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ORIGINAL ARTICLE

Predicting outcomes in non-muscle invasive (Ta/T1) bladder cancer: the role of molecular grade based on luminal/basal phenotype

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Abstract

Bladder cancer tumors can be divided into two molecular subtypes referred to as luminal or basal. Each subtype may react differently to current chemotherapy or immunotherapy. Likewise, the technology required for comprehensive molecular analysis is expensive and not yet applicable for routine clinical diagnostics. Therefore, it has been suggested that the immunohistochemical expressions of only two markers, luminal (CK20+, CK5/6–) and basal (CK5/6+, CK20–), is sufficient to identify the molecular subtypes of bladder cancer. This would represent a molecular grade that could be used in daily practice. Molecular classification is done using immunohistochemistry to assess luminal-basal phenotype based on tissular expression of CK20 and CK5/6 as surrogate for luminal or basal subtypes, respectively. A series of 147 non-muscle-invasive bladder carcinoma cases was selected, and the tumors were divided into four subgroups based on the presence of CK20 and/or CK5/6, that is, null (CK20−, CK5/6−), mixed (CK20+, CK5/6+), basal (CK20−, CK5/6+), and luminal (CK20+, CK5/6−) categories. Survival analysis was estimated using the Kaplan-Meier method and the log-rank test. Hazard ratios were calculated by Cox multivariate analysis. The molecular grade included cases with null ($n = 89$), mixed ($n = 6$), basal ($n = 20$), and luminal ($n = 32$) phenotypes with differences in recurrence-free, progression-free and cancer-specific survival associated with molecular-grade categories in patients with low- or high-grade Ta, or high-grade T1 tumors. The multivariate analysis identified the luminal phenotype as a predictor of more aggressive neoplasms. Our findings provide a rationale to investigate luminal and basal subtypes of bladder cancer using two gene expression signatures as surrogate markers and show that non-muscle-invasive bladder carcinoma can be stratified into biologically and clinically different subgroups by using an immunohistochemical classifier.

Keywords Bladder cancer · Survival · Prognosis · Luminal · Basal · Molecular grade

Introduction

Bladder cancer is traditionally divided into non-muscle or muscle invasive subtypes [1–10]. While the non-muscle-invasive bladder carcinoma and muscle-invasive bladder carcinoma tumors are distinguishable from each other, at the molecular level, there are two major molecular subtypes referred to as luminal and basal, which show distinct clinical behavior and sensitivity to current chemotherapy and immunotherapy [11–32].

Initial studies based on whole-genome transcriptional analysis identified 5 categories of tumors that demonstrated

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differential expression of cytokeratin (CK), fibroblast growth factor receptor-3 mutational status, cell adhesion gene profiles, and cell cycle regulatory gene profiles [11–32]. The 3 major subtypes, urobasal, genomically unstable, and squamous cell carcinoma-like, can be distinguished by pathologic and immunohistochemical features [11–32]. Patschan et al. [22] showed significant differences in the outcome of T1 patients stratified for molecular subtype. The study identified 3 molecular subtypes (classes 1–3) with high-risk tumors (class 2) harboring frequent TP53 and ERBB2 alterations and APOBEC mutations, low-risk tumors (class 1) enriched for fibroblast growth factor receptor-3 mutations, and class 3 tumors showing basal-like characteristics. [22] Reportedly, molecular subtypes of non-muscle-invasive bladder carcinoma could help to better categorize the prognosis and response to current therapies [11–32]; however, robust predictive data is currently lacking [18–21, 24, 25, 30, 33–39]. Recently, Choi et al. [34] suggested the potential utility of immunohistochemical markers as a surrogate of molecular subtypes. A number of these markers have been identified to support luminal (CK20, GATA3, FOXA1) or basal (CK5/6, p63, CK14) classifications. Interestingly, a two-cytokeratin panel (CK20, CK5/6) was identified with the potential to detect luminal/basal phenotypes in urothelial carcinoma [25, 34].

In this current study, we assessed the expression status and prognostic significance of a two-marker immunohistochemical classifier using CK20 and CK5/6 as surrogate for luminal/basal phenotype in conventional non-muscle-invasive bladder carcinoma patients.

Material and methods

Patients and clinical data

Approval from an institutional review board was obtained. The study cohort was a retrospective series of 147 patients with primary bladder tumors treated with a complete transurethral resection of the bladder between 2003 and 2015. T1 carcinoma patients received an additional transurethral bladder resection to exclude pT2 disease. Patients with high-grade carcinoma received adjuvant treatment with intravesical BCG (Bacillus Calmette-Guerin). Patient's follow-up (number of months from the diagnostic procedure to the date of the most recent cystoscopy, last visit or death) was 76 (median), with a range of 6 to 120 months. Disease recurrence was defined as first tumor relapse in the bladder regardless of tumor stage. Tumor stage progression was defined as a shift to any higher stage (T1–T2–T4) in Ta tumors; stage T2–T4 in T1 tumors, or the appearance of metastasis. Survival time was the period between diagnosis and death. Cancer-related death that was caused by bladder carcinoma.

