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The Relationship of p16, Ki-67, Bcl-2, P53 and CK20 Immune Expressions with Recurrence in Superficial Bladder Tumors

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Abstract

***Background*:** Superficial urothelial carcinoma of the urinary bladder account for 70-80% of all newly diagnosed bladder cancer cases. Of these, approximately 50-75% will experience a recurrence in 12 months and 20 to 25 % will progress to invasive types during the person's lifetime.

Aim: In the present study, we investigated the relationship between the recurrence rate and the expressions of various genes via immunohistochemical analysis in order to assess their applicability as prognostic factors for clinical behavior and differential diagnosis.

Material and Methods: The antibodies that were used in this study; tumor suppressor gene P53, apoptosis activity determinant bcl-2 cell proliferation marker Ki-67, CK20 cytokeratin isotope and tumor suppressor gene P16. Fifty eight cases diagnosed with primary superficial bladder cancer were investigated.

Results: Recurrence was detected in 62.1% of the cases. Progression to a higher grade tumor was detected in 38.9% of the 36 cases that showed recurrence. A marked statistically significant relationship was found between recurrence and diffuse or no staining with CK20, more than 50% heterogeneous expression with P16, and more than 1% expression with bcl-2. Evaluation of the effect of the CK20, bcl-2 and P16 parameters with logistic regression analysis showed that the model was significant ($p<0.05$) and more than 1% staining with bcl-2 had an explanatory power of 8.057 times on the recurrence rate. A significant relationship was found between the pathological stage and histological grade of the cases and the CK 20 and Ki 67 expressions.

Conclusion: In conclusion, determining CK20, bcl–2, and p16 expressions with immunohistochemical methods can provide guidance in predicting the recurrence of superficial bladder tumors. Abnormal CK20 staining and Ki-67 expression is related to tumor grade and can be used to support the diagnosis in controversial cases.

Keywords: superficial urinary bladder cancer, recurrence, immunohistochemical analysis

Introduction

Superficial bladder cancers make up 70-80% of bladder cancers. Recurrence occurs within about 12 months in 50-75% of the cases and progression related to the tumor grade is seen in 15-25% [1]. Various studies on the factors affecting recurrence and progression have reported various results regarding their significance. Although there is consensus on the prognostic effectiveness of certain factors, new parameters are still needed.

The aim of the study was to reveal the relationship of CK20, bcl-2, p53, Ki-67 and P16

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expressions with recurrence rate in superficial bladder tumors and to investigate the significance

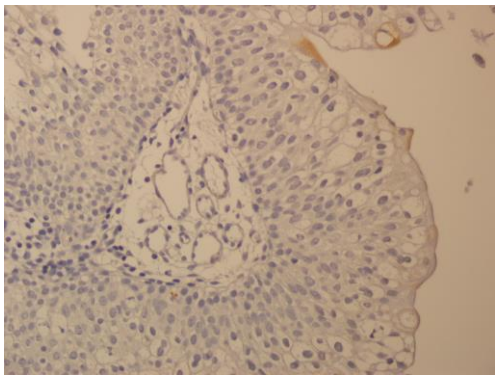

Figure 1: Normal superficial staining with CK20 (400X)

of their use as prognostic factors that may identify clinical behavior.

Patients' and Method

Evaluation of immunohistochemical staining and immune reactivity

The streptavidin biotin-peroxidase immune-histochemical staining method was used to show cytokeratin 20, Ki-67, bcl-2, p53, and p16 expression. The external control was used for each.

Evaluation

The histological types of the cases were reevaluated according to the Urothelial Carcinoma Classification (2004) of the World Health Organization. Cytoplasmic positivity was accepted for CK20 (Figure 1, 2, and 3) bcl-2, (figure 5) and nuclear positivity for p53, Ki-67

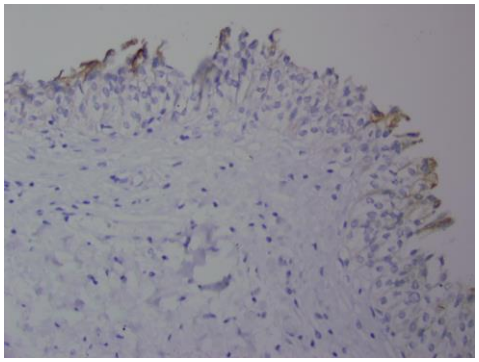

Figure 2: Normal superficial staining with CK20 (400X)

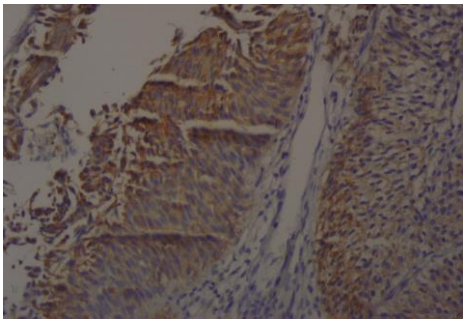

Figure 3: Diffuse staining with CK20 (400X)

(Figure 4) and p16 (Figure 6). The sections were scanned with the 10x lens and counting was performed using the 40x objective in 3 different areas where the staining was most intense for p53 6, Ki-67, and p16 evaluation (Figure 7).

