RESEARCH COMMUNICATION

A Novel Molecular Grading Model: Combination of Ki67 and VEGF in Predicting Tumor Recurrence and Progression in Non-invasive Urothelial Bladder Cancer

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Abstract

Purpose: To assess efficacy of Ki67 combined with VEGF as a molecular grading model to predict outcomes with non-muscle invasive bladder cancer (NMIBC). Materials: 72 NMIBC patients who underwent transurethral resection (TUR) followed by routine intravesical instillations were retrospectively analyzed in this study. Univariate and multivariate analyses were performed to confirm the prognostic values of the Ki67 labeling index (LI) and VEGF scoring for tumor recurrence and progression. Results: The novel molecular grading model for NMIBC contained three molecular grades including mG1 (Ki67 LI≤25%, VEGF scoring≤8), mG2 (Ki67 LI>25%, VEGF scoring ≤ 8; or Ki67 LI ≤ 25%, VEGF scoring > 8), and mG3 (Ki67 LI > 25%, VEGF scoring > 8), which can indicate favorable, intermediate and poor prognosis, respectively. Conclusions: The described novel molecular grading model utilizing Ki67 LI and VEGF scoring is helpful to effectively and accurately predict outcomes and optimize personal therapy.

Keywords: CKi67 - VEGF - recurrence - progression - non-muscle invasive bladder cancer

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Introduction

Non-muscle invasive bladder cancer (NMIBC) is one of the most common malignants in the urological system. In recent years, the transurethral resection (TUR) of tumors and post-operative intravesical instillation has become routine treatment for NMIBC (Evans et al., 2007). However, there are still 30-70% of NMIBC cases which show recurrence and 10% which would develop a muscle invasive disease. Determining the potential prognostic factors of NMIBC can indicate the different risks of recurrence and progression in patients and subsequently help to design the personal therapy strategy. The typical risk factors include tumor grade, size, stage, the multiplicity of lesions, and whether intravesical instillation is performed. The recurrence and progression of the disease cannot be well predicted using the conventional criteria (Evans et al., 2007). Therefore, it is critical to identify new biochemical factors. At present, markers such as fibroblast growth factor receptor-3 (FGFR3), epidermal growth factor receptor (EGFR), retinoblastoma protein (pRB), p53, Ki 67, vascular endothelial growth factor (VEGF), and cytokeratin (CK)-20 (Bryan et al., 2010) are used as indicators. However, none of these markers have been applied to clinical practice alone and proved by multi-center and large scale study (Habuchi et al., 2005). Interestingly, more and more evidence has shown that the combination of biomarkers from the same or different categories has obvious advantages compared to the single markers (Hilmy et al., 2006; Yurakh et al., 2006; Gonul et al., 2008).

So far, both cellular proliferation and angiogenesis have been demonstrated to be important for carcinogenesis. Ki67 is a nuclear protein indicating the extent of cell proliferation and has been used to measure cell growth fraction in immunostaining analysis (Margulis et al., 2009). There is a significant association between the prognosis of bladder cancer and a high level of Ki67 immunoactivity. On the other hand, tumor vascularization plays a fundamental role in neoplastic processes and is essential for tumor progression and the metastatic spread of solid tumors (Vidal et al., 2008). Vascular Endothelial Growth Factor (VEGF) is considered one of the most important angiogenic regulators during tumor angiogenesis (Fauconnet et al., 2009). VEGF is expressed in both invasive and noninvasive bladder tumors, and the increased expression of VEGF is associated with higher tumor stage and progression, but similarly to the circumstance of Ki67, a definite cut-off point for stratifying a better or worse prognosis remains unclear. A recent study has suggested that the overexpression of VEGF and Ki67 both occurr during the process of immature capillary proliferation of malignant carcinoma (van der Loos et al., 2009), indicating the correlation

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between Ki67 and VEGF during tumor development.

In this study, we aimed to clarify the prognostic value of Ki67 and VEGF in NMIBC and to develop a new reasonable evaluation system for predicting the prognosis. We analyzed the immunoactivity of Ki67 and VEGF in 72 NMIBC patients treated by the Department of Urology, the First Affiliated Hospital of Sun Yat-Sen University, between 2004 and 2009. Lesions were classified based on the World Health Organization (WHO) criteria (2004 version).

