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Original Article

Histological and immunohistochemical markers for progression prediction in transurethrally resected high-grade non-muscle invasive bladder cancer

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Abstract: High-grade non-muscle-invasive bladder cancer (Non-MIBC) has a high risk of stage progression to muscle-invasive bladder cancer (MIBC) and could be managed either conservatively by transurethral resection of bladder tumor (TURBT) or more aggressively by radical cystectomy. The selection of patients who may benefit from early radical intervention is a challenge. To define useful prognostic markers for progression, we analyzed clinicopathological features and immunohistochemical expression patterns of E2F1, p27, survivin, p53, EZH2, IMP3, TSC1/hamartin, fatty acid synthase, androgen receptor, 14-3-3σ, MAGEA4, and NY-ESO-1 on 118 cases of high-grade Non-MIBC. During the mean follow-up period of 64.3 months, progression occurred in 18 patients (15.3%). Histologically, large amount of invasive component (> 50%) was noted in 35 cases (29.7%) and was strongly associated with progression. Among the 12 biomarkers, high expressions of E2F1 and nuclear p27 were noted in 46 cases (40.0%) and 14 cases (12.7%), respectively, and were associated with frequent progression. Using multivariate analysis, the proportion of invasive component and high E2F1 expression were independent prognostic factors for the prediction of progression. Our results indicated that large amount of invasive carcinoma component and high expressions of p27 and E2F1 were predictive markers for progression in Non-MIBC. Therefore, we suggest that these parameters, especially proportion of invasive carcinoma component and E2F1 expression, should be evaluated during pathologic examination and considered during selection of the appropriate management strategy for high grade Non-MIBC patients.

Keywords: Bladder cancer, non-muscle-invasive bladder cancer, progression, urothelial carcinoma, high-grade

Introduction

Bladder cancer is the most common malignancy of the urinary tract and the sixth most common cancer in men worldwide [1]. Approximately 75% of patients with bladder cancer initially present with non-muscle-invasive bladder cancer (Non-MIBC) that is either confined to the mucosa as a papillary tumor (stage Ta) or carcinoma in situ (CIS, stage Tis) without stromal invasion, or invasion limited to the submucosa (stage T1) [2]. Non-MIBC is a heterogeneous group of tumors with different rates of progression to MIBC, ranging from 0.8% to 45% in 5 years [3]. Previous studies suggest that important risk factors for the progression are the presence of concomitant CIS, higher grade, and T1 stage [3, 4]. In addition, multiplicity, large tumor size (≥ 3 cm), and a history of recurrence are considered as risk factors [3, 4].

Non-MIBC is generally managed by bladder-conserving transurethral resection of bladder tumor (TURBT), while in MIBC, the urinary bladder is removed by radical cystectomy. Because of the high risk of progression, the management of high-grade Non-MIBC is challenging. Some patients are treated with a combination of TURBT with or without intravesical instillation of Bacillus Calmette-Guérin (BCG), whereas others have been treated more aggressively with cystectomy [5, 6]. Therefore, discrimination of

Table 1. Primary antibodies and subcellular location of antigen

Antibody	Dilution	Company	Subcellular location
p53	1:1500	Dako Corp., Carpentaria, CA	Nucleus
p27	1:100	Santa Cruz Biotechnology, Inc., CA	Nucleus, cytoplasm
Androgen receptor	1:100	Epitomics, CA	Nucleus
E2F transcription factor 1 (E2F1)	1:200	Invitrogen, Carlsbad, CA	Nucleus
Enhancer of zeste homolog 2 (EZH2)	1:25	BD Biosciences Pharmingen, San Diego, CA	Nucleus
Survivin	1:50	Santa Cruz Biotechnology, Inc., CA	Nucleus, cytoplasm
Insulin-like growth factor 2 mRNA binding protein 3 (IMP3)	1:500	Dako Corp., Carpentaria, CA	Cytoplasm
Fatty acid synthase (FASN)	1:500	Novus Biologicals, Littleton, CO	Cytoplasm
Tuberous sclerosis gene 1 (TSC1)/Hamartin	1:150	Abcam, CA	Cytoplasm
MAGEA4	1:200	Abcam, CA	Cytoplasm
NY-ESO-1	1:500	Invitrogen, Carlsbad, CA	Cytoplasm
14-3-3σ	1:50	Santa Cruz Biotechnology, Inc., CA	Cytoplasm

