Original paper

Evaluation of different p16 immunostaining methods and the prognostic role of p16/Ki-67 combined expression in non-muscle invasive bladder cancers

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There are many scoring methods evaluating the expression of p16 in the bladder immunohistochemically. In this study our aim was to determine an optimal p16 scoring method by discussing different staining methods related with p16 expression in bladder cancers and to establish the association of p16 and Ki-67 expressions, alone or in combination, with recurrence and progression. Ninety patients undergoing their first transurethral resection for bladder cancer and newly diagnosed papillary urothelial carcinoma (pTa and pT1) were included in the study. Four different scoring methods were used for p16 (p16a, p16b, p16c, p16d). The patients were divided into two groups based on recurrence and progression. There was a statistically significant difference between recurrence and abnormal p16d staining ($p = 0.005$). In other staining patterns of p16, there was no statistically significant difference in terms of recurrence or progression.In the multivariate logistic regression analysis, combined Ki-67 ≥ 10 and abnormal p16d staining was found to be the only independent predictive factor for recurrence (OR = 2.26, 95% CI: 0.13-46.41, $p = 0.035$) and no independent predictive factor for progression was found. Determining an adequate expression scoring by taking normal transitional epithelial staining pattern as a reference would be an objective approach in p16 evaluation. Moreover, it was found that evaluating p16d and Ki-67 in combination would be significant in predicting recurrence in pTa and pT1 urothelial carcinomas.

Key words: bladder cancer, Ki-67, p16INK4A, progression, recurrence.

Introduction

Bladder cancer is a complex and multi-factorial cancer involving both environmental and genetic factors [1]. During diagnosis, nearly 75% of bladder cancers are superficial which are confined to the mucosa or lamina propria [2-4]. However, although superficial, nearly 30-80% of these tumors show recurrence and 1-50% show progression [2-6]. Tumor diameter, number, grade and stage as used in routine practice, may fail to determine the clinical course of these tumors most of the time [7]. Thus, there is a continuing need for new molecular and immunohistochemical markers to determine the clinical progression and treatment protocols in these patients.

As with many cancers, genetic mutations occurring in cell cycle proteins are frequently observed in bladder cancer. p16, a cyclin-dependent kinase inhibitor, is also considered to be a tumor suppressor gene. In many cancers, p16 is inactivated through muta-

tion, methylation, or deletion. p16 acts at the G1-S checkpoint by increasing Rb protein levels, while loss of p16 causes inadequate cell proliferation through loss of Rb function [8-11]. Many studies have shown that inactivation of the p16/cyclin D1/Rb pathway during oxidative stress plays an important role in the development of urothelial carcinoma (UC) in the bladder [12, 13]. There are many scoring methods for evaluating the expression of p16 in the bladder and other tumors immunohistochemically; however, no standard scoring system or optimal p16 staining method is available.

Ki-67 is a nuclear antigen expressed in the G1, S, and G2, but not the G0, phases of the cell cycle. Tumor recurrence and progression are significantly associated with the Ki-67 index in non-muscle invasive bladder cancers [14-16].

The aim of this study was to determine the most appropriate p16 scoring method by comparing various staining methods related to p16 expression in bladder cancers and to assess the association between p16 and Ki-67 expression, alone or in combination, with recurrence and progression.

Material and methods

Patient and tumor characteristics

In total, 90 consecutive patients undergoing their first transurethral resection (TUR) for bladder cancer and newly diagnosed papillary UC (pTa and pT1) as a result of pathology between 2005 and 2013 were included. Approval for this study was obtained from the Ethics Committee of our university. Clinical data (age, gender, grade, stage, smoking, tumor size and number, presence of CIS, re-TUR, postoperative immunotherapy, and chemotherapy) of the patients and histopathological preparations were received from Abant Izzet Baysal University Urology and Pathology Department and evaluated by two pathologists (GO and MA) blinded to the patient characteristics. All tumors were graded in accordance with World Health Organization (WHO)/International Society of Urological Pathology (ISUP) (2004). Tumor stage was classified according to the Union for International Cancer Control (UICC) 2009 TNM classification.

Patient follow-up

Recurrence was defined as the relapse of UC of the bladder at any pathological stage after the initial surgery. Progress to a higher stage or metastasis, confirmed histopathologically, was deemed progression. Patients having carcinoma *in situ*, undergoing a second TUR, or receiving intracavitary chemotherapy and immunotherapy were excluded from the study.

Immunohistochemistry

Immunohistochemistry (IHC) was performed using a streptavidin-biotin-peroxidase technique (Bond fully integrated IHC and *in situ* hybridization (ISH) system; Leica) with a monoclonal antibody to p16 (Medaysis, 1 : 30 dilution) and Ki-67 (clone MM1; Leica). First, 5-μm sections from paraffin wax-embedded samples were cut and placed on poly-L-lysine-coated slides, deparaffinized with xylene, and then rehydrated. For antigen retrieval, the slides were treated by microwave heating in citrate buffer (pH 6.0) for 10 min. Next, 3% hydrogen peroxide was used to block endogenous peroxidase activity.