Materials and Methods

Patients

We analyzed 58 male and 14 female patients with NMIBC who had been treated in the First Affiliated Hospital of Sun Yat-Sen University, Guangzhou, China. The classification of tumor stage and grade was made according to the International Society of Urological Pathology and World Health Organization Criteria (2004 Version). No special patient selection was performed. The mean age was 61.3 years (27~87 years). After giving their informed consent, all patients underwent transurethral resection (TUR) of the bladder tumor, followed by cystoscopies at 3-month intervals for 2 years and at 6-month intervals thereafter. All patients received immediate adjuvant bladder instillation with mitomycin or epirubicin within 6hr after TUR. The maintenance instillation with mitomycin, epirubicin, or pirarubicin lasted for 1 year according to the following instructions: 30 mg/30 ml for mitomycin, 50 mg/50 ml for epirubicin, and 40 mg/40 ml for pirarubicin; weekly administration for 8 weeks, followed by monthly administration for 6 to 8 months, and every 2 months until 1 year. Cystectomy was performed on 10 patients who had tumor progression and in whom it became a muscle-infiltrating tumor during follow-up. No patient died for non cancer-specific or cancer-specific reasons. The follow-up was completed on May 31, 2011. The mean follow-up for the whole series was 63.4 months (16~93 months). Disease recurrence was defined as biopsy-proven cancer after the initial tumor resection (Wolman et al., 2007), while tumor progression was defined as the development of muscle-invasive or more advanced stage carcinoma, distant metastasis, or death from bladder cancer (Cheng et al., 1999).

Study received the ethics committee approval of the First Affiliated Hospital of Sun Yat-sen University and conformed to ethical guidelines of 1975 Declaration of Helsinki.

Immunohistochemical Study and Scoring

The immunohistochemical (IHC) study was carried out on formalin-fixed, paraffin-embedded tissues. Serial sections of the tumor tissues were obtained from archived paraffin-embedded tissue blocks. In all cases, the primary pathological diagnosis was confirmed by HE staining. Subsequent slides were stained for Ki67 and VEGF. All slides were deparaffinized in xylene and then rehydrated in ethanol, and subsequently heated in a citrate buffer (0.01 mM, pH 6.0) using a steam cooker for 40 min. All sections were treated with 0.3% hydrogen peroxide for 5 min and incubated with the monoclonal primary antibody (Ki67, DAKO M7240 Clone:MIB-1 with 1:50 dilution, VEGF, DAKO M7273 Clone:VG1 with1:50 dilution) at 37°C for 45 min. Secondary biotinylated antibody (DAKO EnvisionTM K5007 HRP/DAB Rabbit/Mouse) was then applied for 30 min. After rinsing, the slides were stained with diaminobenzidine as chromogen and counterstained with routine hematoxylin.

Nuclear staining of Ki-67 was considered positive. Immunoactivity was assessed by Ki67 labeling index (Lebret et al., 2000) (Ki67 LI): the percentage of Ki67-positive cells was determined by scoring 1000 cells. For analytical purposes, the highest category obtained in each patient was considered. We used a semiquantitative analysis system to determine the VEGF immunostaining score. Definite membrane staining or strong cytoplasmic reactivity of VEGF was considered positive (Vidal et al., 2008). In brief, the scoring system was established according to the percentage and intensity of positive cells (Klein et al., 2001). For percentage, 0~4 scores represent <5%, 5~25%, 26~50%, 51~75% and >75% of labeled cells, respectively. For the intensity, 0~3 score indicate weak, middle and strong staining respectively. Multiplication of both scores decided the final quotation, ranging 0~12. A double-blind analysis was performed by two independent pathologists. Each sample was scored twice. All the slides were analyzed without knowledge of clinical data. If the final scores had a more than 3 point discrepancy, a second evaluation would be performed.

Statistical Analysis

Statistical analysis was performed using SPSS version 13.0 software (Chicago, IL, USA). A P value of less than 0.05 was considered significant. Variables in relation to tumor recurrence and progression were assessed using Chi-Square test and two-sided Fisher's exact tests. Significant differences concerning tumor recurrence and progression were calculated using the Kaplan–Meier method and log-rank test. Univariate and multivariate Cox regression models concerning tumor recurrence and progression were adjusted to determine the independent prognostic correlation of the variates analyzed. The case distribution between the conventional pathologic model and the molecular model was tested using Chi-Square test.