In the normal urothelium, CK20 can show occasional strong staining of umbrella cells. Basal and intermediate urothelial cells were always negative. CK20 immune expression was evaluated with a scale between 1 and 4 as used by Desai [2]. Evaluation scores in our study were as follows: 1 = Immune expression in normal superficial cells. 2 = No immune expression; Loss of normal staining 3 = Focal pattern; immune expression in less than 10% of the cells, 4 = Diffuse pattern; immune expression in more than 10% of the cells.

A threshold value of 20-22% has been used in many studies on p53 immune expression [3]. We used: 1 = immune expression of 20% or less 2 = immune expression between 20% and 50% 3 = immune expression of more than 50% for the Ki-67 threshold value of 10% was used in many studies [4].

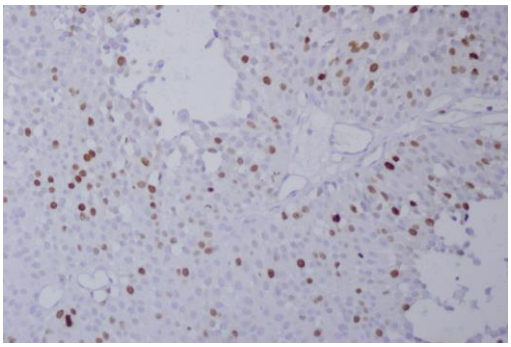

Figure 4: ki-67 immunexpression with more than 50% (400X)

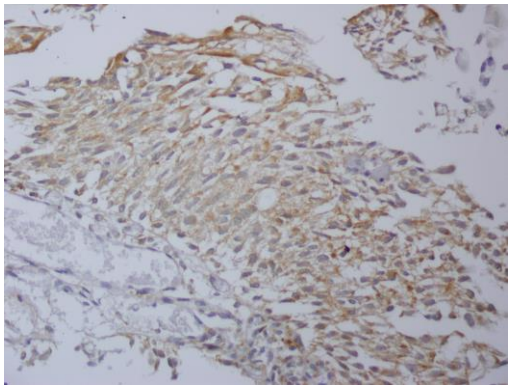

Figure 5: bcl-2 positive cytoplasmic staining (400x)

We used: 1 = 10% basal immune expression, normal expression. 2 = 10-50% immune expression 3 = Immune expression more than 50%. A threshold value of 1% has been accepted in many studies on Bcl-2 immunohistochemical staining [5]. We used: 0 = No staining.1 = Immune expression more than 1%. The study of Shahrokh *et al*., was used as the basis for p16 [6]: 0 = No nuclear immune expression. 1 = Normal heterogeneous immune expression

2 = Strong homogeneous immune expression of more than 50%

Clinical information

Clinical follow-up information of patients were reviewed; age, sex, tumor location, treatment procedure and recurrence status after the diagnosis were investigated. After transurethral resection (TUR), patients treated with 50 mg Epirubicin within 24 hours, with the clinical diagnosis of superficial bladder tumor. Adjuvant chemotherapeutics were used in principle to prevent the recurrence of low-grade tumors and BCG was used for high-grade tumors according to the European Urology Association Guide, taking into account parameters such as the clinical status, side effects and age.

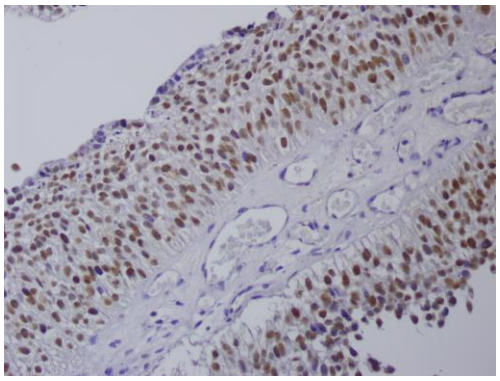

Figure 6: More than 50% positive with p53 (400X)

Clinical data

Samples from 58 patients with the diagnosis of superficial urothelial carcinoma between 1997 and 2008 in our department were included in the study. The mean age of the patients was 64.41 years (between 32 to 88); 13 (22.4%) of those were females and 45 (77.6%) male. Of these cases, 58.6% had a diagnosis of PUCLG-pTa (Non-invasive papillary urothelial carcinoma, low grade), 17.6% of PUCHG-pT1 (Non-invasive papillary urothelial carcinoma, high grade), 6.9% of PUCLG-pT1 (Invasive papillary urothelial carcinoma, low grade), 5.2% of PUCHG-pT1 (Invasive papillary urothelial carcinoma, high grade), and 12.1% had other diagnoses Papillary urothelial neoplasm with low malignant potential (5 cases), Inverted Papilloma (1 case) and Papilloma (1 case). The localization of the tumors was; 32.8% in the right wall, 24.1% in the left wall, 15.5% in the posterior part, 15.1% in the bladder neck and 12.1% in the region of the trigone. Recurrence was seen in 62.1% of the cases.

