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ORIGINAL ARTICLE

Immunohistochemical assessment of basal and luminal markers in non-muscle invasive urothelial carcinoma of bladder

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Abstract

The Cancer Genome Atlas project introduced genomic taxonomy of basal and luminal molecular subtypes in muscle invasive bladder cancer. Fewer studies have addressed the molecular classification in non-muscle invasive bladder cancer (NMIBC). Our aim is to assess the applicability of the proposed phenotypic classification for NMIBC. Three TMAs were constructed from 193 TURBT specimens of 60 bladder cancer patients treated at one of the authors' institutions (1998–2008). Follow-up data on recurrence, grade, or stage progression was obtained. Immunohistochemistry was performed using an automated Ventana System for markers indicative of luminal (GATA3, CK20, ER, Uroplakin II, and HER2/neu) and basal (CK5/6 and CD44) phenotype. Marker expression was evaluated by 3 urologic pathologists. Using unadjusted logistic regression, we found significant association between tumor recurrence at next biopsy and CD44 expression (OR = 2.51, $P = 0.03$), tumor recurrence at any subsequent biopsy and ER expression (OR = 0.24, $P = 0.04$), and tumor grade progression at any subsequent biopsy and HER2/neu expression (OR = 0.24, $P = 0.04$). After adjusting for pathologic stage, we found a significant association between CK5/6 expression and tumor stage progression at either next or any subsequent biopsy (OR = 0.94, $P = 0.006$; and OR = 0.97, $P = 0.02$, respectively). Our findings suggest that individual immunohistochemical markers may be of value as prognostic factors in NMIBC.

Keywords Molecular classification · Basal · Luminal · Immunohistochemistry · Urothelial carcinoma · Non-muscle invasive bladder cancer

Introduction

Urothelial carcinoma of the bladder is the most common malignancy of the urinary tract. According to the American Cancer Society, 80,470 new cases of bladder cancer and 17,670 deaths are estimated to occur in the USA during 2019 [1]. The majority (approximately 75%) of patients are diagnosed with non-muscle invasive bladder cancer (NMIBC) with up to 70% suffering from disease recurrence and 10–20% developing progression [2]. Management is primarily dictated by pathologic stage with transurethral resection (TURBT), with or without intravesical therapy, being the mainstay of treatment for NMIBC. While neoadjuvant therapy and more recently immunotherapy [3, 4] promise to improve outcome in advanced bladder cancer, targeted personalized pharmacological agents remain direly needed.

The National Institutes of Health (NIH) and National Human Genome Research Institute (NHGRI) launched The Cancer Genome Atlas (TCGA) project, generating maps of the main genomic alterations in different types of cancer with

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the goal of improving detection, treatment, and overall outcome. The mapping achieved from the TCGA project in muscle invasive urothelial carcinoma of the bladder (MIBC) has identified two intrinsic molecular subtypes (basal and luminal) [5–7]. McConkey et al. further characterized the basal and luminal molecular classification of MIBC by performing whole genome mRNA expression profiling and unsupervised hierarchical cluster analyses in a cohort of 70 primary MIBC. Similar to its basal breast counterpart, basal MIBC was characterized by p63 activation, squamous and/or sarcomatoid histological differentiation, aggressive disease at presentation, and better response to neoadjuvant chemotherapy. Luminal MIBCs had features of active PPARγ and estrogen receptor (ER) transcription and were enriched for activating *FGFR3* mutations and potential FGFR inhibitor sensitivity. A third type (p53-like subtype) displayed a gene expression signature consistent with active wild-type p53 [8, 9]. In a follow-up TCGA study, five genetically distinct molecular subtypes of MIBC (luminal, luminal papillary, luminal-infiltrated, basal-squamous, and neuronal) were described [10].