Results

Variables in Relation to Tumor Recurrence and Progression

Only cases with intact follow-up information were included in the analysis. Follow-up data were obtained from 72 out of 81 patients (88.89%) who were included in the cohort, while 9 patients were excluded due to incomplete information. The median age at the time of diagnosis was 61 years. The median follow-up was 62 months (range: 16-93 months). 38 (52.78%) patients had one or more recurrences while 10 (13.89%) had tumor progression, with muscle invasive cancer found in the postoperative follow up. 73.61% (n=53) patients were in stage pTa and 26.39% (n=19) in pT1, respectively. The grade was distributed as follows: papillary neoplasm of low malignant potential (PUMLMP) 22.22% (n=16), low

Table 1. Variables in Relation to Tumor Recurrence and Progression

Variables		Recurrence Total	Recurrence No. (%)	X^2	P value	Progression No. (%)	X^2	P value
Whole series		72	38(52.78)			10(13.89)		
Age	<60yr	20	11(55.00)			2(10.00)		
	≥60yr	52	27(51.92)	0.055	0.815	8(15.38)	0.35	0.554
Gender	Male	58	30(51.72)			8(13.79)		
	Female	14	8(57.14)	0.133	0.715	2(14.29)	0.002	0.962
Tumor grade	PUMLMP	16	3(18.75)			0(0.00)		
	LGPUC	38	22(57.89)			6(15.79)		
	HGPUC	18	13(72.22)	11.151	0.004**	4(22.22)	9.865	0.007**
Tumor stage	T1	19	12(63.16)			6(31.58)		
	Ta	53	26(49.06)	1.116	0.291	4(7.55)	6.754	0.009*
Multiplicity	single	49	24(48.98)			7(14.29)		
	multiple	23	14(60.87)	0.888	0.346	3(13.04)	0.02	0.887
Tumor size	<3cm	43	22(51.16)			6(13.95)		
	≥3cm	29	16(55.17)	0.112	0.738	4(13.79)	0.001	0.985
Ki67	Low (LI≤25%)	38	13(34.21)			2(5.26)		
immunoreactivity	High (LI>25%)	34	25(73.53)	11.131	0.001**	8(23.53)	5.006	0.025*
VEGF	+/++ (0<score≤8)	39	16(41.03)			5(12.82)		
immunoreactivity	+++ (score>8)	33	22(66.67)	4.715	0.030*	5(15.15)	0.055	0.815

**P<0.01, *P<0.05

Table 2. Univariate Analysis for Prognosis of NMBIC

	Recurrence Risk ratio(95% CI)	Recurrence P value	Progression Risk ratio(95% CI)	Progression P value
Age	0.991(0.510-1.925)	0.991	0.730(0.204-2.610)	0.628
Gender	1.438(0.600-3.448)	0.415	0.862(0.183-4.070)	0.852
Tumor grade	1.251(.0749-2.091)	0.392	1.334(0.485-3.671)	0.577
Tumor stage	1.097(0.530-2.267)	0.803	3.786(1.077-13.306)	0.038*
Multiplicity	1.145(0.724-1.935)	0.527	0.723(0.117-3.572)	0.946
Tumor size	1.084(0.582-2.366)	0.712	0.896(0.218-3.862)	0.866
Intravesical instillation	1.218(0.517-2.684)	0.876	0.921(0.417-2.985)	0.933
Ki67 immunoreactivity	2.390(1.220-4.682)	0.011*	10.729(1.358-84.752)	0.024*
VEGF immunoreactivity	1.837(1.155-2.921)	0.010*	1.370(0.595-3.154)	0.459

*P<0.05

Table 3. Multivariate Analysis for Prognosis of NMBIC

	Recurrence Risk ratio(95% CI)	Recurrence P value	Progression Risk ratio(95% CI)	Progression P value
Tumor stage	----	----	4.607(1.254-16.933)	0.021*
Ki67 immunoreactivity	2.021(1.018-4.010)	0.044*	12.182(1.527-97.151)	0.018*
VEGF immunoreactivity	1.661(1.034-2.669)	0.036*	----	----

*P<0.05

grade papillary urothelial cancer (LGPUC) 52.78% (n=38) and high grade papillary urothelial cancer (HGPUC) 25.00% (n=18).

The association between the clinicopathological characteristics and the recurrence and progression is shown in Table 1. For recurrence, significant associations were found for tumor grade (P=0.004), and immunopositivity of Ki67 (P=0.001) and VEGF (P=0.030); while for tumor progression, tumor stage (P=0.009) together with tumor grade (P=0.007), tumor stage(P=0.009) and Ki67 immunopositivity (P=0.025) were considered significant factors.