The sections were incubated with primary antibodies, including those against p16 and Ki-67, for 1 h at room temperature. After washing in phosphate-buffered saline, the samples were incubated with a biotin-conjugated secondary antibody and then with a streptavidin-biotin system for 30 min at room temperature. The reactions were visualized by immersion of the specimens in diaminobenzidine tetrahydrochloride. The sections were counterstained with hematoxylin, then rinsed and mounted.

Immunohistochemical scoring system

While there was cytoplasmic p16 expression in tumors that exhibited strong staining, there was no cytoplasmic expression in p16-negative tumors. Although this staining was thought to be specific for p16, only nuclear staining was taken into account when scoring p16. To understand the staining pattern of p16 in normal bladder mucosa, 15 normal bladder mucosa samples, along with cervical intraepithelial neoplasia tissue as a positive control, were used. p16 showed mild-to-moderate nuclear and cytoplasmic expression in all of the tissues of normal bladder mucosa (Fig. 1). Four different scoring methods were used for p16, named p16a, p16b, p16c, and p16d.

P16a: p16 nuclear staining percentage was scored between 0 and 3 as follows: no expression: score 0, expression between 0 and 10%: score 1, expression between 10% and 49%: score 2, and strong expression, over 50%: score 3 [8, 17].

P16b: Expression below 10% was regarded as abnormal while expression ≥ 10% was regarded as normal [18-20].

P16c: Expression below 5% was regarded as abnormal while expression ≥ 5% was regarded as normal [1].

P16d: No expression of p16 (loss of p16 in tumor) or strong homogeneous expression over 50% (over expression) was regarded as abnormal and heterogeneous expression (below 50%) was regarded as normal (Figs. 2C, 3C, 4C, 5C) [21-24].

While assessing scoring methods (p16a, p16b, p16c) the percentage of positive staining of tumor

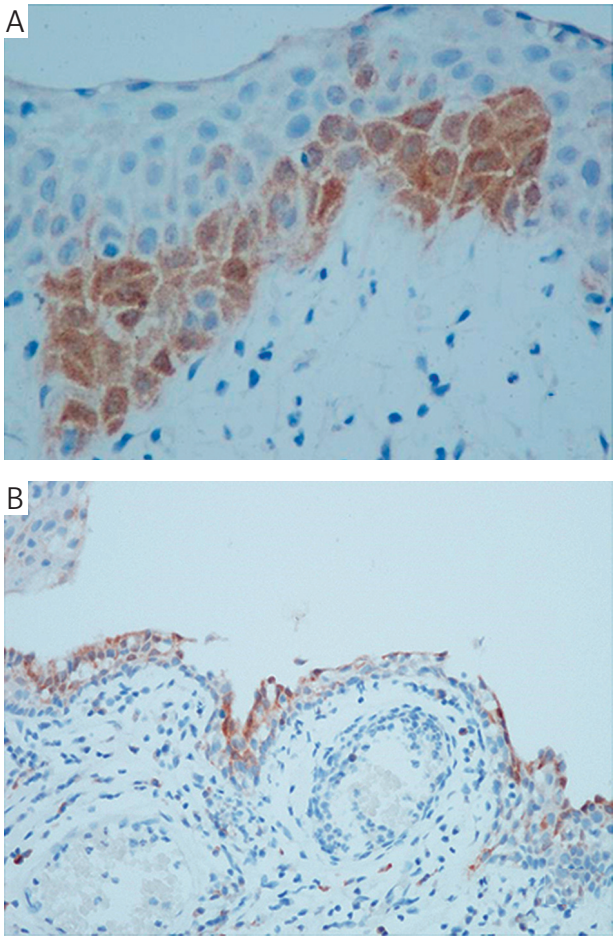

Fig. 1. A) p16 staining in normal transitional epithelium. Nuclear and cytoplasmic expression can be seen (p16, magnification 400×). B) p16 staining in normal transitional epithelium (p16, magnification 200×)

cells was calculated for each case regardless of homogeneous or heterogeneous stainings.

Tonsil tissue was used as a positive control for assessment of Ki-67 staining. While Ki-67 assessing maximum nuclear staining areas were choosen, we counted staining cells (average 4 high power field) and then set its percentage. The cut-off value was 10% for Ki-67. Tumors expressing below 10% were regarded negative while those expressing ≥ 10% were regarded as positive (Figs. 2B, 3B, 4B, 5B) [25].

For p16 and Ki-67 all reactive nuclei were considered positive irrespective of intensity.