Fewer studies have addressed the basal and luminal classification in NMIBC. Hedegaard et al. were able to sub-classify NMIBC into three different classes with basal and luminal-like features and different clinical outcomes [11]. Breyer et al. examined mRNA expression of cytokeratins in transurethral resection of bladder tumors of stage pT1 [12].

The aims of this study include assessing the applicability of the proposed molecular classification in NMIBC, identifying the best markers to achieve a practical and cost-effective immunohistochemical panel to classify these lesions and evaluate such markers as outcome predictors.

Materials and methods

Patient samples and clinical data

We searched the surgical pathology database at the George Washington University for all in-house transurethral resections of bladder tumor (TURBT) diagnosed between 1998 and 2008 with NMIBC including urothelial carcinoma in situ (CIS), non-invasive low- and high-grade papillary urothelial carcinoma (LGTCC and HGTCC, respectively), and pT1. We obtained follow-up data on recurrence, grade, and/or stage progression.

TMA construction

Three tissue microarrays (TMAs) were constructed from 193 TURBTs taken from 60 patients. Paired tumor and benign samples (when available) were spotted 3–6 times each. For each patient represented on the TMA, initial (index) tumor and subsequent recurrent lesion(s) were longitudinally represented for a total of 411 TMA spots. For tumor spots, both invasive and non-invasive components were obtained, when available.

Immunohistochemical analysis

Immunohistochemistry (IHC) was carried out using an automated Ventana System for markers associated with luminal (GATA3, CK20, ER, Uroplakin II, and HER2/neu) and basal phenotype (CK5/6 and CD44). Immunostaining was performed on formalin-fixed paraffin-embedded (FFPE) sections using complete automated IHC platform (BenchMark ULTRA and BenchMark XT, ROCHE, IL). Tissue sections were de-paraffinized and hydrated, and heat-induced epitope retrieval (HIER) was performed for each stain as described in Supplementary Table 1. After cooling and rinsing sections with reaction buffer, primary antibody was applied. Signals were detected with HRP labeled secondary antibody followed by DAB chromogen as per manufacturer's instructions (Ultra View DAB, ROCHE Cat # 760-500).

Three genito-urinary pathologists (GJN, MCRP, and ACT) evaluated all tumor spots and corresponding benign tissue. CK5/6, CK20, CD44, GATA3, and Uroplakin II staining was evaluated for percentage of extent (0–100%) in each spot. In each tumor, the median extent in examined spots was then adopted as the marker score for subsequent analysis. For HER2/neu, each spot was assessed using two previously established scoring systems for breast and gastric carcinoma (0, 1+, 2+, and 3+) [13, 14]. Given the similarity of scores obtained by both systems, the median value among spots scored with the breast system was used as the HER2/neu score.

Additionally, we adopted a dichotomous categorization of each tumor using CD44 and CK5/6 scores as a surrogate for the basal category and CK20 and Uroplakin II for the luminal category. Each tumor was assigned to a basal or luminal category utilizing two methods for comparison. The first method classified a tumor based on the highest score of any of the above 4 surrogate markers (e.g., a case with CK20 score higher than the remaining 3 markers is assigned as luminal). The second method classified a tumor based on the higher value among the sum of the scores of the two markers in each category (e.g., a case with sum scores of CK5/6 and CD44 > sum scores of CK20 and Uroplakin II was assigned as basal).

Statistical analysis

Data analysis was carried out using paired events. For any given case, the paired event consisted of two consecutive TURBT diagnoses during follow-up. Thus, a case could have one or more paired events. For each marker, the considered outcomes included one of six possibilities: (i) tumor recurrence at next biopsy diagnosis (tumor reappeared showing a similar or lower-grade/stage lesion); (ii) tumor recurrence at any subsequent biopsy diagnosis (tumor recurred at least once during