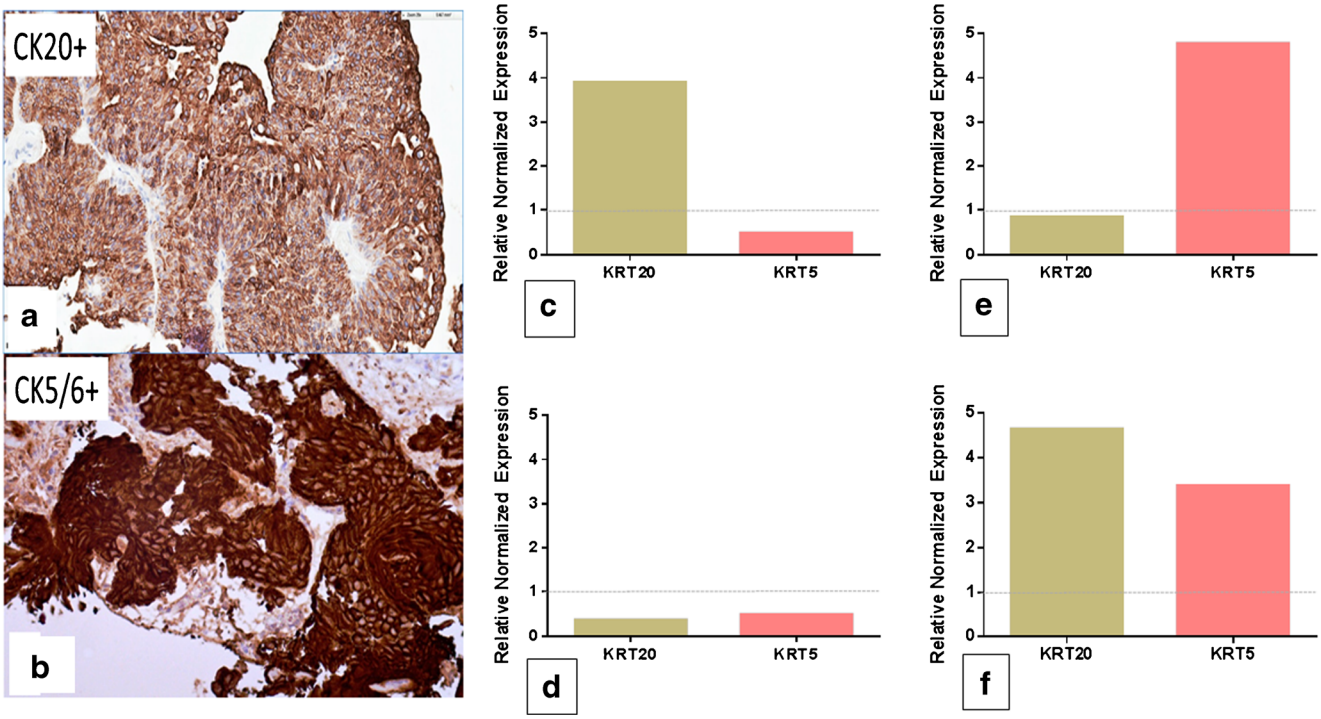

Fig. 1 Molecular-phenotype grade of NMIBC based on the phenotypic expression of CK20 as surrogate of luminal molecular subtype (**a**) or CK5/6 as surrogate of the basal molecular subtype(**b**) (anti-CK20, 100X; anti-CK5/6, 100X). qRT-PCR analysis of KRT20 and KRT5 expression in selected cases showing KRT20 upregulation and KRT5 downregulation (case 6) supporting luminal subtype(**c**), KRT20 and KRT5 downregulation (case 89) supporting null subtype (**d**), KRT20 downregulation and KRT5 upregulation (case 11) supporting basal subtype (**e**), and KRT20 up-regulation and KRT5 up-regulation (case 45) supporting mixed subtype (**f**)

Table 1 Normalized expression KRT 20 and KRT 5 by qRT-PCR in 10 selected cases representative of different molecular-phenotype grade categories also investigated by immunohistochemistry showing similar results with both methods. Level of expression above 1 represent upregulation of the marker and level of expression below 1 represents downregulation of the marker (see also "Material and methods" section and Fig. 1)

Case #	KRT 20*	KRT 5*	Grade/stage	Suggested molecular subtype
1	0.01730	0.65474	LGTa	Null
6	3.90218	0.49040	LGTa	Luminal
9	0.16999	12.38655	LGTa	Basal
11	0.84945	4.78284	HGT1	Basal
17	8.33793	0.06792	HGT1	Luminal
34	2.36639	0.43249	HGT1	Luminal
45	4.65062	3.38195	HGT1	Mixed
60	0.00272	10.01338	LGTa	Basal
83	1.76103	0.37990	HGTa	Luminal
89	0.36501	0.48424	LGTa	Null

*Normalized expression ΔΔCq for KRT 20 and KRT 5

KRT 20, keratin 20; *KRT 5*, keratin 5; *grade/stage*, pathologic grade and stage following the WHO 2016 classification and the AJCC 8th edition

Pathologic evaluation

Pathologic re-assessment of all primary tumors and their recurrences was done by three pathologists blinded to the clinical status. Forty randomly selected bladder carcinoma cases served as a preliminary sample set to facilitate agreement on grading and staging. Sections were graded in the worst differentiated area. If a discrepancy occurred, a review round was organized to obtain consensus on a diagnosis. Pathologic