Prognostic Factors for Recurrence and Progression in Uni-/Multivariate Analysis

Univariate Cox regresion analysis was performed to explore the relationship between the variables and the tumor prognosis. The levels of Ki67 LI (P=0.011) and VEGF scoring (P=0.010) were both significantly associated with tumor recurrence. Meanwhile, tumor stage (P=0.038) and Ki67 LI (P=0.024) were involved in tumor progression. Other variables such as age, gender, tumor grade, multiplicity, tumor size and intravesical instillation did not show a significant relationship with the tumor prognosis (Table 2).

Further validation was performed using multivariate Cox regression analysis (Table 3). Both Ki67 LI (RR 2.021; 95% CI 1.018-4.010; P=0.044) and VEGF scoring (RR 1.661; 95% CI 1.034-2.669; P=0.036) were demonstrated to be independent predictors. On the other hand, tumor stage (RR 4.607; 95% CI 1.254-16.933; P=0.021) and Ki67 (RR 12.182; 95% CI 1.527-97.151; P=0.018) retained to be independent predictors for progression.

The Kaplan–Meier survival analysis revealed that the Ki67 LI (P=0.004) and VEGF scoring (P=0.036) were

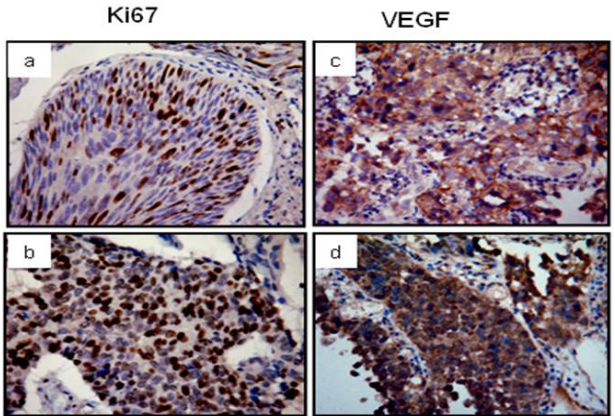

Figure 1. Immunohistochemical Staining of Ki67 and VEGF in NMBIC. a. Ki67 Labeling index of 40%; b.Ki67 Labeling index of 90%; c.VEGF scoring 6; d.VEGF scoring 9 (a-d, ×100)

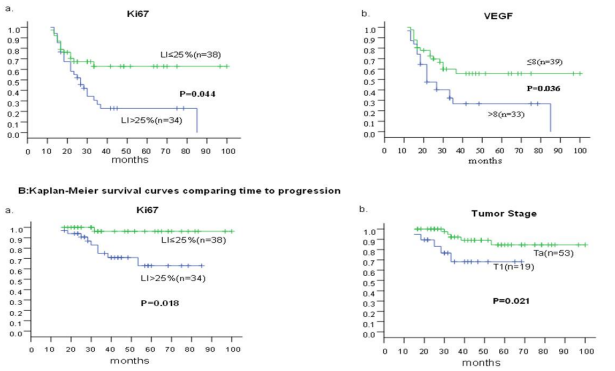

Figure 2. Kaplan-Meier Survival Curves Measured in Months. A. Kaplan-Meier survival curves comparing time to recurrence: a. Green line represents Ki67 LI ≤ 25%, blue line shows Ki67 LI > 25%; b. Green line represents VEGF scoring ≤ 8, blue line shows VEGF scoring > 8. B. Kaplan-Meier survival curves comparing time to progression: a. Green line represents Ki67 LI ≤ 25%, blue line shows Ki67 LI > 25%; b. Green line represents Ta stage, blue line shows T1 stage

significantly correlated with the average survival time compared to recurrence. In addition, compared to the progression, the average survival time in patients having Ki67 LI of > 25% and tumor stage T1 was significantly lower than for those with low Ki67 LI (P=0.018) and Ta stage (P=0.021). These results indicated the Ki67 LI and VEGF scoring can be used to indicate the recurrence in NMIBC while Ki67 LI and tumor stage can be used for predicting the progression (Figure 2).