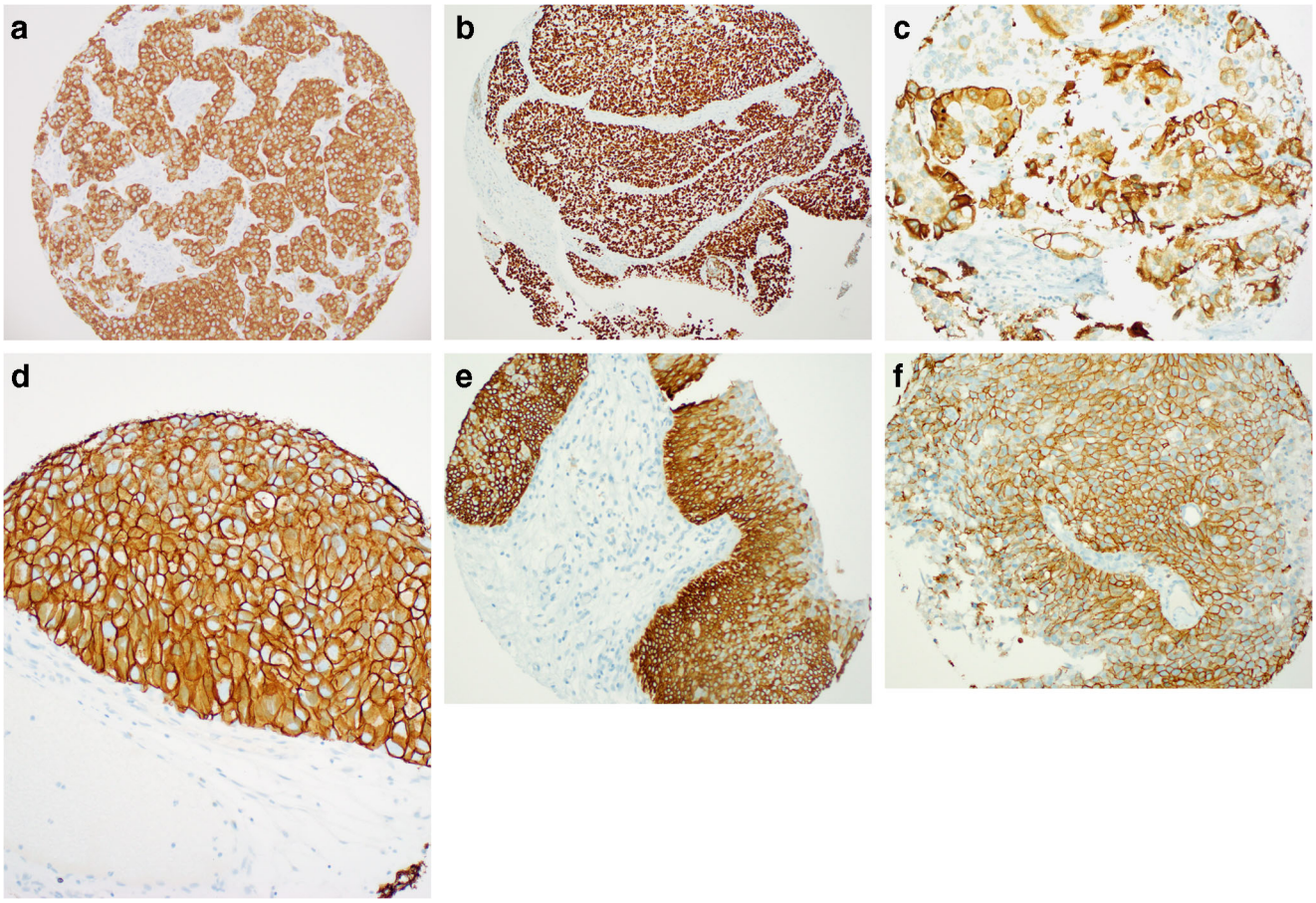

Fig. 1 Microphotographs illustrate basal and luminal immunohistochemical markers in non-muscle invasive bladder cancer. **a** Strong and diffuse cytoplasmic CK20 staining (100% of tumor cells). **b** Diffuse nuclear staining for GATA3 (100% of the tumor cells). **c** Focal membranous and cytoplasmic Uroplakin II staining (80% of the tumor cells). **d** Strong, diffuse, and complete circumferential staining of cell membranes for HER2/neu (100% of the tumor cells). **e** Strong and diffuse cytoplasmic CK5/6 staining (90% of tumor cells). **f** Moderate diffuse membranous staining for CD44 (80% of the tumor cells). All images are at × 200 magnification