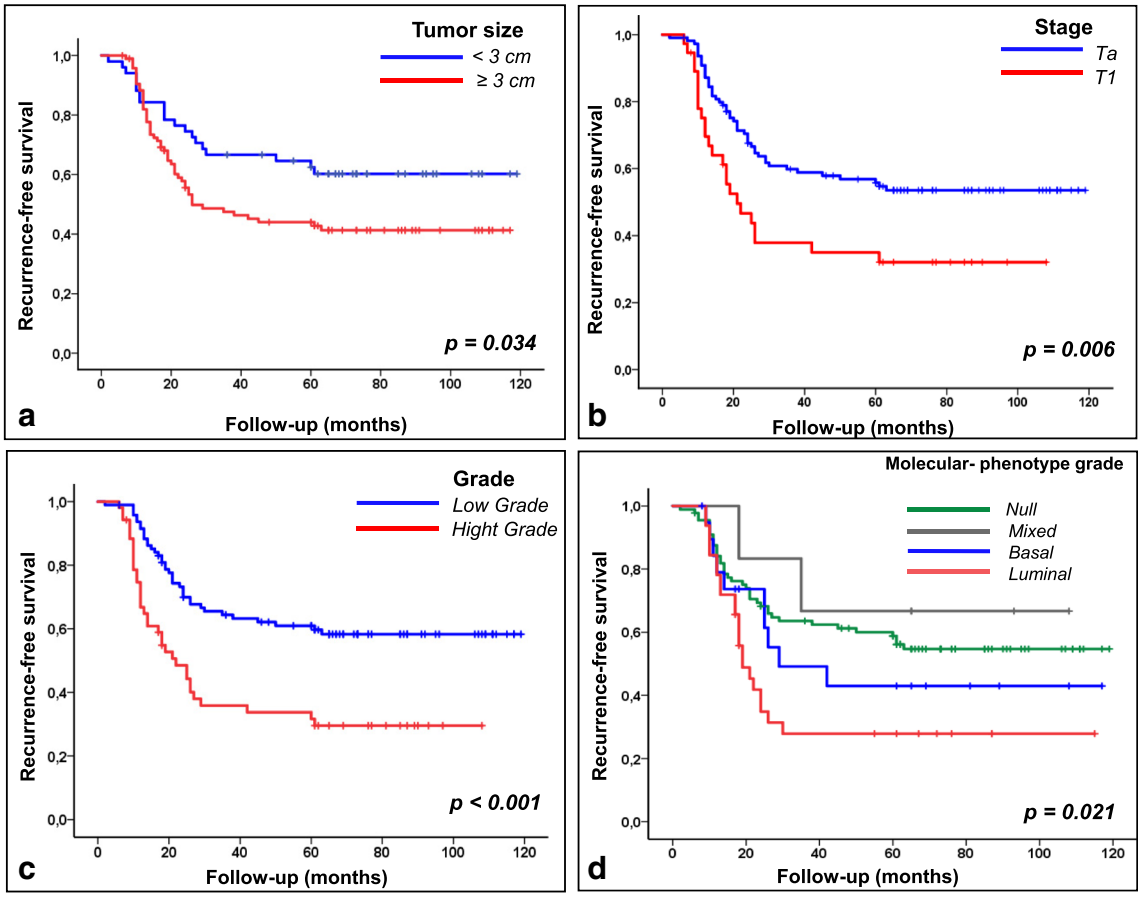

Fig. 2 Parameters related to recurrence-free survival in NMIBC patients. Similar to other classic predictors in the study (**a**, **b**, and **c**), luminal molecular-phenotype grade predicted most aggressive tumors (**d**); meanwhile, mixed molecular phenotype resulted in less aggressive tumors (**d**)

grade and stage were determined according to the WHO/AJCC/TNM 2016 revision. [9]

Qualitative and quantitative assessment of molecular-phenotype grade

A representative 4-μ thick paraffin block was cut from each tumor, deparaffinized in xylene, rehydrated in graded ethanol, and then washed with PBS. For antigen retrieval, the sections were boiled in 10 mM citrate buffer (pH 6.0). Sections were incubated with primary mouse monoclonal antibodies (CK20 [Ks20.8, prediluted; DAKO, Carpinteria, CA] and CK5/6 [D5/16B4, 1:50; Cell Marque, Rocklin, CA]) incorporating positive and negative controls. Immunohistochemical stains were performed using the EnVision system (DakoCytomation, Denmark) with 30 min at room temperature with diaminobenzidine chromogen substrate solution (0.6 mg/ml in tris buffer saline, pH 7.6 with 12 ml 30% hydrogen peroxide). Sections were counterstained with Mayer's hematoxylin. Three pathologists evaluated the immunohistochemical slides independently using a Zeiss Scope A1 optical microscope (Jena, Germany). The same area on each slide was examined using random fields delineated by a 1-cm^2 graded ocular grid attached to the eyepiece of the microscope. A negative immunohistochemical pattern was assigned to cases showing negative-to-rare (< 1%) single randomly distributed cells (in both CK20 and CK5/6 cases) or rare (< 1%) single cells at the basal portion of the papillae (CK5/6) (Fig. 1). The typical positive case was characterized as presenting several positive cells seen at deeper levels of the papillae exhibiting high intensity. Four patterns of expression were identified (null (CK20−, CK5/6−), mixed (CK20+, CK5/6+), basal (CK20−, CK5/6+), luminal (CK20+, CK5/6−)).

RNA extractions, cDNA synthesis, and qRT-PCR assays

To increase the specificity of the immunohistochemical analysis, KRT20 and KRT5 were also investigated in 10 selected cases using qRT-PCR. [40]