Statistical methods

Statistical analyses were performed using IBM Statistical Package for the Social sciences (SPSS) Statistics for Windows, version 20.0 (IBM Corp., Armonk, NY). Continuous variables were tested for normality by the Kolmogorov–Smirnov test. Normally distributed data are presented as means ± standard deviation. The rates and proportions of discrete variables were determined

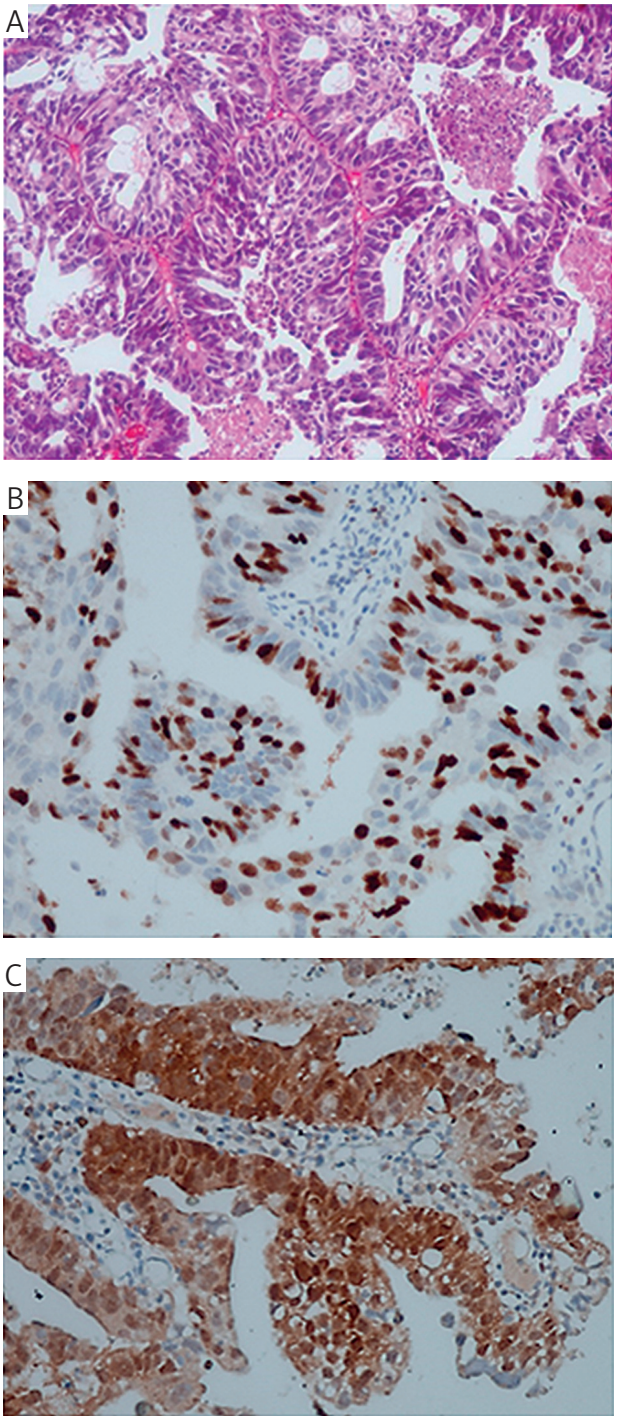

Fig. 2. A) High grade pT1 papillary urothelial carcinoma (HE, magnification 100×). B) High Ki-67 index (60%) in the tumor (Ki-67, magnification 200×). C) Abnormal expression of p16 (Scor 3) according to p16d scoring method. Tumor shows above 50% nuclear and cytoplasmic expression with p16 (p16, magnification 200×)

using the χ^2. The median with data range (minimum to maximum) was used for non-normally distributed data. Correlations between tumor grade, stage and staining of p 16d and Ki-67 were evaluated using Spearman's rank correlation coefficient. Potential predictors of recurrence