follow-up); (iii) tumor grade progression at next biopsy diagnosis (tumor reappeared showing a higher-grade lesion); (iv) tumor grade progression at any subsequent biopsy diagnosis (tumor showed grade progression at least once during follow-up); (v) tumor stage progression at next biopsy diagnosis (tumor reappeared showing a higher-stage lesion); (vi) tumor stage progression at any subsequent biopsy diagnosis (tumor showed stage progression at least once during follow-up).

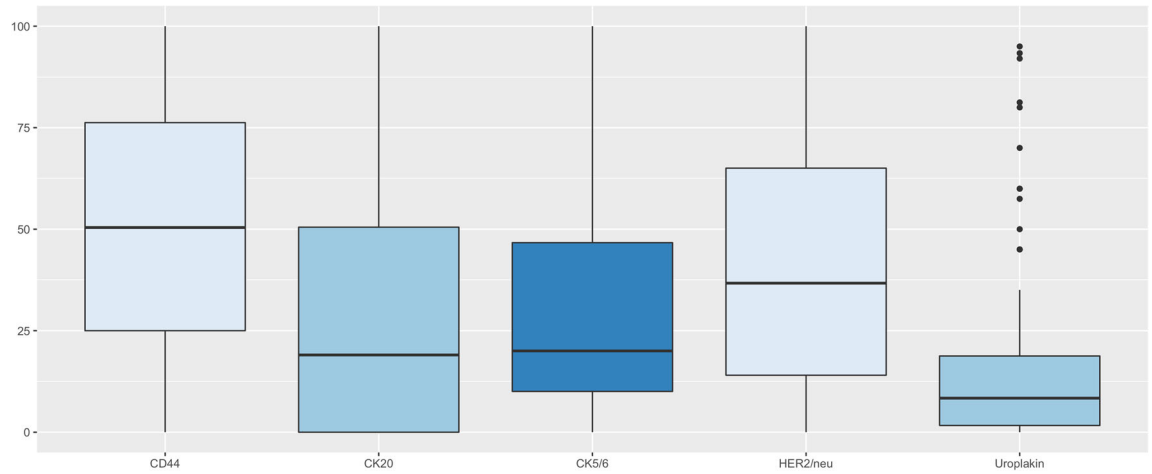

Fig. 2 Boxplot illustrating immunohistochemical marker expression distribution

Table 1 Summary of association analysis of marker expression extent per spot with pathologic features

	Histologic category (% mean)					Stage (% mean)				
	CIS	LGTCC	HGTCC	Invasive	*P* value	Tis	Ta	T1	T2	*P* value
CK5/6	30	37	25	24	0.01	25	31	23	32	0.04
CD44	71	61	45	48	0.0008	60	55	47	41	0.06
CK20	37	23	31	31	0.14	26	26	33	29	0.45
GATA3	100	99	99	99	0.8	100	99	99	100	0.32
ER	1	1	1	4	0.19	2	1	3	3	0.36
HER2/neu	51	36	38	45	0.16	33	36	44	59	0.03
Uroplakin II	33	13	14	17	0.23	14	12	20	12	0.57

**CIS*, carcinoma in situ; *LGTCC*, low-grade urothelial carcinoma; *HGTCC*, high-grade urothelial carcinoma

Correlation between markers was estimated using Pearson's product-moment correlation coefficient. Expression levels were compared for each outcome using the Mann-Whitney *U* test (a.k.a. Wilcoxon rank sum test). Odds ratios and corresponding 95% confidence intervals for each outcome were estimated using conditional binary logistic regression.

