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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Research

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Abstract

Background: Clinical application of a readily accessible biomarker for identification of bladder cancer patients most likely to respond to neoadjuvant chemotherapy (NAC) could help clinicians avoid unnecessary chemotherapy and prevent its subsequent complications in patients unlikely to drive clinical benefit. The primary objective of this study was to investigate the association of immunohistochemical markers of tumor subtype with response to NAC and survival of muscle-invasive bladder cancer (MIBC) patients.

Methods: MIBC patients treated with NAC in two tertiary referral hospitals were retrospectively included in the study. The tissue microarrays (TMAs) were assembled from transurethral resection of bladder tumor (TURBT) specimens and immunohistochemistry (IHC) was performed on the TMA slides. The association of independent variables, including singular IHC markers, combined IHC markers, and clinical covariates with clinical complete response (CR) to NAC, and overall survival (OS) was assessed using logistic regression and Cox proportional hazard regression analysis, respectively. Kaplan-Meier curves were plotted for different IHC-based subtypes.

Results: Data from 140 MIBC patients treated with NAC were retrospectively reviewed. A total of 63 patients with available TURBT specimen 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 the CR to NAC ($p = 0.037$). After a median follow-up of 41 (range, 12-76) months, no statistically significant differences in OS were found between different IHC-based subtypes (Log rank $P = 0.721$). In Cox proportional hazard regression analysis, age >65yr was independently associated with poorer OS after NAC (hazard ratio [HR], 2.26; 95% confidence interval [CI], 1.02–5.05), but failed to remain significant after adjusting for creatinine clearance (HR, 1.54; 95% CI, 0.58–4.10).

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

Introduction

Muscle-invasive bladder cancer (MIBC) is one of the leading causes of genitourinary cancer-related mortality [1]. Neoadjuvant chemotherapy (NAC) is recommended for MIBC patients, but only less than half 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 the 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., basal bladder tumors showed the most improvement in survival with
NAC compared with surgery alone, and thus the authors suggested that the patients with basal subtype should be prioritized for NAC [9]. Similar results have been shown by other investigators [10, 11].

Although the potential application of gene expression-based molecular subtyping has been indicated in identification of chemosensitive patients, but the application of whole transcriptome profiling for all patients in the clinical practice may be an expensive and time-consuming procedure. Since the immunohistochemistry (IHC) is an inexpensive, readily accessible, and reliable technique in practice, thus, 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 as well as KRT20 for luminal tumors showed promising differential expression patterns [11–13].

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

**Patients And Methods**

**Study design and patients**

To perform this retrospective cohort study, data from 140 consecutive patients with MIBC treated with platinum-based NAC in two tertiary referral hospitals between 2009 and 2019 were reviewed. After excluding 72 patients whose formalin-fixed paraffin-embedded (FFPE) samples were not available, and 5 patients with missing data on the status of treatment response, a total of 63 patients were included in a complete-case analysis. Patients were staged using TURBT and computed tomography (CT) of the chest, abdomen, and pelvis. Patient characteristics and treatment outcomes were obtained by retrospective review. All tissue samples were re-reviewed by an expert uro-pathologist. The study methodology was approved by the ethics committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.UNRC.REC.1398.15). The informed consent requirement was waived. All procedures performed in the study involving human samples were in accordance with the 1964 Helsinki Declaration and its later amendments.

**Outcome assessment**

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

**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, and well-preserved areas rich in tumor cells were identified. 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 1 mm diameter cores 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 non-cancerous bladder tissues were also included in the TMA blocks as a control group.

**IHC 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 were then rehydrated by incubating 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% H2O2 for 10 minutes. Samples were immunostained at 4ºC in blocking solution with primary antibodies. After washing with PBS (3 times/5 min), the sections were incubated with appropriate secondary antibody (Detection kit; MAD-000237QK, master diagnostica, Spain) for 45 minutes. Then, the TMA slides were visualized with 3, 3’-diaminobenzidine (DAB) substrate as chromogen for 10 minutes at the room temperature. The sections were counterstained with haematoxylin, 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 semi-quantitative scoring system in a coded manner, 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 intensity score ≥ 2 and tumor positivity ≥ 20% were considered positive, as previously defined [12].

The following primary antibodies were selected to be used 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; desmin (MAD-001011QD) as P53-like marker. In order 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, Spain.
Statistical analysis

The associations between the CR and independent variables were evaluated using logistic regression analysis. Interaction terms between basal and luminal markers were assessed as potential combined IHC markers. Patient survival was estimated using Kaplan-Meier method. A log-rank test was used to compare the OS between the patient groups. A Cox proportional hazard regression analysis was used to identify the significant determinant factors for the OS. A bootstrapping technique was applied using 1000 random data sets generated from the original data. Median follow-up time was calculated using a reverse Kaplan-Meier method. All statistical tests were two-sided and \( p \) values \( \leq 0.05 \) were considered as statistically significant. The statistical analyses were performed using IBM SPSS, version 23 (IBM Corp., Armonk, NY, USA).