Results

No statistically significant difference was found between the recurrence rates and histopathologic diagnoses, presence of invasion, localization and

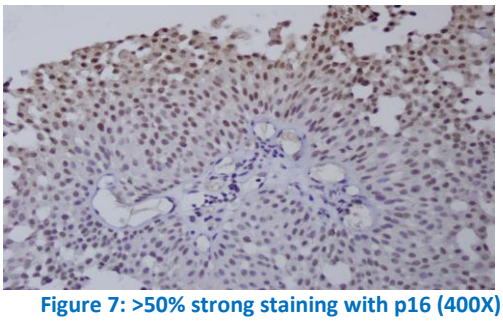

Figure 7: >50% strong staining with p16 (400X)

Table 1: The evaluation of immune markers and the presence of recurrence

		(+) n (%)	(-) n (%)	
CK20	**Normal expression**	9 (%36,0)	16 (%64,0)	***0,001***
	No staining	11 (%91,7)	1 (%8,3)	
	Focal pattern	7 (%58,3)	5 (%41,7)	
	Diffuse staining	9 (%100)	0 (%0)	
Kİ-67	**< 10% staining**	19 (%52,8)	17 (%47,2)	***0,086***
	10-50 % staining	12 (%70,6)	5 (%29,4)	
	> 50% staining	5 (%100)	0 (%0)	
P53	**<20% staining**	4 (%36,4)	7 (%63,6)	***0,125***
	20-50% staining	6 (%60,0)	4 (%40,0)	
	>50% staining	26 (%70,3)	11 (%29,7)	
Bcl-2	**No staining**	5 (%29,4)	12 (%70,6)	***0,001***
	More than%1 staining	31 (%75,6)	10 (%24,4)	
P16	**Normal expression**	10 (%43,5)	13 (%56,5)	***0,018****
	>50% strong staining	26 (%74,3)	9 (%25,7)	

Chi-Square test is used ** p<0.05* *** p<0.01*

also depth of invasion (p>0.05). The recurrence rate was higher in low-grade cases than in high-grade ones but the difference was not statistically significant although close to significance (p>0.05)

The evaluation of immune markers and the presence of recurrence

A marked statistically significant relationship was found between recurrence rates and CK20 immune expression levels (p<0.01) table 1. The recurrence rate was statistically significantly higher in those with no staining or diffuse staining with CK20. A statistically significant relationship was found between the recurrence rates and Bcl-2 and p16 immune expression level (p<0.01).

The evaluation of immune markers according to tumor grade

A marked statistically significant difference was present between the CK20 staining levels of the cases according to grade (p<0.01). The rate of 10% staining and diffuse pattern staining with CK20 was significantly higher in high-grade table 2 cases than in low-grade cases. A marked statistically significant difference was present between the Ki-67 staining levels of the cases according to grade (p<0.01). While Ki-67 staining of more than 10% was common in low-grade cases, a Ki-67 staining of more than 50% was significantly more common in high-grade cases than in low-grade cases.

The evaluation of immune markers and presence of invasion

A statistically significant difference was present between CK20 staining levels according to invasion depth (p<0.05). A normal CK20 staining rate was common in cases diagnosed with PTa while a focal and diffuse PT1 staining pattern was significantly more common in PT1 cases. A statistically significant difference was present between the Ki-67 staining levels according to invasion depth (p<0.05). A normal Ki-67 staining rate was common in cases diagnosed with PTa while a staining rate of more than 50% was significantly more common with PT1.

The evaluation of immune markers according to the presence of high-grade lesion in recurrence

No statistically significant difference was found between CK20,Ki-67, p53, bcl-2, and p16 immune expression levels of the cases that were and were not high-grade in their recurrence (p>0.05).