A Molecular Grading Model by Combining Ki67 and VEGF

As mentioned above, we found that Ki67 LI and VEGF scoring can be used as two independent molecular criteria for the prediction of NMIBC recurrence. By dichotomizing the data at the median value, we found that using Ki67 LI of 25% as a threshold would lead to a sensitivity of 73.52% (1-9/34) and a specificity of 73.53% (25/34) in predicting recurrence. For VEGF scoring, a sensitivity of 67.65% (1-11/34) and a specificity of 66.67% (22/33) were observed under a threshold score value of 8. More interestingly, the sensitivity and specificity for the prediction of tumor recurrence could increase to

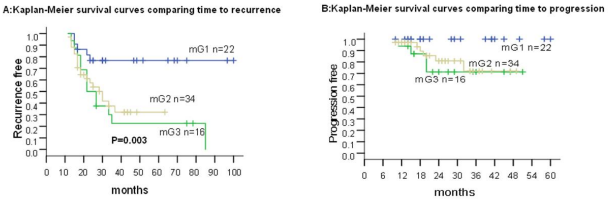

Figure 3. Kaplan-Meier Survival Curves Measurement Using the Molecular Grading System. A Kaplan-Meier survival curves comparing time to recurrence: Blue line represents mG1 phase, grey line is mG2 phase and green line is mG3 phase. B Kaplan-Meier survival curves comparing time to progression: Blue line represents mG1 phase, grey line is mG2 phase and green line is mG3 phase.

Table 4. Molecular Grading Model by Ki67 and VEGF

	Ki67	VEGF	Recurrence -free for 5 years	Risk for recurrence
Molecular Grade 1(mG1)	≤25%	≤8	77.3%*	Low
Molecular Grade 2(mG2)	≤25%	>8	41.2%*	Intermediate
	>25%	≤8		
Molecular Grade 3(mG3)	>25%	>8	18.8%*	High

*P<0.05

Table 5. Cases Distribution in Two Systems

	PUNLMP	LGPUC	HGPUC	Total
mG1	8	10	4	22
mG2	8	22	4	34
mG3	0	6	10	16
Total	16	38	18	72

P<0.05

Table 6. Comparation Between Two Systems

Grading system	Source of Variability	No. of Patients	% Agreement
Molecular grade	Two scorers/two slides	72	87*
Pathologic grade	Two pathologists/one slide	72	63*

*P<0.05

91.18% (1-3/34) and 81.25% (13/16), respectively, with a combined threshold. For this reason, we developed a molecular grading model based on the combination of Ki67 LI and VEGF scoring to evaluate the potential risk of NMIBC recurrence (Table 4).

Based on a previous report (Li et al., 2004) and the median value we observed in our experiment, we considered Ki67 LI=25% and VEGF scoring = 8 as two board lines in the molecular grading system. We typically divided the system into three grades: mG1 (Ki67 LI≤25%, VEGF scoring≤8), mG2 (Ki67 LI>25%, VEGF scoring≤8; or Ki67 LI≤25%, VEGF scoring>8), and mG3 (Ki67 LI>25%, VEGF scoring>8). The corresponding Kaplan-Meier analysis was performed using this novel molecular grading system (Figure 3 A). From our data, the molecular stages can represent a low, medium, and high recurrence risk, respectively (P=0.003). Another evaluation for the risk of tumor progression in NMIBC was also done, in which mG1 showed no progression while both mG2 and mG3 indicated a similar course of progression (Figure 3B).

We further compared the patient distribution with the

conventional pathologic dependent system, as shown in Table 5. We also compared the sensitivity, specificity and reproducibility between the two systems. Our results indicated that both specificity and reproducibility showed significant differences (Table 6).