To prevent family-wise error rates due to multiple comparisons, *P* values were adjusted using Bonferroni's method. For hypothesis testing, statistical significance was established at adjusted ≤ 0.05 for 2 tails of distribution. Data was analyzed, and plots were generated using R version 3.5.1 (2018-07-02) from the R Foundation for Statistical Computing (Vienna, Austria). All the data, code, and results are freely available as supplementary materials in a GitHub repository at https://github.com/alcideschaux/BL-NMIBUC

Results

Patient demographics and clinical-pathologic features

A total of 411 TMA spots were evaluated for each biomarker. The 60-patient dataset included 41 men and 19 women. Age at diagnosis ranged from 47 to 89 years (mean and median 68 years). Follow-up ranged from 2.1 to 274.9 months (mean, 42.7 months; median, 39.4 months). The dataset included pathologic features of 193 tissue samples. Distribution by pT stage was as follows: 9 TURBTs were pTis, 102 pTa, 66 pT1, 10 pT2; pT stage was not available for 6 cases.

Regarding outcome, at any subsequent biopsy diagnosis, tumor recurrence was encountered in 52, grade progression in 5, and stage progression in 6 out of 60 patients. Tumor recurrence at next biopsy diagnosis was detected in 102, grade progression in 6, and stage progression in 9 out of 193 TURBTs.

Biomarkers correlation with pathologic features

Most tumors lacked ER expression (90% of TMA spots were negative, with the remaining spots showing < 50% expression). Conversely, most tumors were GATA3 positive (95% of TMA spots showed 100% positivity, with the remaining spots showing > 50% positivity). CK20, CK5/6, and Uroplakin II showed a right-skewed distribution, with most values under 50% positivity. The distribution of CD44 and HER2/neu was more symmetrical. Figure 1 shows microphotographs illustrating the different IHC staining patterns for the markers used in this study. Figure 2 depicts a graph with the distribution of marker expression.

Correlation of marker expression score and pathologic features are summarized in Table 1. Basal marker CK5/6 was statistically associated with histologic category and stage; basal marker CD44 was associated with the histologic category and luminal marker HER2/neu with stage. CK5/6 expression was higher in non-invasive low-grade urothelial carcinoma and stage T2. CD44 expression was highest in CIS. Among luminal markers, HER2/neu was highly expressed in stage T2 tumors. Detailed findings can be found in the GitHub repository.

A negative correlation was seen between basal and luminal marker expression. As shown in Supplementary Table 2, we found a significant but weak positive correlation among basal markers. The same was true among luminal markers.

Comparative analysis of markers expression in a given patient throughout sequential biopsies did not show any significant trends between the primary index and subsequent tumors (results not shown; see supplementary materials included in the GitHub repository).

Association of marker expression and outcome

As depicted in Supplementary Fig. 1, we found no statistically significant association of marker expression scores with outcome parameters. While a higher expression level of CD44

Table 2 Univariate regression analysis findings of the relationship of individual markers with outcome measures. Marker levels were categorized as a high expression using the median as the cutoff point