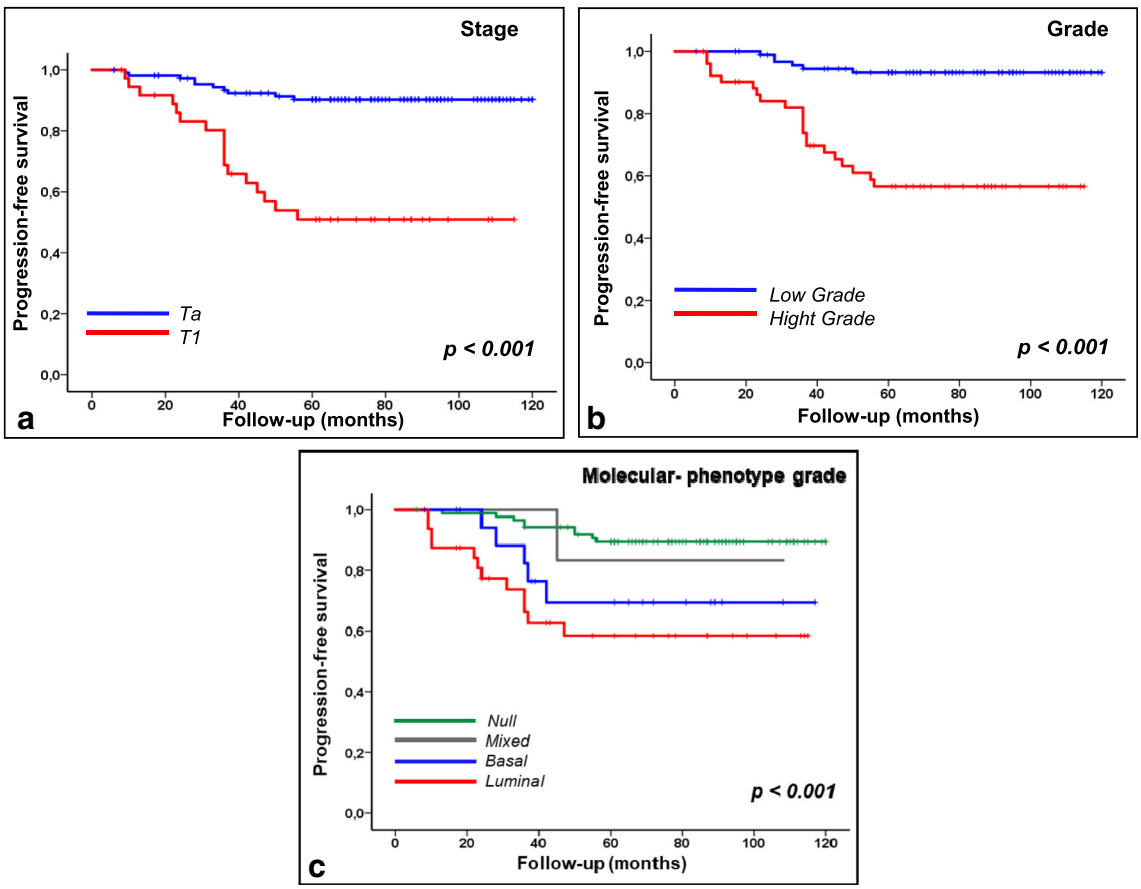

Fig. 3 Parameters related to progression-free survival in NMIBC patients. Similar to the other classic predictors in the study (**a**, **b**), luminal molecular-phenotype grade was predictive of more aggressive tumors; meanwhile, null molecular phenotype resulted in less aggressive neoplasms (**c**)

Total RNA was isolated using the RNeasy Mini kit (Qiagen, CA). The RNA concentration and purity were tested using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Reverse transcription was performed with 500 ng total RNA using a SensiFAST cDNA Synthesis Kit (Bioline). Primers were KRT20F: GGACGACACCCAGCGTTTAT and KRT20R: CGCTCCCATAGTTCACCGTG; KRT5F: CCAAGGTTGATGCACTGATG and KRT5R: TGTCAGAGACATGCGTCTGC.

Each PCR was performed with a 20 μL final volume containing 4 μL cDNA, 0.8 μL (each) primers, 4.4 μL diH2O, and 10 μL 1 × SYBR Green PCR Master Mix (iTaq Universal SyBR Green Supermix, Bio-Rad). Relative gene expression was performed by RT-PCR using CFX Connect™ detection system and Ct (threshold cycle) values were quantified and converted to raw data with CFX Manager software (Bio-Rad Laboratories, Inc., Hercules, CA). [40].

The thermal cycling conditions included a denaturation program (95 °C for 5 min), and an amplification program repeated 40 times (95 °C for 5 s and 60 °C for 30 s). Fold change was calculated as $2-\Delta\Delta Ct$, where $\Delta\Delta Ct$ was the normalized cycle threshold value relative to endogenous controls: GAPDHF: TGTCCCCACTGCCAACGTGTCA and GAPDHR: AGCGTCAAAGGTGGAGGAGTGGGT [40]. Gene expression was calculated using control samples (healthy adjacent urothelial tissue). A relative quantification above 1 represents upregulation and below 1 represents downregulation (Fig. 1, Table 1).

Statistical analysis

The aim of the study was to search for differences between the four molecular grades (null, mixed, basal, and luminal) regarding disease-free, progression-free, and overall cancer-specific survival in non-muscle-invasive bladder carcinoma.

Survival analysis was conducted using the Kaplan-Meier method and differences among groups were tested for significance using the log-rank test. Hazard ratios were calculated with 95% confidence intervals using the Cox regression, and in all calculations, a p value of less than or equal to 0.05 was considered indicative of a statistically significant difference.

All statistical analysis was performed using the Statistical Package for Social Sciences (IBM® SPSS® Statistics, Armonk, NY, USA) version 24.0 for Windows® Software.

Results

Mean patient age at diagnosis of the 147 (17 were female) cases was 66.88 years (range 29–93 years). Ninety-five (64.6%) patients had low-grade non-invasive (Ta) carcinoma; 15 patients (10.2%) had high-grade non-invasive (Ta) carcinoma; and 37 (25.2%) patients had T1 high-grade invasive carcinoma. Fifty-one patients had < 3 cm tumors. Tumor size ranged from 1 to 8 cm (mean 3.27 cm). The molecular grade