Discussion

Most of urothelial bladder cancers show localized lesions at the initial diagnosis and have a mildly benign clinical outcome. However, after transurethral resections of visible superficial tumors, more than 50% patients show recurrence within 2 years and up to 20~30% of cases will develop into muscle invasive disease (Chade et al., 2009). Until today, it has been a big obstacle in estimating the recurrence and progression of bladder cancer using the conventional criteria. However, with further investigation of tumorgenesis, it was realized that the changes at the molecular level are usually much earlier than those at the morphological level. For this reason, detailed molecular insights into the biological behavior of bladder cancer might promote more precise prediction. Several biomarkers, including Ki67 and VEGF, have shown potential in predicting the prognosis of NMIBC. Ki67 is an indicator of cell proliferation and a measure of cell growth fraction presented during the G1, S, G2, and M stages of the cell cycle (Yurakh et al., 2006). It is an established independent predictor of recurrence and progression for many malignancies (Li et al., 2004; Viale et al., 2008). In previous studies, increased Ki67 expression was proven to be related to tumor grade, stage, recurrence, progression and the survival of bladder cancer (Margulis et al., 2006; Yurakh et al., 2006; Gonul et al., 2008; Margulis et al., 2009; van Rhijn et al., 2010). It is an independent risk factor for prognosis (Burger et al., 2007). Vascular endothelial growth factor (VEGF) is a potent stimulator of endothelial cell proliferation in vitro and in vivo16. It is a positive regulator of angiogenesis and plays a prominent role in tumor angiogenesis, being highly expressed in almost all tumors (Kilicarslan et al., 2003; Sakamoto et al., 2008) including NMIBC. In bladder cancer, VEGF expression can be detected in both invasive and noninvasive disease, and the increased expression of VEGF is associated with increasing tumor stage and progression (Oka et al., 2005). Although fruitful work has been done in this research field, a clear threshold to stratify patients at different risk of disease recurrence and progression is still lacking. Van der Loos CM et al. recently suggested that overexpression of both VEGF and Ki67 can be detected during the immature capillary proliferation of tumors, indicating a correlation of Ki67 and VEGF in tumor development (van der Loos et al., 2009). But, to our knowledge, there are few studies on the combination of these two biomarkers for predicting the prognosis of NMIBC. Here, we demonstrated that Ki67 LI>25% and VEGF scoring > 8 could serve as a threshold to predict higher risk of tumor recurrence.

In this study, our results confirmed that both Ki67 and VEGF immunopositivity are associated with tumor recurrence, and Ki67 LI is further considered a significant factor for tumor progression, which is consistent with previous studies (El-Chennawi et al., 2009; Margulis et al., 2009). More importantly, the results of univariate and multivariate analysis revealed that Ki67 and VEGF are both risk factors of tumor recurrence for NMIBC. However, multianalysis based on Cox regression models failed to identify VEGF as a risk factor for tumor progression. By dichotomizing the data of the median value (Li et al., 2004), we established a new molecular grading system through utilizing a labeling index of Ki67 and a semiquantative scoring system of VEGF. In most previous approaches, different standards and cut-off points of Ki67 and VEGF were proposed to predict the cancer prognosis, which, however, failed to reach an agreement (Santos et al., 2003). Our study kept the threshold as Ki67 LI 25%, which was proposed by van Rhijn BW (van Rhijn et al., 2003; van Rhijn et al., 2010). For VEGF expression, Suguru Shirotake et al. proposed that NMIBC with greater VEGF expression and stronger microvessel density (MVD) had an earlier recurrence and a significantly higher recurrence rate (Shirotake et al., 2011). Others also agreed that bladder tumors with overexpression of VEGF were biologically aggressive (Al-Abbasi et al., 2009). Klein et al. developed a semiquantitative scoring system of VEGF based on immunostaining and suggested that a threshold value of 6.0 is a prognostic indicator in papillary thyroid carcinoma (Klein et al., 2001). However, to our best knowledge, few studies have applied this VEGF scoring system to predict the prognosis of NMIBC. In the present study, we showed for the first time that a VEGF score of > 8 implied a higher risk for recurrence. It is easy to establish a quantitative threshold and estimate the risk of recurrence via such a simple semiquantitative scoring system. However, the practicability of this threshold needs further verification.

Our findings indicated that single variables such as Ki67 LI and VEGF scoring can only predict 73.53% and 66.67% of recurrence cases individually. However, with combination, the sensitivity and specificity for the prediction of tumor recurrence can increase to 91.18% and 81.25%, respectively, making it feasible to combine the two markers for establishing a molecular grading model for NMIBC to predict prognosis. Patients were distinguished on the basis of three molecular grades (mG1, mG2 and mG3). Higher molecular grade corresponded to higher risk of recurrence. No progression case was found in patients of mG1, but patients of mG2 and mG3 showed no obvious difference in assessment for progression, probably because of the limited case number of progression.

Compared with the conventional system, the new system showed a different distribution of patients. Namely, our new molecular dependent grading system showed higher specificity and reproducibility. Nevertheless, the sensitivity of the two systems did not show statistically significant difference. One reason might lie in the limitation of small cohort and relatively short follow-up. As known, protein aberrance usually plays a very important role in tumorgenesis. More importantly, those changes occurred earlier than morphological abnormality. We expect that the new molecular grading system could enhance the efficiency of the conventional grading system in judging

the outcomes of NMIBC. Also, further investigation to identify more sensitive biomarkers is eagerly required by pathologists and clinicians.

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