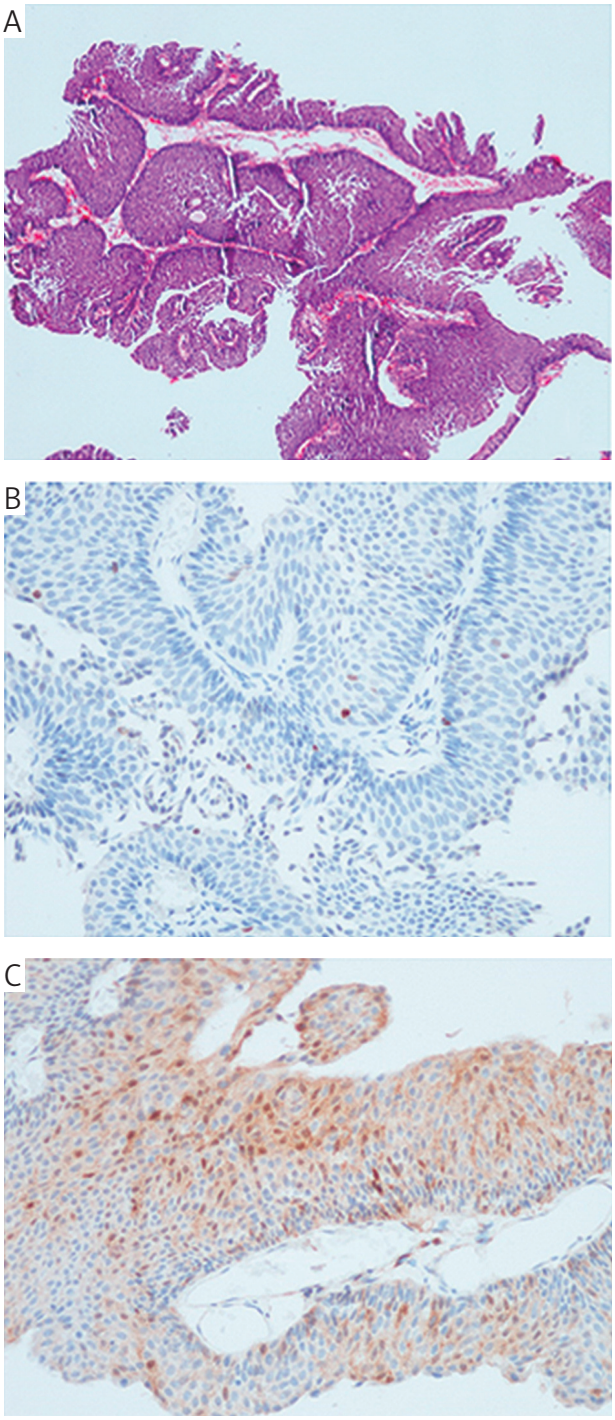

Fig. 3. A) Low grade pTa papillary urothelial carcinoma (H/E, magnification 40×). B) Low Ki-67 index (1%) in the tumor (Ki-67, magnification 200×). C) Normal p16 immunostaining (Scor 2) according to p16d scoring method. Tumor shows very low cytoplasmic expression but the nuclear staining is evident (p16, magnification 200×)

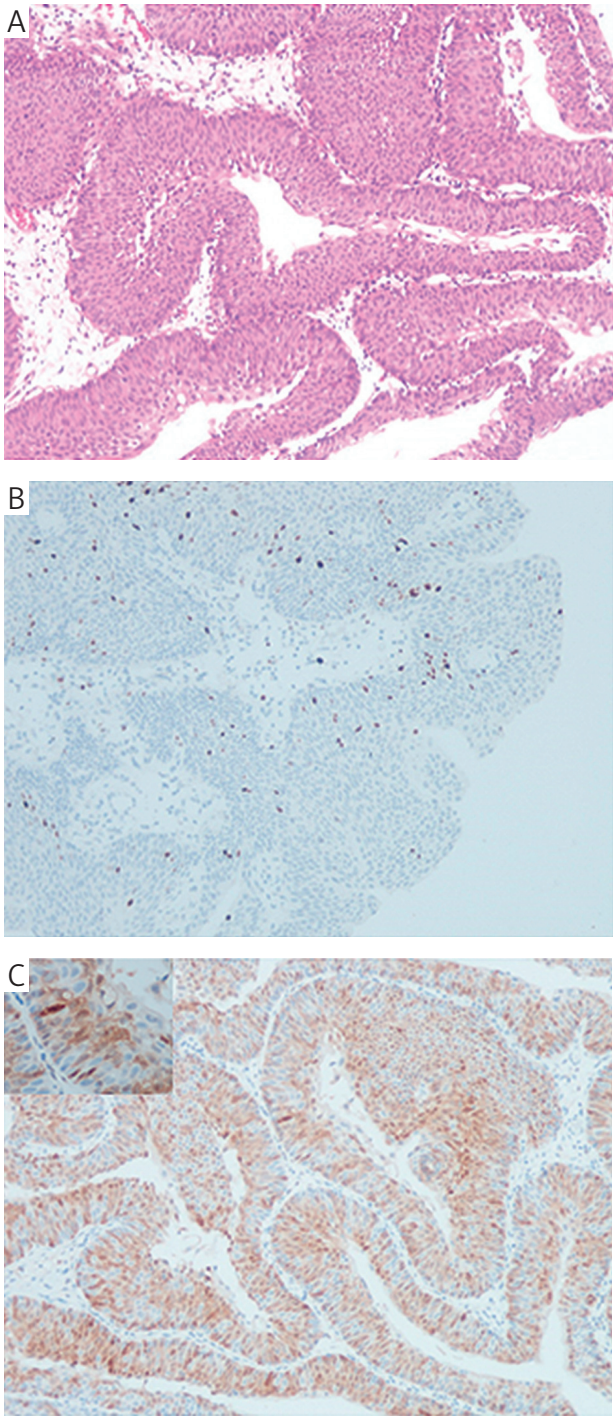

Fig. 4. A) Low grade pT1 papillary urothelial carcinoma (HE, magnification 100×). B) Ki-67 index is below 10% (7%) in the tumor (Ki-67, magnification 100×). C) Normal p16 staining (Scor 2) according to p16d scoring method. Nuclear staining is lower than cytoplasmic staining in the tumor (p16, magnification 100×) (top left: p16, magnification 400×)

and progression in individual patients with superficial UC of the bladder were initially compared, and variables that showed a p value of < 0.05 were included in a logistic regression model. Results were expressed as odds ratio (OR) and 95% confidence interval (CI). The two-sided p value of < 0.05 was considered to indicate statistical significance. Relation between Ki-67 and p16d staining with recurrence time was evaluated with log rank test. Recurrence rates were determined with Kaplan Meier analysis. A 5% type-1 error level was used to infer statistical significance.