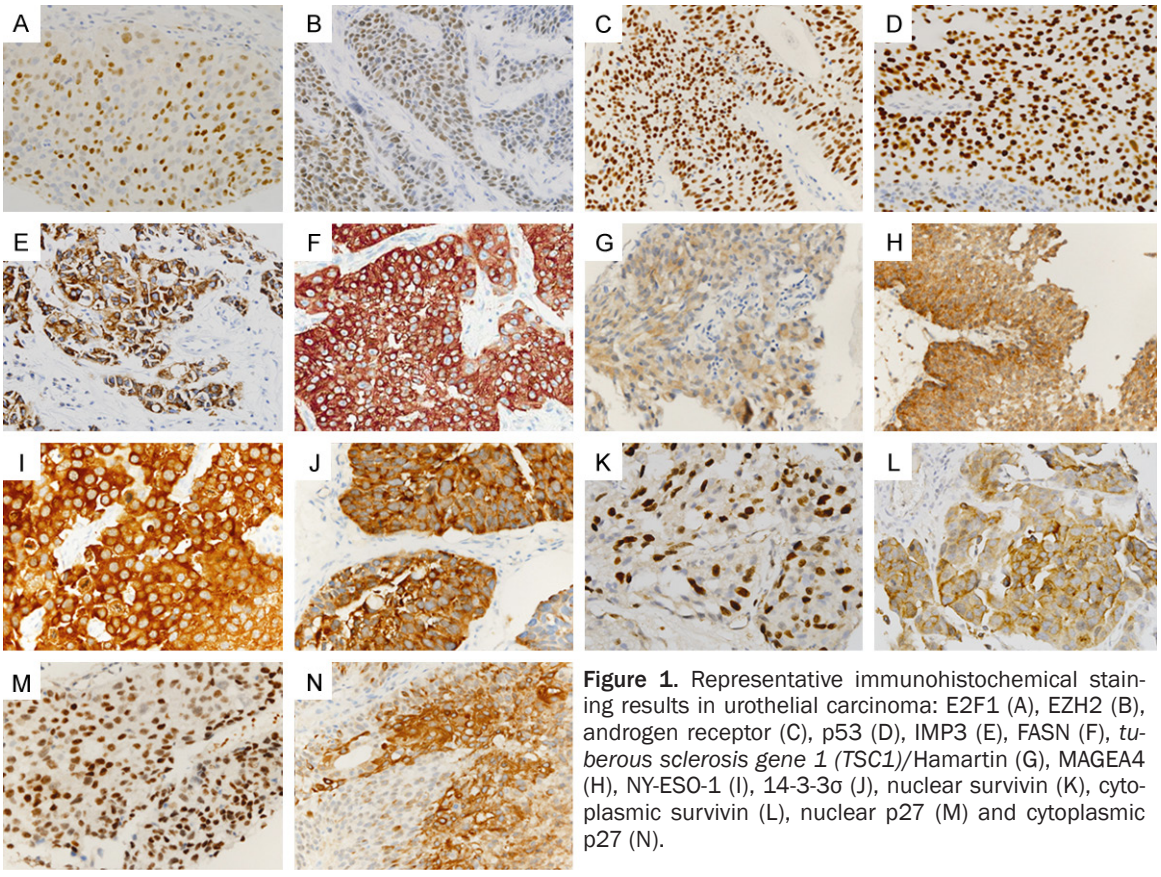

Figure 1. Representative immunohistochemical staining results in urothelial carcinoma: E2F1 (A), EZH2 (B), androgen receptor (C), p53 (D), IMP3 (E), FASN (F), *tuberous sclerosis gene 1 (TSC1)*/Hamartin (G), MAGEA4 (H), NY-ESO-1 (I), 14-3-3σ (J), nuclear survivin (K), cytoplasmic survivin (L), nuclear p27 (M) and cytoplasmic p27 (N).

high-grade Non-MIBC cases with progressive potential to MIBC is crucial, considering the benefit of early radical intervention, but should be cautious given the cost of removing a patient's urinary bladder. Nevertheless, few tools are available to predict progression in Non-MIBC patients.