Results

Patient characteristics

A total of 140 consecutive adult MIBC patients treated with NAC between April 2009 and April 2019 were retrospectively evaluated to be included in the study. A total of 72 patients whose formalin-fixed paraffin-embedded (FFPE) samples were not available, and 5 patients for whom the response status was not documented were excluded from the analysis. Then, 63 patients were considered for the complete-case analysis and followed until April 2020. Patient characteristics were summarized in Table 1. Patients with and without CR were similar with regard to all clinical covariates.

Expression of IHC 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 Figure 1.

Since KRT14 stains were only positive in 10 cases, and GATA3 stains were only negative in 8 cases, these two markers were not considered to represent an effective differentiation marker in our study. In addition to 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 Additional file 1: 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 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. 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 further analyzed. 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 Additional file 2: Supplementary Figure 1.

### Tumor response

After chemotherapy, clinical CR was achieved in 20 (31.7%) patients. The associations of singular and combined IHC markers with the 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 the Table 2, 40% of patients with the CR were classified in the basal group, compared to only 16.3% of patients without CR. The relationships between CR and additional potential combined markers identifying basal and luminal subtypes are indicated in Additional file 1: Supplementary Table 1.

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

### Survival outcome

After a median follow-up of 41 (range, 12-76) months, 30 patients died. Achievement of a clinical CR after chemotherapy was significantly associated with better survival in our population ($p = 0.004$). Kaplan-Meier survival curves for distinct groups including basal, luminal, double negative, and double positive categories, based on IHC expression of KRT5/6 and KRT20, are shown in Figure 2. The median OS was 28 (95%CI [confidence interval], 7.5-48.5), 39 (95%CI, 12.2-65.8), 55 (95%CI, 21.9-88.1), and 34 (95%CI, 23.1-44.9) months for patients categorized as luminal, basal, double negative, and double positive group, respectively. No statistically significant difference in the OS was found between the IHC-based subtypes, using the Log rank test ($P = 0.721$). In Cox proportional hazard regression analysis, age >65yr was independently associated with poorer OS after the NAC (hazard ratio [HR], 2.26; 95% CI, 1.02–5.05), but failed to remain significant after adjusting for creatinine clearance (HR, 1.54; 95% CI, 0.58–4.10). The relationships between different covariates and the OS are shown in Table 3.

### Discussion

Identifying patients who have a higher likelihood of response based on molecular subtype classification seems to be a promising strategy to improve survival benefit and prevent unnecessary toxicity [2]. Several molecular classifications for bladder cancer have been introduced so far. In a study by University of North Carolina (UNC), muscle-invasive tumors were grouped into basal and luminal subtypes [17]. The MD Anderson (MDA) classification also added a third group, named P53-like [11]. Several additional molecular classifications with some overlaps have been proposed up to now [7, 16, 18]. Previous studies,
using whole transcriptome profiling, have 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 and those without NAC, the analysis of non-NAC-treated patients indicated that cases with basal subtype have a shorter OS compared with the luminal subtypes (HR, 2.22; \( p = 0.002 \)), representing the intrinsic aggressiveness of basal tumors [9]. In contrast, the OS of patients with basal and luminal subtypes was not significantly different among the NAC-treated group (HR, 0.84; \( p = 0.61 \)). Thus, the authors showed that the impact of NAC on the OS was greatest in patients with basal tumors. Also, McConkey et al., in a small study of 60 patients enrolled in a neoadjuvant trial, showed that the basal tumors had improved survival compared to luminal and p53-like tumors [10]. Furthermore, the association of distinct molecular subtypes with response to NAC has been 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 the NAC. The rapid proliferation of basal tumors, and thus, their particular sensitivity to frontline chemotherapy are the possible explanation [2].

The basal and luminal subtypes were originally defined using the global transcriptomics, but their phenotype may also be recognized using the IHC [12, 13, 16]. In the clinical practice, application of a limited number of IHC markers may confer multiple advantages over whole transcriptome profiling and may be considered as an attractive surrogate. In this study, bladder tumors were assigned to distinct subtypes by using a set of markers as previously described [12]. Since there was a significant overlap between IHC markers identifying subtypes in previous reports [12], a negative marker for both basal and luminal subtypes may help to better discrimination. Guo et al. concluded that the IHC staining with GATA3 and KRT5/6 is a simple classifier of molecular subtypes which is effective in over 80% of the cases, and may strongly translate the transcriptomic classifiers into IHC assays [13]. In our study, there were no significant associations between the CR and singular IHC markers, but interestingly, KRT5/6(+)/KRT20(-) signature, as 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 due to extremely high and low positive cases for 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 have been assessed by Tanaka et al. [19]. More recently, consistent with our experience, Front et al. have shown that the 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; \( p = 0.017 \)) [20]. Compared with our study, similar results were also observed by Front et al. regarding the subtype-related survival outcomes. The authors stated that the lack of significant survival differences between patients with basal and luminal tumors can reflect the clinical benefit from NAC. Also, our results are in line with Seiler et al. study, where they demonstrated that the OS of patients with basal and luminal subtypes was not significantly different among the NAC-treated group [9].
There are several limitations in our study in addition to its retrospective nature. First, despite being cheap, fast and universally available, IHC technique harbors many limitations, including lack a uniformly approved scoring system and the variation in sensitivity and specificity of different antibodies used. Some protocols and strategies were established by the Lund group to uniformly determine molecular subtypes using the IHC and to increase its generalizability [21]. Second, although TMA is a valid method in 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 the clinical CR after the NAC received chemoradiation instead of surgery procedure. However, in the study conducted by Tanaka et al., the correlation of each IHC-based subtype with pathologic CR rate was completely analogous to that with clinical CR rate [19]. Lastly, owing to the relatively small study population, our statistical power was not strong. Of note, in spite of significant \( p \) value observed, the fairly wide range of 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 to stratify NAC administration may be a readily available and cost-effective biomarker, although prospective validation in a large dataset is needed before clinical implementation.