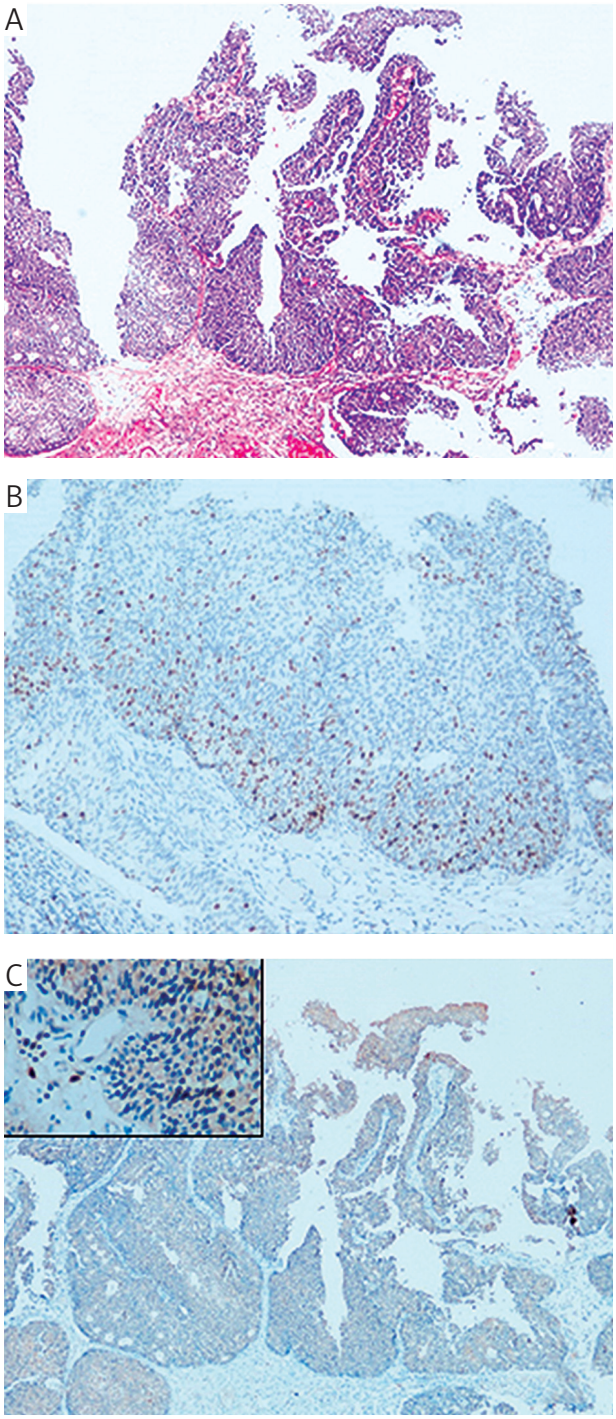

Fig. 5. A) Low grade pT1 papillary urothelial carcinoma (HE, magnification 40×). B) Ki-67 index is above 10% (17%) in the tumor (Ki-67, magnification 100×). C) Abnormal expression of p16 (Scor 0). None of the tumor cells show p16d expression, but the positive p16 staining of fibroblasts can be seen as a positive internal control (p16, magnification 40×) (top left: p16, magnification 400×)

Results

Of all the patients, 83 were males and 8 were females. The patients were divided into two groups based on recurrence and progression. The average follow-up term was 32.8 (IQR 36.2-103.6) months.

Using Spearman's correlation test, Ki-67 was significantly positively correlated with tumor stage and tumor grade ($r = 0.372$ and 0.443, respectively; $p < 0.001$). Abnormal p16d staining was also significantly positively correlated both with tumor stage and tumor grade ($r = 0.307$, $p = 0.003$ and $r = 0.279$, $p = 0.008$, respectively). There wasn't any statistically significant correlation between the other staining pattern of p16 (p16a, p16b, p16c) and tumor grade-stage. In the recurrence group, abnormal p16d staining was observed in 28 (45.2%) patients. There was a statistically significant between relation recurrence and abnormal p16d staining ($p = 0.005$). However, there was no significant relation between abnormal p16d staining and progression ($p = 0.183$). Regarding the other p16 staining patterns (p16a, p16b, p16c), there was no statistically significant difference in terms of recurrence or progression.

In the recurrence group, Ki-67 was < 10 in 12 (19.4%) patients and ≥ 10 in 50 (80.6%) patients. However, no such difference was detected in the progression group ($p = 0.374$). In the group having both Ki-67 ≥ 10 and abnormal p16d staining, 22 (81.5%) patients showed recurrence while in the patient group having both Ki-67 < 10 and normal p16d staining, 5 (18.5%) patients had recurrence. There was a statistically significant difference in favor of the first group ($p < 0.001$). However, there was no difference in terms of progression ($p = 0.126$). In the group with Ki-67 ≥ 10 and normal p16d staining, there were 29 (56.9%) patients. When compared with the patient group with Ki-67 ≥ 10 and abnormal p16d, there was a statistically significant difference in favor of this group ($p = 0.035$, I).