There are recent studies describing the molecular mechanisms behind the progression of bladder cancer [7-14]. They have suggested various proliferation- and progression-related proteins as prognostic markers, including E2F1, p27, p53, EZH2, IMP3, and survivin [7-10]. High expressions of fatty acid synthase (FASN) and

Table 2. Clinicopathological features of 118 cases of high-grade non-muscle invasive bladder cancer

Variables		Number of cases (%)
Sex	Male	104 (88.1)
	Female	14 (11.9)
Multiplicity	Absence	51 (43.2)
	Presence	67 (56.8)
Tumor size	Not recorded	14 (11.9)
	< 1.5 cm	16 (13.6)
	1.5 cm-3 cm	48 (40.7)
	> 3 cm	40 (33.9)
pT stage	Ta	17 (14.4)
	T1	101 (85.6)
Proportion of invasive component	≤ 50	84 (71.2)
	> 50	34 (28.8)
Carcinoma in situ	Absence	79 (66.9)
	Presence	39 (33.1)
Lymphovascular invasion	Absence	116 (98.3)
	Presence	2 (1.7)
Muscularis propria	Not included	34 (28.8)
	Included	83 (70.3)
	Not assessable[a]	1 (0.8)
Stage progression	Absence	100 (84.7)
	Presence	18 (15.3)

[a]Not assessable: due to cautery artifact, fragmentation, or incorrect orientation of tumor tissues.

the androgen receptor have been related to poor disease-specific survival in bladder cancer [11, 12]. TSC1/hamartin, a tumor suppressor involved in the development of various malignancies, controls cell proliferation partly by up-regulating p27 and 14-3-3σ. Low expression of TSC1/hamartin tends to lead to a high risk of progression in Non-MIBC [13]. MAGEA4 and NY-ESO-1 are cancer/testis (CT) antigens, normally expressed only in human germ cells in the testis, but their expression is increased in various types of human cancers including urinary bladder tumors [14].

In this study, we tried to identify clinicopathological features and immunohistochemical (IHC) markers from a panel of biomarkers for the prediction of tumor progression in high-grade Non-MIBC. Several of these were found to be significant predictive markers for progression. Because immunohistochemistry is widely used in pathology laboratories worldwide, the results from our study can be easily applied in the clinic for patient management.

Materials and methods

Study samples

This retrospective study was approved by the Asan Medical Center Institutional Review Board. A total of 403 patients who underwent TURBT between January 1996 and December 2006 at the Asan Medical Center and whose tumor tissues were available for tissue microarray (TMA) construction were included. Cases were reviewed for various pathological features and graded according to the 2004 World Health Organization Tumor Classification and assigned tumor, node, metastasis stages according to the American Joint Committee on Cancer Staging System, 7th edition [15, 16]. Patients' clinical information including age, sex, tumor recurrence and progression was obtained from electronic medical records or hospital charts.

After microscopic examination for diagnostic reassessment and histological tumor grading, 167 patients with high-grade Non-MIBC were selected. Among them, 49 patients were excluded because of residual tumor detection, immediate radical cystectomy within a month after initial TURBT, or a short follow-up period of less than a month. No cases with isolated Tis were included in this study. A total of 118 cases were finally included in this study. Tumor progression was defined as an increase of the T stage from Non-MIBC (Ta or T1) to MIBC (T2) on follow-up TURBT or radical cystectomy, which was performed more than 1 month after initial TURBT. The Cutoff value for the proportion of invasive carcinoma component was determined by ROC curve analysis.

Tissue microarray block construction and immunohistochemistry

TMA blocks with 0.6-mm diameter cores were constructed from formalin-fixed, paraffin-embedded bladder cancer tissue blocks of

Table 3. Correlation between clinicopathological features and stage progression in 118 cases of high grade non-muscle invasive bladder cancer

Variables		Cases without progression	Cases with progression	*P*-value
Sex	Male	88 (88.0)	16 (88.9)	> 0.999
	Female	12 (12.0)	2 (11.1)	
Multiplicity	Absence	47 (47.0)	4 (22.2)	0.070
	Presence	53 (53.0)	14 (77.8)	
Tumor size (cm)	< 1.5	14 (15.7)	2 (13.3)	0.494
	1.5-3	39 (43.8)	9 (60.0)	
	> 3	36 (40.5)	4 (26.7)	
pT stage	Ta	14 (14.0)	3 (16.7)	0.723
	T1	86 (86.0)	15 (83.3)	
Invasive component (%)	≤ 50	75 (75.0)	9 (50.0)	0.046
	> 50	25 (25.0)	9 (50.0)	
Carcinoma in situ	Absence	67 (67.0)	12 (66.7)	> 0.999
	Presence	33 (33.0)	6 (33.3)	
Lymphovascular invasion	Absence	99 (98.3)	17 (94.4)	0.283
	Presence	1 (1.0)	1 (5.6)	