**Abbreviations**

NAC, neoadjuvant chemotherapy; MIBC, muscle-invasive bladder cancer; TMA, tissue microarray; TURBT, transurethral resection of bladder tumor; IHC, immunohistochemistry; OS, overall survival; CR, complete response; HR, hazard ratio; CT, computed tomography; H&E, hematoxylin and Eosin; DAB, diaminobenzidine; UNC, University of North Carolina; MDA, MD Anderson; BASQ, basal/squamous

**Declarations**

**Ethics approval and consent to participate**

This study was approved by the Ethics Committee of Urology and Nephrology Research Center, Shahid Beheshti University of Medical Sciences (IR.SBMU.UNRC.REC.1398.15) and the informed consent requirement was waived.

**Consent for publication**

Not applicable.
Availability of data and materials

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

Conception and design: AR, MG, BM, AB; acquisition of data: AR, MG, PMT, AJ, MS; analysis and interpretation of data: AR, MG, PMT, BM, AB; writing, review, and/or revision of the manuscript: AR, MG, PMT, AJ, MS, BM, AB. All authors read and approved the final manuscript.

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**Tables**
Table 1.
Baseline characteristics of patients

| Characteristics          | CR (n = 20) | No CR (n = 43) |
|-------------------------|-------------|----------------|
| Age, Mean (SD), years   | 68.4 (9.5)  | 68.6 (10.5)    |
| Gender, n (%)           |             |                |
| Female                  | 3 (15.0)    | 4 (9.3)        |
| Male                    | 17 (85.0)   | 39 (90.7)      |
| T stage, n (%)          |             |                |
| T2                      | 14 (70.0)   | 26 (60.4)      |
| T3                      | 5 (25.0)    | 14 (32.6)      |
| T4a                     | 1 (5.0)     | 3 (7.0)        |
| Tumor grade, n (%)      |             |                |
| Low                     | 0           | 2 (4.7)        |
| High                    | 20 (100)    | 41 (95.3)      |
| N status, n (%)         |             |                |
| Negative                | 15 (75.5)   | 36 (83.7)      |
| Positive                | 5 (25.0)    | 7 (16.3)       |
| Chemotherapy regimen, n (%) |       |                |
| Gem/Cis                 | 11 (55.0)   | 23 (53.5)      |
| Gem/Carbo               | 9 (45.0)    | 20 (46.5)      |
| Creatinine clearance,   |             |                |
| Mean (SD), mL/min       | 49.7 (15.4) | 55.7 (20.0)    |
| Previous BCG therapy, n (%) |       |                |
| No                      | 18 (90.0)   | 32 (74.4)      |
| Yes                     | 2 (10.0)    | 11 (25.6)      |
| Smoking status, n (%)   |             |                |
| No                      | 12 (60.0)   | 30 (69.8)      |
| Yes                     | 8 (40.0)    | 13 (30.2)      |
CR, complete response; SD, Standard Deviation; Gem, Gemcitabine; Cis, Cisplatin; Carbo, Carboplatin; BCG, Bacillus Calmette-Guerin
Table 2.
Association of covariates with the clinical complete response to chemotherapy

| Covariate                  | CR (n=20) | No CR (n=43) | Odds ratio (95% CI) | Bootstrap p |
|----------------------------|-----------|--------------|---------------------|-------------|
| **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 | 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       |
| Female gender              | 15        | 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       |
| N 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, Bacillus Calmette-Guerin
Table 3.
Cox regression analysis for relationship between covariates and overall survival

| Covariate                      | Hazard ratio (95% CI) | Bootstrap P |
|--------------------------------|-----------------------|-------------|
| **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**   |
| Female gender                  | 0.25 (0.34-1.83)      | 0.099       |
| T stage, T3-T4a                | 1.26 (0.60-2.63)      | 0.531       |
| N 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, Bacillus Calmette-Guerin; CR.
Figures

**Figure 1**

Representative images of immunohistochemical staining for selected basal (KRT14 and KRT5/6) and luminal (KRT20, and GATA3) markers
Figure 2

Kaplan-Meier curves for overall survival based on immunohistochemical expression of KRT5/6 and KRT20. The patients were classified into 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.

Supplementary Files

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- AdditionalFile2.pdf
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