In the recurrence group, the tumor size was < 3 cm in 24 (39.3%) patients and ≥ 3 cm in 37 (60.7%). A statistically significant difference was found between recurrence and tumor size ($p < 0.001$). In the progression group, the tumor size was < 3 cm in 5 (31.2%) patients and ≥ 3 cm in 11 (68.8%). However, there was no statistically significant difference between progression and tumor size ($p = 0.071$, Table I). Recurrence rate was significantly higher in patients with multiple tumors than in those with a single tumor (51.6% vs. 48.4%, $p = 0.003$). There was also a positive correlation between tumor number and progression ($r = 0.320$, $p = 0.002$). The progression rate was significantly higher in patients with multiple tumors than in patients with a single tumor (75% vs. 25%, respectively, $p = 0.002$). Similarly, Spearman's correlation test revealed that the stage and grade of tumor were positively correlated with recurrence ($r = 0.253$, $p = 0.016$ and $r = 0.288$, $p = 0.006$, respectively). In the recurrence group, 23 (37.1%) patients were pTa and 39 (62.9%) were pT1. There was a statistically sig-

Table I. Comparison of patients according to presence of recurrence and progression

		Recurrence			Progression		
		present N = 62	absent N = 28	p	present N = 16	absent N = 74	p
Sex (F/M)		3/59	4/24	0.121	1/15	6/68	0.801
Age (year)		68.35 ±10.4	67.54 ±13.4	0.754	68.31 ±6.3	68.05 ±12.2	0.935
P16a		8 (13.1%)	1 (3.6%)	0.102	2 (9.6%)	7 (12.5%)	0.418
P16b		28 (45.2%)	13 (46.4%)	0.911	8 (50.0%)	33 (44.6%)	0.694
P16c		46 (74.2%)	16 (57.1%)	0.106	14(87.5%)	48 (64.9%)	0.076
P16d		28 (45.2%)	4 (14.3%)	**0.005**	8 (50%)	24 (32.4%)	0.183
Ki-67	**> 10**	12 (19.4%)	13 (46.4%)	**0.008**	3 (18.8%)	22 (29.7%)	0.374
	≤ 10	50 (80.6%)	15 (53.6%)		13 (81.2%)	52 (70.3%)	
Ki-67 ≥ 10 and abnormal p16d		22 (81.5%)	2 (15.4%)	**< 0.001**	6 (85.7%)	18 (54.5%)	0.126
Ki-67 < 10 and normal p16d		5 (18.5%)	11 (84.6%)		1 (14.3%)	15 (45.5%)	
Ki-67 ≥ 10 and abnormal p16d		22 (81.5%)	2 (15.4%)	**0.035**	6 (85.7%)	18 (54.5%)	0.413
Ki-67 ≥ 10 and normal p16d		29 (56.9%)	13 (86.7%)		7 (53.8%)	35 (66.0%)	
Tumor size	**≤ 3 cm**	24 (39.3%)	22 (78.6%)	**0.001**	5 (31.2%)	41 (56.2%)	0.071
	> 3 cm	37 (60.7%)	6 (21.4%)		11(68.8%)	32 (43.8%)	
Number of tumor multiple	**single**	30 (48.4%)	23 (82.1%)	**0.003**	4 (25%)	49 (66.2%)	**0.002**
	32 (51.6%)	5 (17.9%)			12 (75%)	25 (33.8%)	
Smoking		45 (91.8%)	12 (57.1%)	**0.001**	15 (100%)	42 (76.4%)	**0.037**
Stage	**pTa**	23 (37.1%)	18 (64.3%)	**0.016**	7 (43.8%)	34 (45.9%)	0.873
	pT1	39 (62.9%)	10 (35.7%)		9 (56.2%)	40 (54.1%)	
Grade	**low**	25 (40.3%)	20 (71.4%)	**0.006**	5 (31.2%)	40 (54.1%)	0.098
	high	37 (59.7%)	8 (28.6%)		11(68.8%)	34 (45.9%)	

Table II. Multivariate logistic regression analysis of independent predictive factors for recurrence

		Multivariate analysis		
	Reference	OR	95% CI	p
Smoking		1.27	(0.66-18.99)	0.139
Tumor size	≥ 3 cm	0.85	(0.54-10.2)	0.257
Stage	pT1	0.71	(0.23-3.78)	0.924
Ki-67	≥ 10	1.79	(1.01-33.56)	0.062
P16d	abnormal staining	2.14	(0.58-12.1)	0.118
Ki-67 ≥ 10 and abnormal p16d		2.26	(0.13-46.41)	0.035

nificant difference between stage and recurrence (p = 0.016). In the recurrence group, 25 (40.3%) patients were low grade and 37 (59.7%) patients were high grade. There was a statistically significant difference between grade and recurrence (p = 0.006). Similarly, there was a significant difference between smoking and both recurrence and progression (p < 0.001 and p = 0.037, respectively, Table I).