TURBT specimens using a tissue microarrayer (Beecher Instruments, Silver Spring, MD). Three representative cores were obtained for each case to be included in TMA blocks. One core of normal tonsil was included in each TMA block as a positive control.

The primary antibodies used in this study and subcellular location of corresponding antigens are summarized in **Table 1**. IHC staining was performed using an automated staining system (BenchMark XT; Ventana Medical Systems, Tucson, AZ) and an ultraView Universal DAB detection kit (Ventana Medical Systems). The results of IHC staining for p53, p27, EZH2, E2F1, IMP3, survivin, and FASN obtained in our previous study ("Kim K." et. al, 2014, submitted) were utilized to examine their clinical significance as predictive markers of progression in high-grade Non-MIBC. Representative expression patterns of those markers are presented in **Figure 1**. Nuclei were counterstained with hematoxylin.

Assessment of immunohistochemical results

The TMA slides were evaluated by three independent pathologists (KEK, HJG, and YMC) who were blinded to the associated clinical and pathological information. The percentage of stained tumor cells in the entire area of three cores was recorded. Cutoff values for high expression of each protein were determined by ROC curve analysis as follows: E2F1 (5%), p53 (30%), nuclear p27 (30%), cytoplasmic p27 (30%), EZH2 (30%), IMP3 (30%), cytoplasmic survivin (5%), nuclear survivin (30%), TSC1/hamartin (30%), FASN (30%), androgen receptor (30%), 14-3-3σ (30%), MAGEA4 (30%), and NY-ESO-1 (30%).

Statistical analysis

The relationships between protein expression or clinicopathological parameters and progression were evaluated by cross-correlation analysis, a Cox proportional hazards model and Kaplan-Meier analysis. The hazard ratio, along with the 95% confidence interval, was assessed for each factor. All tests were two-sided, and *P*-values less than 0.05 were considered statistically significant.

Results

Clinicopathological features of high grade non-MIBC cases

The clinicopathological features of 118 cases are summarized in **Table 2**. The median age was 70 years (range, 32-93) at the initial TURBT with a 7.4:1 male-to-female ratio. The cases showed the following high risk features: multiplicity (67 cases, 56.8%), tumor size of ≥ 3 cm (40 cases, 33.9%), stage T1 (101 cases, 85.6%), and concurrent CIS (39 cases, 33.1%). The majority of cases (83 cases, 70.9%) included the muscularis propria in the TURBT specimens. The mean proportion of invasive component of the tumor was 34.2% (0-100%). During the mean follow-up period of 64.3 months (range, 1.8-188.5 months), 46 cases (39.0%) experienced tumor recurrence in the urinary bladder at a mean of 11.4 months (range, 1.2-38.5 months). Stage progression occurred in 18 patients (15.3%) at a mean of 17.7 months (range, 2.8-65.3 months) after the initial TURBT.

Table 4. Correlation between protein expression and stage progression in 118 cases of high grade non-muscle invasive bladder cancer

Variables		Cases without progression	Cases with progression	*P-value*
P53	Low	74 (76.3)	12 (70.6)	0.760
	High	23 (23.7)	5 (29.4)	
Nuclear p27	Low	84 (90.3)	12 (70.6)	0.040
	High	9 (9.7)	5 (29.4)	
Cytoplasmic p27	Low	85 (91.4)	15 (88.2)	0.651
	High	8 (8.6)	2 (11.8)	
Androgen receptor	Low	86 (89.6)	15 (88.2)	> 0.999
	High	10 (10.4)	2 (11.8)	
E2F1	Low	64 (65.3)	5 (29.4)	0.007
	High	34 (34.7)	12 (70.6)	
EZH2	Low	87 (90.6)	15 (88.2)	0.670
	High	9 (9.4)	2 (11.8)	
Nuclear survivin	Low	55 (57.9)	10 (55.6)	> 0.999
	High	40 (42.1)	8 (44.4)	
Cytoplasmic survivin	Low	85 (89.5)	14 (77.8)	0.234
	High	10 (10.5)	4 (22.2)	
IMP3	Low	93 (94.9)	15 (88.2)	0.276
	High	5 (5.1)	2 (11.8)	
FASN	Low	46 (46.0)	5 (27.8)	0.199
	High	54 (54.0)	13 (72.2)	
TSC1/Hamartin	Low	86 (87.8)	12 (70.6)	0.130
	High	12 (12.2)	5 (29.4)	
MAGEA4	Low	29 (30.9)	2 (11.8)	0.145
	High	65 (69.1)	15 (88.2)	
NY-ESO-1	Low	96 (97.0)	100 (100)	> 0.999
	High	3 (3.0)	0 (0)	
14-3-3σ	Low	41 (42.7)	6 (35.3)	0.606
	High	55 (57.3)	11 (64.7)	