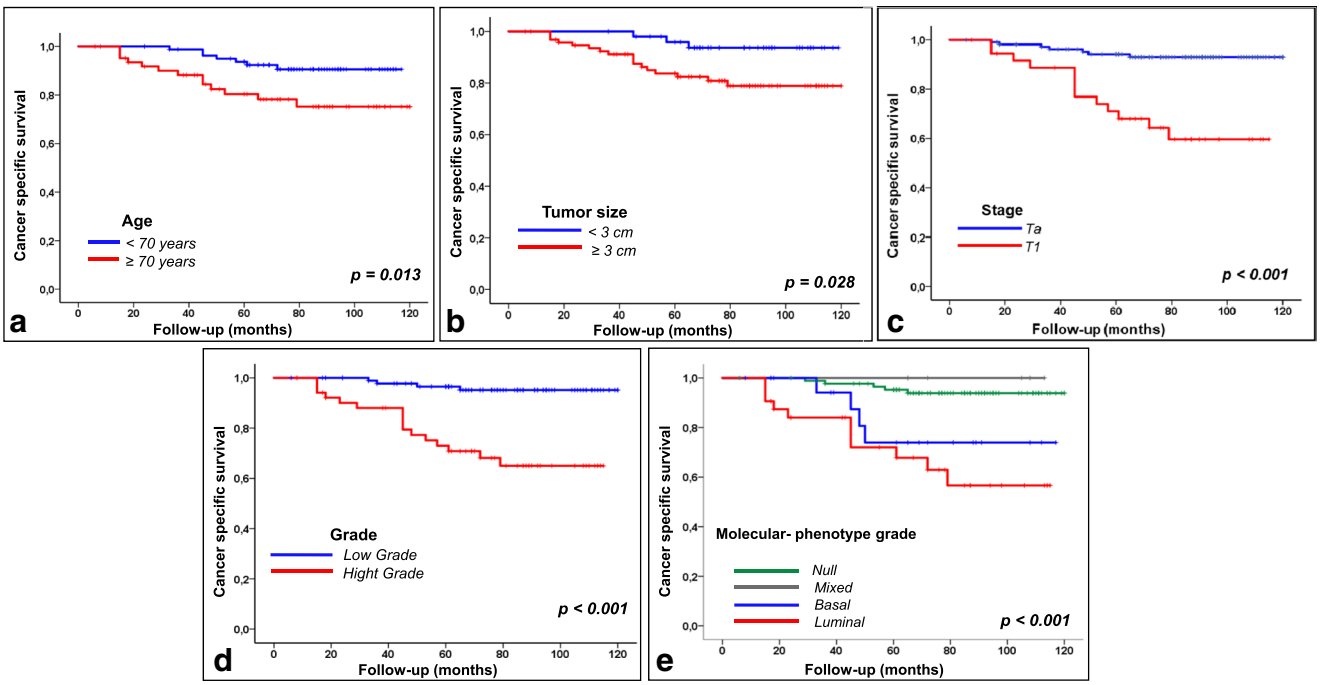

Fig. 4 Parameters related to cancer-specific survival in NMIBC patients. Similar to the other classic predictors in the study (**a**, **b**, **c**, and **d**), luminal molecular-phenotype grade was predictive of more aggressive tumors (**e**); meanwhile, mixed molecular phenotype resulted in less aggressive neoplasms (**e**)

Table 2 Characteristics of 147 patients and survival analysis of clinicopathologic variables in study including molecular-phenotype grading

Variable[&]	Overall $N = 147$ (%)	Recurrence-free survival $N = 74$ (%)		Progression-free survival $N = 120$ (%)		Cancer-specific survival $N = 127$ (%)	
			p value*		*p* value*		*p* value*
Age (years)[#]			0.691		0.150		0.013
< 70	83 (56.5)	39 (47.0)		70 (84.3)		76 (91.6)	
≥ 70	64 (43.5)	35 (54.7)		50 (78.8)		51 (79.7)	
Gender			0.424		0.062		0.112
Female	17 (11.6)	8 (47.1)		17 (100)		17 (100)	
Male	130 (88.4)	66 (50.8)		103 (79.2)		110 (84.6)	
Tumor size (cm)[$]			0.034		0.162		0.028
< 3	51 (34.7)	31 (60.8)		44 (86.3)		48 (94.1)	
≥ 3	96 (65.3)	43 (44.8)		76 (79.2)		79 (82.3)	
Stage			0.006		< 0.001		< 0.001
Ta	110 (74.8)	61 (55.5)		100 (90.9)		103 (93.6)	
T1	37 (25.2)	13 (35.1)		20 (54.1)		24 (64.9)	
Grade			< 0.001		< 0.001		< 0.001
LG	95 (86.4)	57 (60.0)		89 (93.7)		91 (95.8)	
HG	52 (13.6)	17 (32.7)		31 (59.6)		36 (69.3)	
Molecular grade			0.021		< 0.001		< 0.001
Null	89 (60.5)	50 (56.4)		80 (89.9)		84 (94.4)	
Mixed	6 (4.1)	4 (66.7)		5 (83.3)		6 (100)	
Basal	20 (13.6)	10 (50.0)		15 (75.0)		16 (80.0)	
Luminal	32 (21.8)	10 (31.3)		20 (62.5)		21 (65.6)	

*Log-rank test; #mean age (range) 66.88 ± 10.8 (29–93); $mean tumor size (cm) (range) 3.27 ± 1.56 (1–8); &Follow-up (months) median (range) 76 (6–120)

SD standard deviation

included patients with null ($n = 89$), mixed ($n = 6$), basal ($n = 20$), and luminal ($n = 32$) phenotypes, with differences in recurrence-free, progression-free, and cancer-specific survival associated with molecular-grade categories (Figs. 2, 3, and 4) (Table 2).

T status and grade were also associated with recurrence-free, progression-free, and cancer-specific survival (Table 2). Tumor size was associated with recurrence-free and cancer-specific survival but not to progression-free survival, and patient age was associated with cancer-specific survival only (Table 2; Figs. 2, 3, and 4). Phenotype grade was also associated with recurrence-free, progression-free, and cancer-specific survival in patients with low-grade Ta (Table 3; Fig. 5) or high-grade Ta non-invasive (Table 4; Fig. 6) tumors, or T1 high-grade invasive (Table 5; Fig. 7) tumors.