	Marker	Odds ratio	Lower CI	Upper CI	*P* value
Tumor recurrence at next biopsy diagnosis	CK5/6	1.54	0.68	3.46	0.30
	CD44	2.51	1.1	5.85	0.031
	CK20	0.86	0.38	1.95	0.73
	ER	0.89	0.31	2.66	0.83
	HER2/neu	0.73	0.33	1.62	0.44
	GATA3	1.08	0.25	5.53	0.92
	Uroplakin II	0.54	0.21	1.32	0.19
Tumor recurrence at any subsequent biopsy diagnosis	CK5/6	1.55	0.41	5.88	0.51
	CD44	2.51	0.66	12.2	0.20
	CK20	0.56	0.12	2.13	0.42
	ER	0.24	0.06	1.04	0.044
	HER2/neu	0.28	0.04	1.16	0.11
	GATA3	0.73	0.11	14.3	0.78
	Uroplakin II	1.13	0.23	4.34	0.87
Tumor grade progression at next biopsy diagnosis	CK5/6	1.02	0.16	8.04	0.98
	CD44	1.99	0.19	43.9	0.58
	CK20	0.44	0.06	2.8	0.39
	ER	0.00	NA	8.74e+112	0.99
	HER2/neu	0.19	0.01	1.36	0.15
	GATA3	0.00	NA	2.77e+96	0.99
	Uroplakin II	0.26	0.03	1.64	0.15
Tumor grade progression at any subsequent biopsy diagnosis	CK5/6	0.93	0.28	3.34	0.91
	CD44	0.64	0.16	2.38	0.51
	CK20	0.70	0.21	2.39	0.57
	ER	1.17	0.17	5.09	0.84
	HER2/neu	0.24	0.05	0.86	0.04
	GATA3	0.00	NA	1.04e+68	0.99
	Uroplakin II	1.03	0.28	4.95	0.97
Tumor stage progression at next biopsy diagnosis	CK5/6	0.17	0.01	1.19	0.12
	CD44	1.57	0.25	12.3	0.63
	CK20	2.01	0.25	41.6	0.55
	ER	0.00	NA	1.52e+120	1.0
	HER2/neu	0.53	0.07	3.34	0.50
	GATA3	3.22	0.15	25.8	0.32
	Uroplakin II	0.62	0.1	4.88	0.61
Tumor stage progression at any subsequent biopsy diagnosis	CK5/6	0.84	0.29	2.53	0.75
	CD44	2.20	0.73	7.54	0.17
	CK20	2.20	0.7	8.4	0.20
	ER	0.78	0.12	3.17	0.76
	HER2/neu	0.60	0.2	1.74	0.35
	GATA3	2.10	0.29	9.81	0.39
	Uroplakin II	0.74	0.24	2.55	0.61

**CI*, 95% confidence interval

was observed in association with TURBTs with tumor stage progression at the next biopsy diagnosis, the finding was not statistically significant ($P = 0.13$).

High expression of ER (OR = 0.24, $P = 0.04$) showed less likelihood for tumor recurrence and high expression of HER2/neu (OR = 0.24, $P = 0.04$) for grade progression, both at any