Clinicopathological factors for the prediction of stage progression in high grade non-MIBC cases

The cases with progression showed more invasive component (> 50%), which was also strongly associated with progression in a Cox regression analysis (**Tables 3** and **5**). Gender, tumor size, initial T stage of TURBT specimens, concurrent CIS were not associated with progression (**Tables 3** and **5**). Histological variants, including micropapillary carcinoma, did not meet statistical significance (data not shown).

Protein expression for prediction of stage progression in high grade non-MIBC cases

E2F1, EZH2, androgen receptor, and p53 localized to the nucleus, whereas IMP-3, FASN, hamartin, MAGEA4, NY-ESO-1 and 14-3-3σ localized to the cytoplasm (**Figure 1A-J**). Survivin and p27 were found in both the nucleus and the cytoplasm (**Figure 1K-N**).

Cases with progression had more frequent and high expression of E2F1 and nuclear p27 than cases without progression (**Table 4**). In a Cox regression analysis, cases with high expression of E2F1 and nuclear p27 were associated with frequent progression; this was supported by a Kaplan-Meier analysis (**Table 5**; **Figure 2C**, **2D**). In a multivariate analysis, the proportion of the invasive component and E2F1 expression were independent prognostic factors for the prediction of progression (**Table 5**). The expressions of other markers (cytoplasmic p27, nuclear and cytoplasmic survivin, p53, androgen receptor, EZH2, IMP-3, FASN, MAGEA4, TSC1/hamartin, NY-ESO-1, and 14-3-3σ) were not associated with progression in high grade (**Tables 4** and **5**).

Discussion

Here, we showed that proportion of invasive carcinoma component is a histological feature independently predictive of progression from Non-MIBC to MIBC. Among the 12 IHC markers, high expressions of nuclear p27 and, especially, E2F1 are strongly associated with tumor progression.

To the best of our knowledge, our study is the first to analyze the prognostic significance of the proportion of the invasive component in Non-MIBC. By definition, all Ta tumors are composed of non-invasive papillary urothelial carcinoma [16]. During TURBT specimen review, we noticed that many T1 tumors were also composed of mostly non-invasive papillary urothelial carcinoma. Since clinical behavior of malignant tumors is usually determined by the invasive component, we decided to evaluate whether the proportion of invasive component in T1 tumors is important for the tumor progression or not. Although the proportion of the invasive component was not measured directly, previous studies had suggested that the presence of a non-papillary solid tumor as a prognostic factor of progression and disease-specific sur-

Table 5. Prognostic parameters to predict stage progression in 118 cases of high grade non-muscle invasive urothelial carcinomas

	Univariate analysis			Multivariate analysis		
Variables	*P* value	HR	95% CI	*P* value	HR	95% CI
Sex	0.840	1.163	0.267-5.061			
Multiplicity	0.141	2.308	0.759-7.019			
Tumor size (cm)	0.795	1.218	0.275-5.400			
pT stage	0.902	1.081	0.312-3.739			
CIS	0.868	0.920	0.345-2.453			
Invasive component (> 50%)	0.004	3.908	1.544-9.888	0.004	4.306	1.577-11.758
E2F1	0.009	4.050	1.424-11.521	0.038	3.204	1.066-9.623
p27 (nucleus)	0.028	3.230	1.137-9.172	0.120	2.401	0.796-7.241

Abbreviations: HR, hazard ratio; CI, confidence interval; LVI, lymphovascular invasion; CIS, carcinoma in situ.