Using the Cox multivariate analysis, pathologic and molecular grades were identified as independent

Table 3 Survival analysis of patients with TaLG bladder carcinoma according to molecular-phenotype grade

Molecular grade	Overall TaLG $N = 95$ (%)	RFS $N = 57$ (%)	*p* value*	PFS $N = 89$ (%)	*p* value*	CSS $N = 91$ (%)	*p* value*
			0.183		0.005		< 0.001
Null	66 (69.5)	43 (65.2)		63 (95.5)		64 (97.0)	
Mixed	3 (3.2)	2 (66.7)		3 (100)		3 (100)	
Basal	5 (5.3)	2 (40.0)		3 (60.0)		3 (60.0)	
Luminal	21 (22.1)	10 (47.6)		20 (95.2)		21 (100)	

*Log-rank test

RSF, recurrence-free survival; *PFS*, progression-free survival; *CSS*, cancer-specific survival

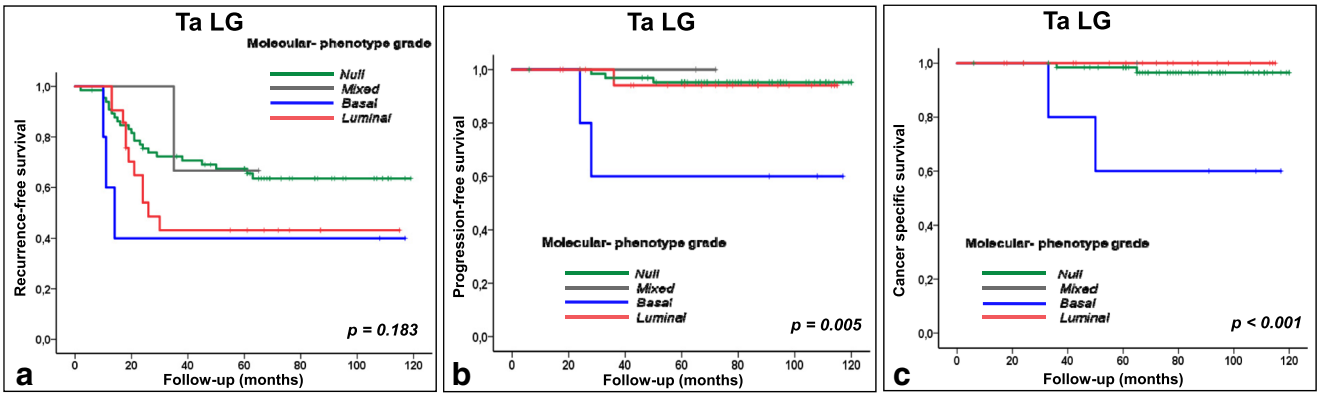

Fig. 5 Molecular-phenotype grade was a predictor of RFS, PFS, and CSS in patients with non-invasive (Ta) low-grade urothelial carcinoma, with basal phenotype resulting in more aggressive neoplasms (RFS, PFS, CSS) and mixed phenotype resulting in the less aggressive phenotype (RFS, PFS, CSS)

predictors of recurrence-free, progression-free, and cancer-specific survival, with the high pathologic grade and the luminal molecular grade associated with more aggressive neoplasms. Age was also identified as an independent predictor of cancer-specific survival in our cohort series (Table 6).

Discussion

Recent studies suggest that bladder cancers can be divided into two candidates intrinsic molecular subtypes referred to as luminal and basal [11–32, 35]. Luminal tumors have an expression signature similar to intermediate/superficial layers of the urothelium. Basal tumors show an expression signature similar to the basal layer of the urothelium. [13] The immunohistochemical expressions of two markers, luminal (GATA3) and basal (CK5/6), were sufficient to identify the molecular subtypes of bladder cancer with 90% accuracy in one study [13]. Immunohistochemistry of CK5 (basal) and CK20 (luminal) expression can identify upper tract urothelial carcinomas with worse cancer-specific survival [25].

Recently, Patschan et al. [22] identified outcome differences in T1 patients stratified as 3 molecular subtypes (classes 1–3). High-risk tumors (class 2) harbored frequent *TP53* and *ERBB2* alterations and APOBEC-related mutations, whereas low-risk tumors (class 1) were enriched for fibroblast growth factor receptor-3 mutations. Class 3 tumors showed basal-like characteristics.

Our paper aimed to corroborate the proposal by Choi et al. [34] on the use of CK20 and CK5 as surrogate for luminal-basal by immunohistochemistry and therefore to establish its potential use in routine histopathology. We also attempted to corroborate the findings by Breyer et al. [40] using the same RT-PCR technology to molecularly validate the correspondence between immunohistochemistry and RT-PCR data in selected cases with the purpose of adding robustness to our study. Differences among these publications include the inclusion of muscle-invasive bladder carcinoma by Choi et al. [34] and the restriction to pT1 carcinomas by Breyer et al. [40] Interestingly, our results show an association between luminal-basal subtypes and aggressiveness in non-muscle-invasive bladder carcinoma confirms that the use of the immunohistochemical expression of CK20/CK5–6 can be used to classify the molecular subtypes, [25, 34] thus supporting the potential prognostic use of molecular grading in non-muscle-invasive bladder carcinoma.