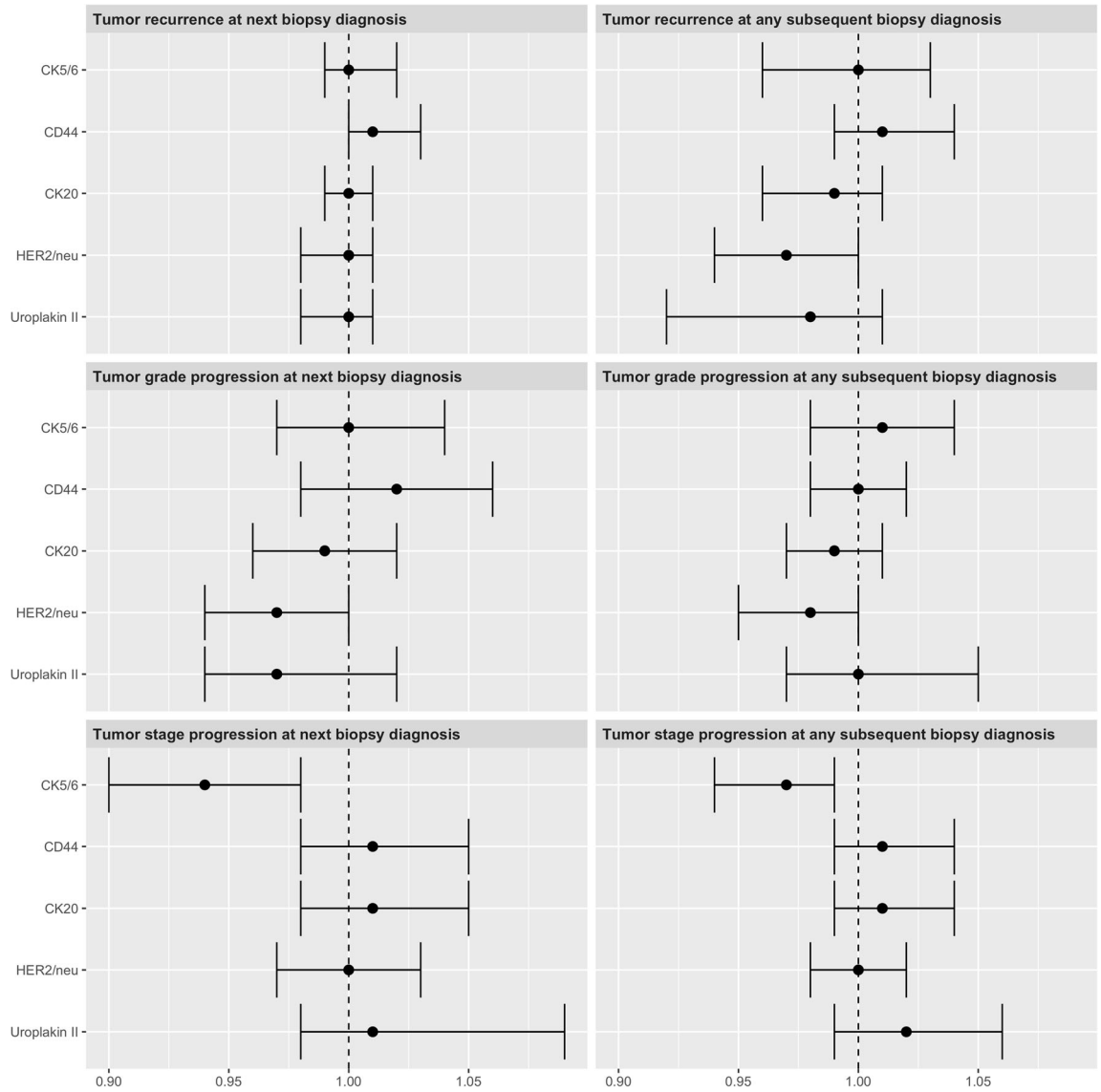

Fig. 3 Forest plots depicting findings of regression analysis of the relationship between biomarker expression and outcome adjusted for the pathologic stage. Outcome odds ratio (dots) with 95% confidence intervals (error bars) are shown for each marker. The dashed line corresponds to an odds ratio of 1. Only CK5/6 was significantly associated with tumor stage progression at the next biopsy and at any subsequent biopsy diagnosis

subsequent biopsy diagnosis. High expression CD44 (OR = 2.51, $P = 0.03$) was associated with a higher likelihood for tumor recurrence at the next diagnosis. Table 2 summarizes the unadjusted odds ratios using the median expression score as the cutoff for positivity in each marker. After adjusting for pathologic stage, only CK5/6 was associated with lower odds ratios for tumor stage progression at next and at any subsequent biopsies (OR = 0.94, $P = 0.006$; and OR = 0.97, $P = 0.02$, respectively) (Fig. 3).

Finally, the association between basal vs luminal markers and outcome using the two methods defined in material and methods showed no significant correlation with the highest score of any of the 4 surrogate markers (Supplementary Fig. 2) nor the higher value among the sum of the scores of the two markers in each category (Supplementary Fig. 3).