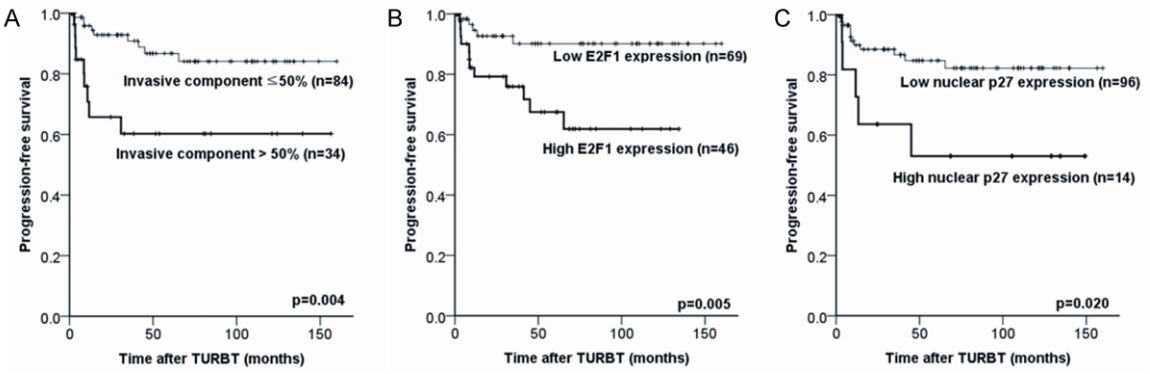

Figure 2. Kaplan-Meier analysis for predicting progression. The invasive tumor component (A), E2F1 expression (B), and nuclear p27 expression (C) are associated with frequent progression.

vival in Non-MIBC, in which non-papillary solid pattern was usually the growth pattern of the invasive component [17, 18].

E2F1 is a tumor suppressor that plays a critical role in cell-cycle progression and induction of apoptosis in response to DNA damage under normal conditions, but increased levels of E2F1 promote cellular proliferation [19]. Dysregulated E2F1 also mediates tumor progression through the upregulation of epidermal growth factor receptor (EGFR) and activation of the cytoplasmic Ras/mitogen-activated protein kinases (MAPK)/extracellular signal-regulated kinase (ERK) and phosphoinositide-3-kinase (PI3K)/AKT signaling cascades [19]. In fact, high expression of E2F1 and its associated target genes predicted progression from Non-MIBC to MIBC [7].

p27, a cyclin-dependent kinase inhibitor, inhibits cyclin E-CDK2 in the nucleus and regulates the G1-S transition of the cell cycle. Low protein levels and cytoplasmic mislocalization of p27 result in loss of its antiproliferative role and lead to increased cell proliferation and cell migration. Accordingly, low p27 expression was suggested as a poor prognostic factor in various malignant tumors including bladder cancer [5, 8, 20]. In contrast, this study suggests that the nuclear localization of p27 is directly correlated with progression. Lopez-Beltran *et al.* also failed to find the correlation between p27 expression and survival for NMIBC [21]. Therefore, a further study is required to confirm the prognostic significance of nuclear p27 expression in high grade Non-MIBC.

Against our expectations, the T stage, CIS, and multiplicity did not predict stage progression in this study. It can be partly explained by the fact that this study included only high-grade cases of Non-MIBC and a relative small number of Ta cases (17 cases, 14.4%). One previous study analyzing all grades (grades 1, 2, and 3) of Non-MIBC reported that the T stage was a statisti-

cally significant predictor for recurrence but not progression, where they suggested that this discrepancy stems from an incomplete resection of tumors during TURBT [22].

Although this study showed prognostic significance of histological features (proportion of invasive component) and IHC markers (E2F1 and nuclear p27) in Non-MIBC, its limitations include the retrospective design and reliance on experience from a single institution. Furthermore, remarkable advances in molecular technologies and elucidation of the mechanisms of carcinogenesis and tumor progression have led to the discovery of new molecular markers, including mutant forms of FGFR3 as well as USP18 and DGCR2 expressions [23-25]. Therefore, further multi-institutional studies with newly discovered molecular markers will help validate and strengthen the clinical utility of these results and improve the accuracy of predicting progression in Non-MIBC.

Disclosure of conflict of interest

None.

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