According to the univariate analysis, abnormal p16d staining, Ki-67 ≥ 10, tumor size ≥ 3 cm, multiple tumors, smoking, pT1 stage, and high-grade tumor were definite risk factors for recurrence. As there was a significant correlation between tumor number and size and between tumor grade and stage, only tumor size and stage were evaluated in the multivariate analysis. In the multivariate logistic regression analysis, combined Ki-67 ≥ 10 and abnormal p16d staining was found to be the only independent predictive factor for recurrence (OR = 2.26, 95% CI: 0.13-46.41, p = 0.035) and no independent predictive factor for progression was found (Table II).

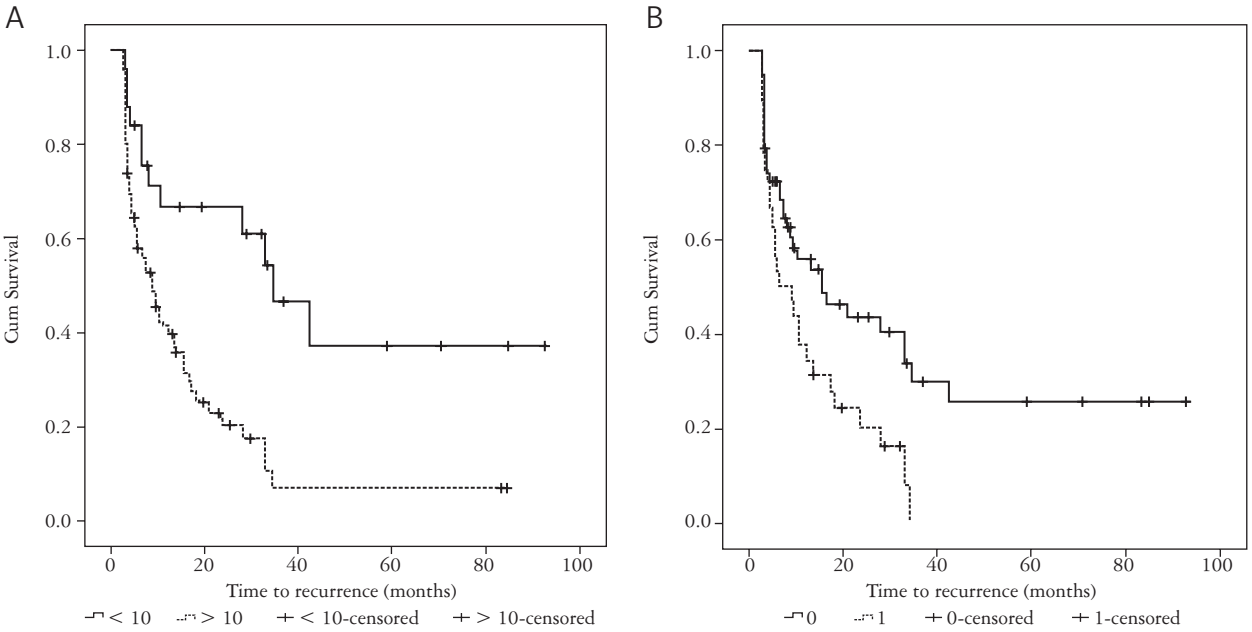

Fig. 6. A) The relationship between with Ki-67 and recurrence time. B) The relationship between with p16 and recurrence time

Based on a Kaplan-Meier analysis, the median time to recurrence was 34.7 (95% CI: 20.4-49) months in the group in which Ki-67 staining was ≥ 10, and 9.1 (95% CI: 5.5-12.7) months in the group where Ki-67 staining was < 10. In terms of median time to recurrence, there was a statistically significant difference between the two groups ($p < 0.001$, Fig. 6A).

Based on this analysis, the median time to recurrence was 6.6 (95% CI: 1.2-11.9) months in the group with abnormal p16d staining and 15.6 (95% CI: 3.6-27.6) months in the group with normal p16d staining. In terms of median time to recurrence, there was a statistically significant difference between the group with abnormal p16d staining and that with normal p16d staining ($p = 0.015$, Fig. 6B).

Discussion

Many studies have shown that Ki-67 and p16 staining are independent immunohistochemical markers predictive of recurrence and progression in bladder cancer [15, 19, 20, 26, 27]. As with many tissue section studies, there are various approaches to immunohistochemical evaluation of p16 in the bladder. p16, which is more difficult to evaluate than cell cycle proteins, such as Ki-67 and p53, is a valuable marker for predicting the clinical course [1, 19].

The p16 protein is a component of the p16/cyclin D1/Rb pathway and controls the G1-S transition of the cell cycle. In most tumors, the control of G1 progression and initiation of S phase have deteriorated, which causes unlimited entry into the cell cycle and cell proliferation [28]. It has been shown that abnormal expression of p16 is associated with several cancers, such as lung, laryngeal, and tonsillary carcinomas [29-31]. Various scoring methods have been used in studies comparing p16 expression and prognostic parameters in various neoplasms [32-34]. In bladder cancer, there is no consensus about the regarding the p16 staining pattern.