Discussion

The knowledge gathered on intrinsic bladder cancer subtypes has shown strong potential for guiding personalized therapy in MIBC. As indicated above, only few studies have addressed intrinsic molecular classification in NMIBC. Hedegaard et al. performed a comprehensive transcriptional analysis of 460 early-stage urothelial carcinomas from a prospective European multicenter trial cohort including 3 CIS, 345 pTa,

and 112 pT1 cases. By using a 117 gene signature, three molecular classes of NMIBC were distinguished with significantly different risk for progression. Class 1 and class 2 both showed a luminal gene expression pattern. Class 2 tumors were further characterized by the activation of EMT-related transcription factors and higher expression of stem cell–associated markers *ALDH1A1*, *ALDH1A2*, *PROM1* (*CD133*), *NES,* and *THY1* (*CD90*). Class 3 tumors showed gene expression signature similar to that of basal MIBC with high expression of CK5 and CD44. Class 1 tumors were primarily pTa tumors with a favorable outcome. Although low-grade tumors were found across all three classes, a higher proportion displayed luminal markers. Class 2 tumors demonstrated a higher likelihood for progression through a pathway similar to CIS lesions. GATA3 was highly expressed throughout the cohort, including class 3 tumors [11]. In our study, CK5/6 and CD44 were also significantly co-expressed, supporting a basal-like molecular signature in a subset of NMIBC. Similar to Hedegaard's study, we found GATA3 expression throughout, including basal-like tumors. We also found HER2/neu expression to be associated with favorable outcome (lower odds ratios of grade progression at any subsequent biopsy diagnosis).

Breyer et al. assessed *CK5* and *CK20* mRNA expression as markers of luminal and basal subtypes in pT1 tumors by performing RT-qPCR in a cohort of 284 patients. mRNA expression combination of high CK20 and low CK5 characterized the luminal subtype and was associated with worse progression-free and recurrence-free survival [12]. Similar to Brayer et al., we found CK5/6 expression to be associated with lower odds ratios of stage progression at next and any subsequent biopsy.

Raspollini et al. examined several biomarkers in NMIBC (T1) including CD44. They found no association between CD44 and survival outcome [15]. In contrast, we found that high expression of CD44 showed higher odds ratios for tumor recurrence at the next biopsy diagnosis.

Our attempt to create a practical dual IHC-based classifier for basal and luminal groups (using CD44 and CK5/6 vs CK20 and Uroplakin II, respectively) was not successful in predicting outcome in NMIBC. Although not widely studied in NMIBC, one possibility could be tumoral molecular heterogeneity. Warrick et al. recently addressed the topic of heterogeneity in MIBC [16]. In their study, 39% of patients with more than one morphologically distinct tumor had molecular heterogeneity among coexisting different tumors. Although TMA assays might not capture the heterogeneity of each tumor, it allows for consistency and standardization of IHC processing variables [17, 18].

Our findings suggest that individual IHC markers may be of value as prognostic indicators in NMIBC. Whether such approach could be contributory in a larger cohort, with additional IHC and molecular markers, remains to be seen. The strengths of our study include being performed at a single institution with long follow-up and the advantage of longitudinal interrogation of individual patient outcomes.

Future studies to validate our results are needed preferably in a multi-institutional cohort with a larger number of patients. The latter will also help address the value of basal/luminal IHC markers in predicting response to intravesical therapy.

Author contributions GJN conceived and designed the study and wrote, edited, and reviewed the manuscript. MCRP collected the data and wrote, edited, and reviewed the manuscript. AC and MLE analyzed the data and wrote, edited, and reviewed the manuscript. ACT collected the data and reviewed the manuscript. DT and WB collected the data and reviewed the manuscript. MKR collected samples and clinical data and reviewed the manuscript. RS performed the immunohistochemical analysis and reviewed the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

All authors gave final approval for publication. GJN takes full responsibility for the work as a whole, including the study design, access to data, and the decision to submit and publish the manuscript.

Informed consent The Institutional Review Board approved this study.

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