and KRT20, are shown in Fig. 2. The median OS was 28 months (95% confidence interval [CI], 7.5–48.5 months), 39 months (95% CI, 12.2–65.8 months), 55 months (95% CI, 21.9–88.1 months), and 34 months (95% CI, 23.1–44.9 months) for patients categorized in the luminal, basal, double-negative, and double-positive group, respectively. No statistically significant difference in OS was found between the IHC-

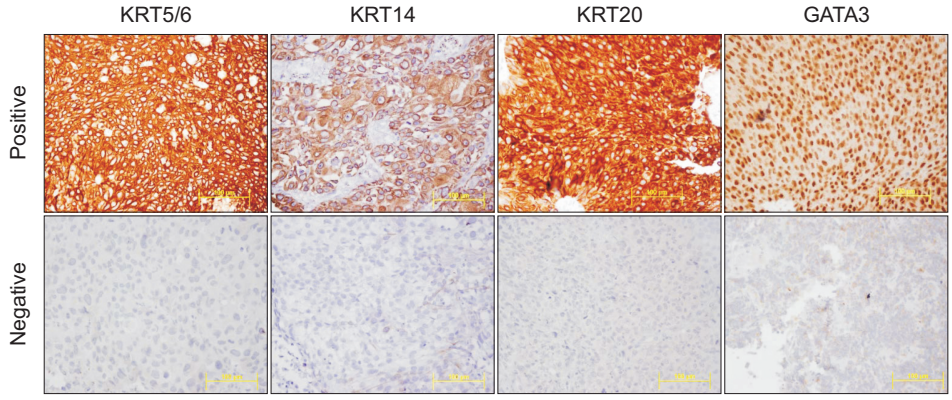

Fig. 1. Representative images of immunohistochemical staining for selected basal (KRT14 and KRT5/6) and luminal (KRT20 and GATA3) markers.

Table 2. Association of covariates with clinical complete response to chemotherapy

Covariate	CR (n=20)	No CR (n=43)	Odds ratio (95% confidence interval)	Bootstrap p-value
	% of patients with covariate			
Singular IHC markers				
KRT5/6(+)	75.0	58.1	2.16 (0.66–7.02)	0.210
KRT14(+)	25.0	11.6	2.53 (0.64–10.03)	0.180
KRT20(+)	55.0	60.5	0.80 (0.27–2.33)	0.711
GATA3(+)	85.0	88.4	0.74 (0.16–3.48)	0.666
Desmin(+)	20.0	18.6	1.09 (0.29–4.17)	0.884
Combined IHC markers				
KRT5/6(+)/KRT20(-) (basal subtype)	40.0	16.3	3.43 (1.03–11.46)	0.037
KRT20(+)/KRT5/6(-) (luminal subtype)	20.0	18.6	1.09 (0.29–4.17)	0.873
KRT5/6(-)/KRT20(-) (double-negative)	5.0	23.3	0.17 (0.02–1.46)	0.058
KRT5/6(+)/KRT20(+) (double-positive)	35.0	41.9	0.75 (0.25–2.25)	0.634
Clinical covariates				
Age >65	65.0	53.5	1.61 (0.54–4.84)	0.389
Woman, gender	15.0	9.3	1.72 (0.35–8.54)	0.487
T stage, T3–T4a	30.0	39.5	0.65 (0.21–2.04)	0.486
Node positive	25.0	16.3	1.71 (0.45–6.27)	0.410
Gem/Cis regimen	55.0	53.5	1.06 (0.37–3.08)	0.906
Creatinine clearance, <60 mL/min	80.0	67.4	1.93 (0.54–6.87)	0.291
Previous BCG therapy	10.0	25.6	0.32 (0.06–1.62)	0.091
Smoking	40.0	30.2	1.54 (0.51–4.65)	0.431

CR, complete response; IHC, immunohistochemistry; Gem, gemcitabine; Cis, cisplatin; BCG, bacille Calmette-Guérin.

