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 mutation.
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 (Medayis, 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
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 chosen, 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 using the $\chi^2$. The median with data range (minimum to maximum) was used for non-normally distributed data. Correlations between tumor grade, stage and staining of p16d and Ki-67 were evaluated using Spearman’s rank correlation coefficient. Potential predictors of recurrence
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.
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 difference 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).

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

<table>
<thead>
<tr>
<th></th>
<th>Recurrence</th>
<th></th>
<th>Progression</th>
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<tbody>
<tr>
<td></td>
<td>Present</td>
<td>Absent</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>N</td>
<td>N = 62</td>
<td>N = 28</td>
<td>N = 16</td>
<td>N = 74</td>
</tr>
<tr>
<td><strong>Sex (F/M)</strong></td>
<td>3/59</td>
<td>4/24</td>
<td>1/15</td>
<td>6/68</td>
</tr>
<tr>
<td><strong>Age (year)</strong></td>
<td>68.35 ± 10.4</td>
<td>67.54 ± 13.4</td>
<td>68.31 ± 6.3</td>
<td>68.05 ± 12.2</td>
</tr>
<tr>
<td><strong>P16a</strong></td>
<td>8 (13.1%)</td>
<td>1 (3.6%)</td>
<td>10.2</td>
<td>7 (12.3%)</td>
</tr>
<tr>
<td><strong>P16b</strong></td>
<td>28 (45.2%)</td>
<td>13 (46.4%)</td>
<td>0.911</td>
<td>8 (50.0%)</td>
</tr>
<tr>
<td><strong>P16c</strong></td>
<td>46 (74.2%)</td>
<td>16 (57.1%)</td>
<td>0.106</td>
<td>14 (87.5%)</td>
</tr>
<tr>
<td><strong>P16d</strong></td>
<td>28 (45.2%)</td>
<td>4 (14.3%)</td>
<td>0.005</td>
<td>8 (50%)</td>
</tr>
<tr>
<td><strong>Ki-67 ≥ 10</strong></td>
<td>12 (19.4%)</td>
<td>13 (46.4%)</td>
<td>0.008</td>
<td>3 (18.8%)</td>
</tr>
<tr>
<td><strong>Ki-67 ≤ 10</strong></td>
<td>50 (80.6%)</td>
<td>15 (53.6%)</td>
<td></td>
<td>13 (81.2%)</td>
</tr>
<tr>
<td><strong>Ki-67 ≥ 10 and abnormal p16d</strong></td>
<td>22 (81.5%)</td>
<td>2 (15.4%)</td>
<td>&lt; 0.001</td>
<td>6 (85.7%)</td>
</tr>
<tr>
<td><strong>Ki-67 &lt; 10 and normal p16d</strong></td>
<td>5 (18.5%)</td>
<td>11 (84.6%)</td>
<td>1 (14.3%)</td>
<td>15 (45.5%)</td>
</tr>
<tr>
<td><strong>Ki-67 ≥ 10 and abnormal p16d</strong></td>
<td>22 (81.5%)</td>
<td>2 (15.4%)</td>
<td>0.035</td>
<td>6 (85.7%)</td>
</tr>
<tr>
<td><strong>Ki-67 ≥ 10 and normal p16d</strong></td>
<td>29 (56.9%)</td>
<td>13 (86.7%)</td>
<td>7 (53.8%)</td>
<td>35 (66.0%)</td>
</tr>
<tr>
<td><strong>Tumor size</strong></td>
<td>≤ 3 cm</td>
<td>24 (39.3%)</td>
<td>0.001</td>
<td>5 (31.2%)</td>
</tr>
<tr>
<td></td>
<td>&gt; 3 cm</td>
<td>37 (60.7%)</td>
<td>6 (21.4%)</td>
<td>11 (68.8%)</td>
</tr>
<tr>
<td><strong>Number of tumor</strong></td>
<td>single</td>
<td>30 (48.4%)</td>
<td>0.003</td>
<td>4 (25%)</td>
</tr>
<tr>
<td></td>
<td>multiple</td>
<td>32 (51.6%)</td>
<td>5 (17.9%)</td>
<td>12 (75%)</td>
</tr>
<tr>
<td><strong>Smoking</strong></td>
<td>45 (91.8%)</td>
<td>12 (57.1%)</td>
<td>0.001</td>
<td>15 (100%)</td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td>pTa</td>
<td>23 (37.1%)</td>
<td>0.016</td>
<td>7 (43.8%)</td>
</tr>
<tr>
<td></td>
<td>pT1</td>
<td>39 (62.9%)</td>
<td></td>
<td>9 (56.2%)</td>
</tr>
<tr>
<td><strong>Grade</strong></td>
<td>low</td>
<td>25 (40.3%)</td>
<td>0.006</td>
<td>5 (31.2%)</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>37 (59.7%)</td>
<td></td>
<td>11 (68.8%)</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>OR (95% CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smoking</td>
<td>1.27</td>
<td>(0.66-18.99)</td>
<td>0.139</td>
</tr>
<tr>
<td>Tumor size</td>
<td>≥ 3 cm</td>
<td>0.85</td>
<td>(0.54-10.2)</td>
</tr>
<tr>
<td>Stage</td>
<td>pTa</td>
<td>0.71</td>
<td>(0.23-3.78)</td>
</tr>
<tr>
<td>Ki-67</td>
<td>≥ 10</td>
<td>1.79</td>
<td>(1.01-33.56)</td>
</tr>
<tr>
<td>P16d</td>
<td>abnormal staining</td>
<td>2.14</td>
<td>(0.58-12.1)</td>
</tr>
<tr>
<td>Ki-67 ≥ 10 and abnormal p16d</td>
<td>2.26</td>
<td>(0.13-46.41)</td>
<td>0.035</td>
</tr>
</tbody>
</table>

Significant 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 ≥ 5 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).
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 tonsillar 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 didn’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 G₀ 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]. Korkolopolou 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.

References


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