Few studies have addressed the role of immunohistochemistry in the treatment of non-muscle-invasive

Table 4 Survival analysis of patients with TaHG bladder carcinoma according to molecular-phenotype grade

Molecular grade	Overall TaHG $N = 15$ (%)	RFS $N = 4$ (%)	p value*	PFS $N = 13$ (%)	p value*	CSS $N = 12$ (%)	p value*
			0.004		< 0.001		< 0.001
Null	6 (40)	0 (0.0)		5 (83.3)		6 (100)	
Mixed	1 (6.7)	1 (100)		1 (100)		1 (100)	
Basal	6 (40)	3 (50.0)		5 (83.3)		5 (83.3)	
Luminal	2 (13.3)	0 (0.0)		0 (0.0)		0 (0.0)	

*Log-rank test

RSF, recurrence-free survival; *PFS*, progression-free survival; *CSS*, cancer-specific survival

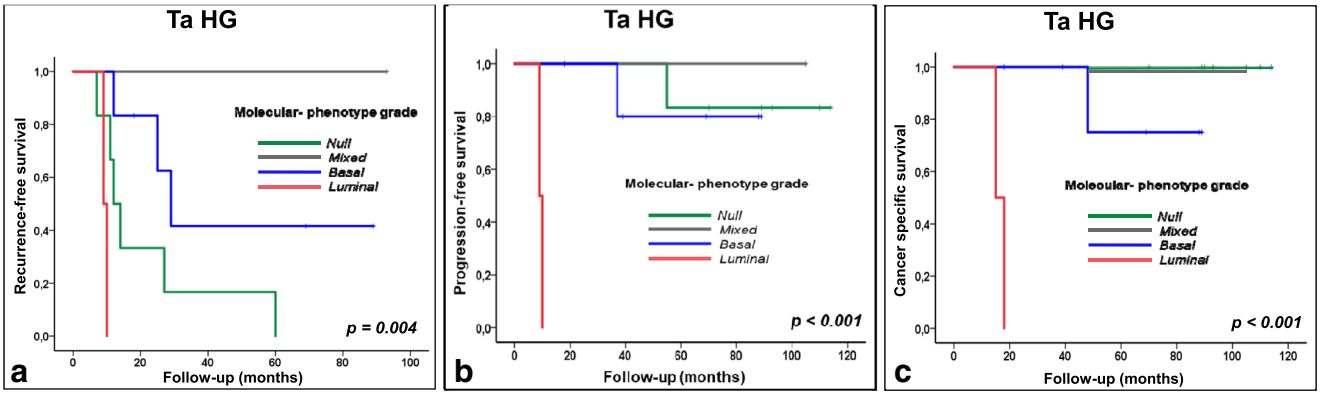

Fig. 6 Molecular phenotype-grade was a predictor of RFS, PFS, and CSS in patients with non-invasive (Ta) high-grade urothelial carcinoma with luminal phenotype resulting in more aggressive neoplasms (RFS, PFS, CSS) and mixed phenotype resulting in the less aggressive disease (RFS, PFS, CSS)

bladder carcinoma following the introduction of molecular taxonomy [13, 25, 34]. A classification system that divides urothelial carcinoma into luminal and basal categories was recently published [34, 36, 37]. Whereas luminal carcinomas expressed genes associated with superficial umbrella cells and appeared similar to superficial papillary tumors, basal carcinomas expressed genes more characteristic of urothelial basal cells, which have a worse prognosis but may be more responsive to neoadjuvant chemotherapy or checkpoint immunotherapy [11–32].

Our study followed a similar rationale in the treatment of non-muscle-invasive bladder carcinoma and showed a characteristic molecular-grade signature with most cases (60.5%) presenting null phenotypes, luminal phenotypes (22%), basal phenotypes (14%), or a mixed category accounting for 4% of the cases. Interestingly, our multivariate analysis revealed that the luminal molecular grade was an independent predictor of most aggressive tumors in non-muscle-invasive bladder carcinoma as seen by its association with recurrence-free, progression-free, and cancer-specific survival, with hazard ratios of 2.3, 8.1, or 9.0, respectively. This is similar to the data reported by Breyer et al. [40] for pT1 bladder carcinomas. Additionally, we have observed a statistical power similar to the molecular grade as compared with other classic pathologic parameters including tumor size and pathologic grade or stage. Null or mixed molecular grades resulted in less aggressive tumors in our series. Molecular grade also had a power similar to the classic clinical parameters such as patient's age and gender, thus supporting molecular grade as potentially relevant in practice. Furthermore, if validated, molecular grade could be implemented as a predictor of response to therapeutic strategies in non-muscle-invasive bladder carcinoma patients. Also, our study showed molecular grade as a predictor of recurrence-free, progression-free, and cancer-specific survival in patients with Ta/T1 high-grade urothelial carcinoma with a luminal phenotype resulting in more aggressive neoplasms, and mixed phenotype resulting in less aggressive neoplasms. Therefore, if validated in non-muscle-invasive bladder carcinoma, the molecular grading could assist in the stratification of patients in categories relevant with prognosis. Furthermore, our study did not specifically address the issue of response to therapy based on luminal/basal phenotype due to the fact that all high-

Table 5 Survival analysis of patients with T1HG bladder carcinoma according to molecular-phenotype grade

Molecular grade	Overall T1HG $N = 37$ (%)	RFS $N = 13$ (%)	p value*	PFS $N = 20$ (%)	p value*	CSS $N = 24$ (%)	p value*
			0.015		< 0.001		< 0.001
Null	17 (45.9)	7 (41.2)		12 (70.6)		14 (82.4)	
Mixed	2 (5.4)	1 (50.0)		1 (50.0)		2 (100)	
Basal	9 (24.3)	5 (55.6)		7 (77,8)		8 (88.9)	
Luminal	9 (24.3)	0 (0.0)		0 (0.0)		0 (0.0)	

*Log-rank test

RSF, recurrence-free survival; *PFS*, progression-free survival; *CSS*, cancer-specific survival