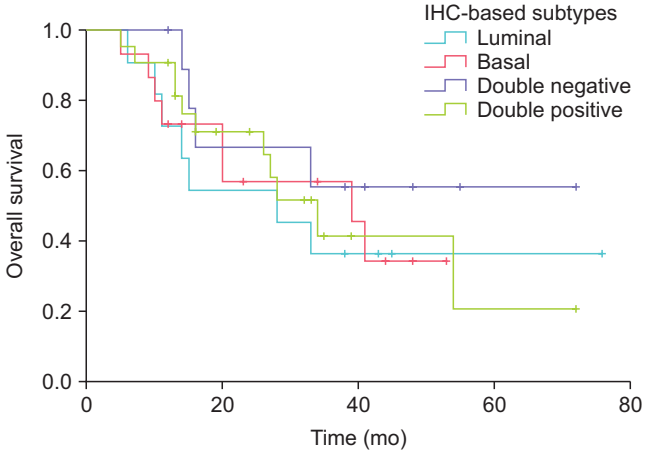

Fig. 2. Kaplan–Meier curves for overall survival based on immunohistochemical expression of KRT5/6 and KRT20. Patients were grouped into the following subtypes: basal, as assessed by KRT5/6(+)/KRT20(-); luminal, as assessed by KRT20(+)/KRT5/6(-); double-negative, as assessed by KRT5/6(-)/KRT20(-); and double-positive, as assessed by KRT5/6(+)/KRT20(+), respectively. IHC, immunohistochemistry.

based subtypes, using the log-rank test (p=0.721). In a Cox proportional hazard regression analysis, age >65 years was independently associated with poorer OS after NAC (hazard ratio [HR], 2.26; 95% CI, 1.02–5.05), but failed to remain significant after adjustment for creatinine clearance (HR, 1.54; 95% CI, 0.58–4.10). The relationships between different

Table 3. Cox regression analysis for the relationship between covariates and overall survival

Covariate	Hazard ratio (95% confidence interval)	Bootstrap p-value
Singular IHC markers		
KRT5/6(+)	1.18 (0.56–2.49)	0.671
KRT14(+)	1.66 (0.67–4.09)	0.336
KRT20(+)	1.25 (0.60–2.60)	0.573
GATA3(+)	1.50 (0.52–4.35)	0.454
Desmin(+)	1.93 (0.87–4.27)	0.053
Combined IHC markers		
KRT5/6(+)/KRT20(-) (basal subtype)	1.16 (0.51–2.62)	0.707
KRT20(+)/KRT5/6(-) (luminal subtype)	1.29 (0.55–3.00)	0.589
KRT5/6(-)/KRT20(-) (double-negative)	0.56 (0.19–1.61)	0.288
KRT5/6(+)/KRT20(+) (double-positive)	1.05 (0.50–2.22)	0.881
Clinical covariates		
Age >65	2.26 (1.02–5.05)	0.033
Woman, gender	0.25 (0.34–1.83)	0.099
T stage, T3–T4a	1.26 (0.60–2.63)	0.531
Node positive	0.99 (0.40–2.42)	0.978
Gem/Cis regimen	0.74 (0.36–1.54)	0.405
Creatinine clearance, <60 mL/min	2.02 (0.85–4.80)	0.081
Previous BCG therapy	0.73 (0.28–1.91)	0.537
Smoking	1.28 (0.61–2.67)	0.491
Clinical CR	0.38 (0.16–0.89)	0.004

IHC, immunohistochemistry; Gem, gemcitabine; Cis, cisplatin; BCG, bacille Calmette-Guérin; CR, complete response.

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covariates and OS are shown in Table 3.

DISCUSSION

Identifying which patients are more likely to respond to treatment on the basis of molecular subtype classification seems to be a promising strategy for improving survival benefit and preventing unnecessary toxicity [2]. Several molecular classifications for bladder cancer have been introduced. In a study by the University of North Carolina, muscle-invasive tumors were grouped into basal and luminal subtypes [17]. The MD Anderson classification added a third group, named p53-like [11]. Several additional molecular classifications with some overlap have been proposed to date [7,16,18]. Previous studies, using whole-transcriptome profiling, indicated that patients with the basal subtype are most likely to benefit from NAC. In a large, multicenter retrospective study comparing patients treated with NAC or not, the analysis of non-NAC-treated patients indicated that OS is shorter in cases with the basal subtype than in cases with the luminal subtype (HR, 2.22; p=0.002), reflecting the intrinsic aggressiveness of basal tumors [9]. In contrast, the OS of patients with basal and luminal subtypes was not significantly different in the NAC-treated group (HR, 0.84; p=0.61). Thus, the authors showed that the impact of NAC on OS was greatest in patients with basal tumors. Also, McConkey et al. [10], in a small study of 60 patients enrolled in a neoadjuvant trial, showed that survival was better in patients with basal tumors than in patients with luminal or p53-like tumors. Furthermore, the association of distinct molecular subtypes with response to NAC was demonstrated in a previous study [11]. Taken together, these observations raise the hypothesis that the natural course of basal disease progression might be affected by NAC. The rapid proliferation of basal tumors, and thus, their particular sensitivity to frontline chemotherapy, is a possible explanation [2].