In some of these studies, p16 was evaluated using a score of 0 to 3 (no staining: score 0, staining between 0 and 10%: score 1, staining between 10 and 49%: score 2, strong staining: score 3) [8, 17]. In other studies, expression below 10% was regarded as abnormal, while that above 10% was considered normal [18-20]. Another study interpreted p16 expression below 5% as negative staining for p16 [1]. Some authors regarded expression loss (no staining) and overexpression (staining > 50%) as abnormal and moderate staining as normal [21-24]. Four of the scoring methods employed in these studies were also used in our work for comparison.

p16 is a marker expressed in both the nucleus and cytoplasm. However, the functional and biological importance of cytoplasmic staining is not yet understood [35]. Some studies on p16 expression take into account only nuclear staining [1, 24, 36], while others consider both nuclear and cytoplasmic staining [18, 19, 23]. Han *et al.* compared endocervical carcinomas and endometrial adenocarcinomas in terms of p16 expression. Use of nuclear staining only as a reference for scoring was claimed to be the most adequate and effective method [35]. Similarly, in a study evaluating p16 expression in ovarian cancer, Kommoss *et al.* took into consideration only nuclear staining in scoring [36]. In our study, p16

expression of all tumor tissues was scored based on the four abovementioned staining patterns by taking only nuclear staining as a reference. The results obtained were assessed in terms of predicting recurrence and progression in superficial bladder tumors.

Krüger *et al.* used a tissue microarray (TMA) technique and used p16b as a reference for scoring (expression < 10% was regarded as abnormal and expression ≥ 10% as normal) and found a significant correlation between p16 and progression; however, staining did not correlate with recurrence [19]. However, we found no statistically significant correlation between p16d and progression, likely due to the limited number of patients showing progression. In our study, there was a statistically significant association between recurrence and p16 expression only when the p16d scoring system was used. Olsson *et al.* [23] also used the same staining pattern (p16d) in stage T1 urinary bladder cancers and they detected normal p16 expression was related to a lower risk of tumor progression. Similar to our study, Lee *et al.* [21] investigated four cell cycle proteins (p16, pRb, p53, cyclin D1) and found no correlation between progression and p16 (p16d method) expression; their results suggested that multiple genetic defects affected the clinical course and metastatic capacity of bladder carcinomas. Shariat *et al* used the staining pattern of p16d and they determined that combined staining of p16 and pRb can be a useful marker in prognosis of bladder cancer [22]. In our study we detected that evaluating p16d and Ki-67 in combination was an independent predictor for recurrence in pTa and pT1 bladder cancers. In another study in which p16 staining < 10% was regarded as abnormal (p16b), a significant correlation was found between p16 expression and recurrence, as in our work, but there was no correlation between p16 and progression [20].

Some of previous studies investigated the association of Human papillomavirus (HPV) and p16 expression in UC. Steinestel *et al.* research on p16(INK4a) immunoexpression followed by detection and subclassification of HPV DNA in total of 45 patients (UC *in situ* and controls). They didnt't find any significant correlation between HPV and p16 overexpression in UC *in situ* [37]. In a different study that analyzed HPV and p16 relationship in UC with squamous differentiation, they claimed p16 expression did not appear to be a strong representative marker for evidence of HPV infection in this type of cancer [38].

Ki-67 is expressed throughout the cell cycle, with the exception of the G_0 phase, and shows proliferative activity in various carcinomas [39]. In our study, there was no significant correlation between Ki-67 and progression, while a significant correlation was found between recurrence and Ki-67 expression. Similar to our results, Mhawech *et al.* classified pT1 tumors and stated that Ki-67 had no importance in predicting progression [25]. In a study of 226 patients with bladder-confined cystectomy, Margulis *et al.* found a significant correlation between recurrence and Ki-67 staining, consistent with our results [14]. In 332 patients with bladder cancer, Ding *et al.* found that Ki-67 expression was an independent predictor of both recurrence and progression [15].

We found a statistically significant correlation between recurrence and cases with both abnormal p16d staining and Ki-67 ≥ 10%. This result was beyond the ability of Ki-67 and p16d individually to predict recurrence. In a study of pTa and pT1 tumors in the bladder, Hitchings *et al.* found a statistically significant correlation between abnormal expression of both p53 and p16 and progression [18]. Korkolopoulou *et al.* also indicated that p53/p16 combined expression was an independent predictor of decreased survival in muscle-invasive tumours in bladder cancers [40]. Additionally with the conclusion of these previous studies our results suggested that using the combination of p16d/Ki 67 markers have more predictive power than either marker alone for recurrence.

Conclusions

Although several studies have investigated the roles of p16 and Ki-67 expression in predicting prognosis in bladder carcinomas, there is still no consensus regarding the most appropriate method of scoring p16 expression. In our study, four different p16 expression scoring methods in bladder carcinomas were compared. We believe that p16d scoring system, which used normal transitional epithelial staining pattern as a reference, is most objective method for predicting bladder tumors recurrence and progression. Moreover, in our study, it was found that evaluating p16d and Ki-67 in combination could facilitate prediction of recurrence in superficial bladder tumors. Further studies of p16 expression patterns in a larger series of patients, including a greater number with progression and employing other cell cycle proteins in combination, are needed.

The authors declare no conflict of interest.

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