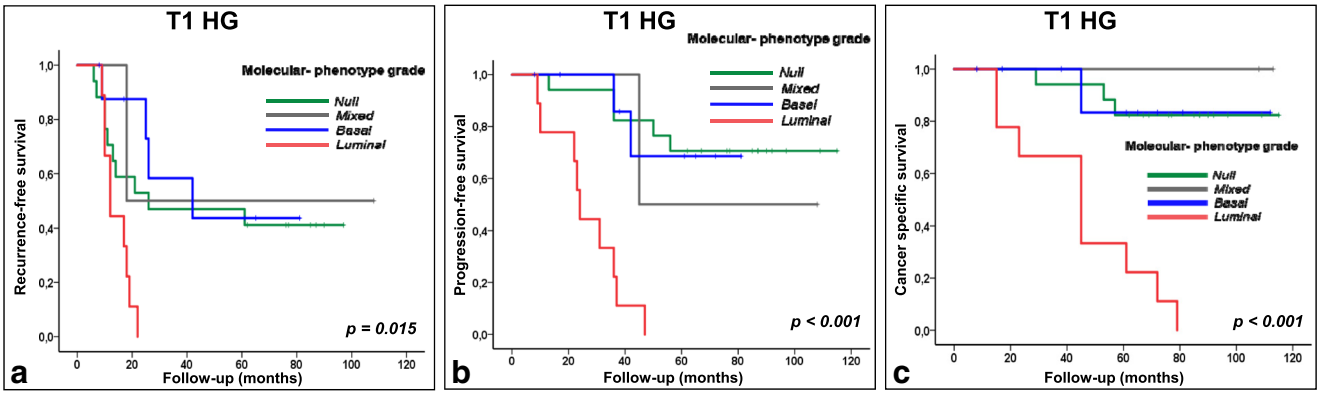

Fig. 7 Molecular phenotype grade was a predictor of RFS, PFS, and CSS in patients with invasive (T1) high-grade urothelial carcinoma with luminal phenotype resulting in more aggressive neoplasms (RFS, PFS, CSS) and mixed phenotype resulting in the less aggressive disease (RFS, PFS, CSS)

grade Ta/T1 cases had been treated by intravesical bacillus Calmette-Guerin (as per guidelines); however, our finding that the luminal (chemo-resistant) phenotype was independently associated with more aggressive behavior than basal (chemo-sensitive) phenotype might suggest that basal tumors would be more responsive to current intravesical bacillus Calmette-Guerin protocols; a finding that needs to be substantiated in larger prospective series. This is actually not surprising since the cancer genome atlas derived data on muscle-invasive bladder carcinoma suggest that luminal cases have limited response to systemic immunotherapy with checkpoint inhibitors. [35] An additional topic of interest is the fact that our study presents data related to conventional urothelial carcinoma of the non-muscle invasive subtype, and therefore, it does not include the analysis of luminal-basal phenotype in the so-called bladder carcinoma with variant histology; a fact that could be considered a limitation. However, the potential interest of variant histology in modern urinary bladder pathology suggests that an entirely separate study would be necessary to properly address this topic, and this is out of the scope of our study. [41, 42]

In conclusion, our study shows that molecular-phenotype grade is feasible and dividing non-muscle-invasive bladder carcinoma patients in prognostically and potentially predictive categories add additional information regarding pathologic grade and stage. Finally, it is relevant to say that more studies are needed to fully develop the potential of this classification

Table 6 Multivariate survival analysis of patients with non-muscle invasive (Ta/T1) bladder cancer

Variable	Recurrence-free survival $N = 74$		Progression-free survival $N = 120$		Cancer-specific survival $N = 127$	
	p value**	Hazard ratio (CI 95%)	*p* value**	Hazard ratio (CI 95%)	*p* value**	Hazard ratio (CI 95%)
Age (years)	–	–	–	–		
< 70*					0.010	Reference
≥ 70						3.428 (1.345–8.741)
Tumor size (cm)	0.099		–	–	0.493	
< 3*		Reference				Reference
≥ 3		1.578 (0.918–2.713)				1.570 (0.432–5.702)
Stage	0.143		0.676		0.985	
Ta*		Reference		Reference		Reference
T1		0.563 (0.261–1.215)		1.278 (0.404–4.043)		1.013 (0.263–3.906)
Grade	<0.001		<0.001			
LG*		Reference		Reference	<0.001	Reference
HG		3.005 (1.820–4.962)		11.575 (4.411–30.379)		9.604 (3.130–29.471)
Molecular grade	0.004	Reference	<0.001		0.001	Reference
Null*	0.286	0.459 (0.110–1.917)	0.990	Reference	0.983	----
Mixed	0.402	0.730 (0.349–1.526)	0.555	0.987 (0.125–7.818)	0.186	2.474 (0.647–9.462)
Basal	0.002	2.307 (1.350–3.943)	<0.001	1.396 (0.461–4.223)	<0.001	9.036 (3.090–26.420)
Luminal				8.142 (3.259–20.340)		

*Reference category/denominator of the comparison in the Cox regression analysis; **p value, obtained the Wald test of the Cox regression analysis; IC (95%) confidence interval 95% for HR

in practice, and for that, the pathologist is to play a very important role in the near future.

Author contribution ALB conceived and designed the study and wrote, edited, and reviewed the manuscript. ALB, JR, PA, and PLF designed the study and reviewed the manuscript. JR, PA, and PLF performed the statistical analysis and reviewed the manuscript. JF, AC, VH, and AB collected and analyzed data and reviewed the manuscript. AB performed and evaluated RT-PCR methods. RM and LC critically read and edited the final manuscript. All authors gave final approval for publication.

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Compliance with ethical standards

Approval from an institutional review board was obtained.

Competing interests The authors declare that they have no conflict of interest.

Informed consent Written informed consent was obtained from all the patients included in the study.

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