The basal and luminal subtypes were originally defined by using global transcriptomics, but their phenotypes can also be recognized by using IHC [12,13,16]. In clinical practice, application of a limited number of IHC markers may confer multiple advantages over whole-transcriptome profiling and may be considered an attractive surrogate. In this study, bladder tumors were assigned to distinct subtypes by using a set of markers as previously described [12]. Since previous reports showed significant overlap between IHC markers in identifying subtypes [12], a negative marker for both basal and luminal subtypes may aid in better discrimination. Guo et al. [13] concluded that IHC staining with GATA3 and KRT5/6 is a simple classifier of molecular subtypes that is effective in over 80% of cases and that may strongly translate the transcriptomic classifiers into IHC assays. In our study, there were no significant associations between CR and singular IHC markers; but interestingly, the KRT5/6(+)/KRT20(-) signature, a dual-marker combination for the basal subtype, was associated with the response to NAC. However, the association of CR with additional combined markers of basal subtype did not reach statistical significance, probably because of the extremely high and low positive cases for the GATA3 and KRT14 markers, respectively. Further investigation using different antibodies for GATA3 and KRT14 may provide better results.

A restricted number of studies have investigated the relationship between IHC-based subtypes and chemotherapy outcomes. In a retrospective analysis of bladder cancer patients treated with chemoradiation, the impact of IHC-based subtypes on survival and CR to chemoradiation therapy were assessed by Tanaka et al. [19]. More recently, consistent with our experience, Font et al. [20] showed that patients with basal/squamous (BASQ)-like tumors (KRT5/6/KRT14 high; FOXA1/GATA3 low) were more likely to achieve a CR to NAC (odds ratio, 3.96; p=0.017). Font et al. [20] also reported findings similar to ours regarding subtype-related survival outcomes. Those authors stated that the lack of significant survival differences between patients with basal and luminal tumors may reflect the clinical benefit from NAC. Also, our results are in line with those of Seiler et al. [9], who demonstrated that the OS of patients with basal and luminal subtypes was not significantly different in the NAC-treated group.

There are several limitations to our study in addition to its retrospective nature. First, despite being cheap, fast, and universally available, the IHC technique harbors many limitations, including the lack of a uniformly approved scoring system and variation in the sensitivity and specificity of the different antibodies used. Some protocols and strategies were established by the Lund group to uniformly determine molecular subtypes using IHC and to increase its generalizability [21]. Second, although TMA is a valid method for the assessment of bladder cancer samples [22], intratumoral heterogeneity of bladder cancer by molecular subtypes may complicate the assessment of small tissues [23]. Considering this problem, three parallel tissue cores were obtained per tumor in this study to account for intratumoral heterogeneity. Third, the assessment of pathologic CR was not applicable in our study because the majority of patients who achieved clinical CR after NAC received chemoradiation instead of surgery. However, in the study conducted by Tanaka et al. [19], the correlation of each IHC-based subtype

with the pathologic rate of CR was completely analogous to that with clinical rate of CR. Fourth, because of the unacceptable rate of missing data for post-cystectomy pathology, and the inaccessibility of reports on lymph node density and TURBT, detailed pathologic data were not analyzed. Also, the lack of accurate recurrence data in our retrospective study prevented us from using cancer-specific survival as an end point. Fifth, there is no validation cohort or mRNA classification that correlates to IHC-based subtypes. Last, owing to the relatively small study population, our statistical power was not strong. Of note, despite the significant p-value observed, the fairly wide range of the confidence interval for the odds ratio should be considered. Therefore, to confirm the results of our study and to establish the exact role of IHC markers in the management of MIBC patients, further larger studies are highly warranted.

CONCLUSIONS

The combined IHC expression of KRT5/6 and KRT20 is a readily available and cost-effective biomarker for stratifying NAC administration, although prospective validation in a large dataset is needed before clinical implementation.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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AUTHORS' CONTRIBUTIONS

Conception and design: Abolfazl Razzaghdoust, Mahdi Ghajari, Bahram Mofid, and Abbas Basiri. Acquisition of data: Abolfazl Razzaghdoust, Mahdi Ghajari, Peyman Mohammadi Torbati, Anya Jafari, Mohammad-Reza Fattahi, and Maryam Salahi. Analysis and interpretation of data: Abolfazl Razzaghdoust, Mahdi Ghajari, Peyman Mohammadi Torbati, Bahram Mofid, and Abbas Basiri. Writing, review, and/or revision of the manuscript: Abolfazl Razzaghdoust, Mahdi Ghajari, Peyman Mohammadi Torbati, Anya Jafari, Mohammad-Reza Fattahi, Maryam Salahi, Bahram Mofid, and Abbas Basiri.

SUPPLEMENTARY MATERIALS

Supplementary materials can be found via https://doi.org/10.4111/icu.20200425.

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