Table 2: The evaluation of immune markers according to tumor grade

		Low grade n(%)	High grade n(%)	
CK20	**Normal Staining**	21 (%55,3)	0 (%0)	***0,006*** **
	No staining	6 (%15,8)	4 (%30,8)	
	10 % staining	7 (%18,4)	5 (%38,5)	
	Diffuse staining	4 (%10,5)	4 (%30,8)	
Kİ-67	**<10% staining**	26 (%68,4)	3 (%23,1)	***0,001*** **
	10-50% staining	12 (%31,6)	5 (%38,5)	
	>50%staining	0 (%0)	5 (%38,5)	
P53	**<20% stainimg**	8 (%21,1)	1 (%7,7)	***0,534***
	20-50 % staining	6 (%15,8)	2 (%15,4)	
	>50% staining	24 (%63,2)	10 (%76,9)	
Bcl-2	**No staining**	10 (%26,3)	2 (%15,4)	***0,423***
	>1% staining	28 (%73,7)	11 (%84,6)	
P16	**Normal staining**	16 (%42,1)	4 (%30,8)	***0,470***
	>50% strong staining	22 (%57,9)	9 (%69,2)	
Chi-Square test is used		*** p<0.01*		

Evaluation of immune markers according to the diagnosis and stage

The normal CK20 immune expression rate was high in cases diagnosed with PUCLG-PTa, PUN, Papilloma, and Inverted Papilloma while the focal immune expression rate was high in PUCLG-PT1 and PUCHG-PT, and the diffuse pattern rate in PUCHG-PT1.There was a marked statistically significant difference between Ki-67 immune expression levels among the cases ($p<0.01$). A statistically significant difference was found between bcl-2 immune expression levels according to histopathological diagnosis ($p<0.05$).

Statistical evaluation; Logistic regression

Evaluating the effects of the CK20, bcl-2 and P16 parameters with logistic regression analysis showed that the model was significant ($p<0.05$) and the Negelkerke R square value was 0.225 while the model had a moderate explanatory coefficient (62.2%) (Table 3).The effect of the bcl-2 parameter on the model was found to be statistically significant (p: 0.041; $p<0.05$ More than 1% staining with bcl-2 had an 8.057 times explanatory power for the recurrence risk.

Statistical Examinations

The NCSS (Number Cruncher Statistical System) 2007 and PASS 2008 statistical software (Utah, USA) were used for statistical analyses when evaluating study findings. The Chi-Square test and Fisher's Exact test were used in addition to descriptive statistical methods (mean, standard deviation, frequency) when evaluating study data. Logistic analysis was applied for multivariate analysis. Significance was evaluated at the level of $p<0.05$.

Discussion

Bladder cancer is the most common malignancy of the urinary tract. The worldwide age standardized rate (ASR) is10.1 per 100 000 for men and 2.5 per 100 000 for women [7]. Various factors such as tumor diameter, prior recurrence rate, and category are involved in recurrence [8]. We studied the CK20, p53, bcl-2, Ki-67, and p16 markers that can be used to determine recurrence in superficial bladder cancer.

Only the superficial cells express CK20 in normal urothelium. Studies reported the dedifferentiation of CK20 in recurrent disease,

Table 3: Results of logistic regression analysis

Variables in the Equation

		B	S.E.	Sig.	Exp(B)	95,0% C.I.for EXP(B) Lower	Upper
Step 1[a]	CK20(1)	,494	,794	,534	1,639	,346	7,773
	BCL2(1)	2,086	1,024	,041	8,057	1,084	59,892
	P16(1)	-,750	,953	,431	,472	,073	3,058
	Constant	-1,383	,667	,038	,251		

a. Variable(s) entered on step 1: CK20, BCL2, P16.

especially pTa/pT1 urothlelial carcinoma [9, 10]. Desai *et al*., evaluated diffuse CK 20 expression as well as no expression as abnormal staining in their study. They found a higher recurrence rate in 63.2% of the cases with diffuse or no staining. They also found a significant relationship between CK20 expression and tumor histopathological grade [2]. We found the recurrence rate to be significantly higher in cases with diffuse or no CK 20 immune expression compared to those with a normal pattern, in our study.

A marked statistically significant relationship was also found between abnormal CK20 staining and tumor grade ($p<0.01$) and the presence of invasion.CK20 immune expression could therefore be useful indetermining presence of invasion and histological grade and can also used as an important marker in the prediction of recurrence. We believe that identifying diffuse staining as well as no staining with CK20 as abnormal staining patterns will be more accurate, as also suggested in other studies.

Bcl-2, an apoptosis inhibitor, is another factor used in studies to identify the prognosis of bladder carcinoma. Ricardo Gonzalez-Campora *et al*., found high recurrence and mortality rates in Bax-/bcl-2 positive cases and low rates in negative cases [5]. We found a marked statistically significant difference between the recurrence rates according to the bcl-2 immune expression level in our study ($p<0.01$). The recurrence rate was statistically significantly high in cases with an immune expression rate of more than 1%. Goyal *et al*., found that bcl-2 positivity was seen in 85% of muscle invasive papillary urothelial carcinoma as compared to only 52.9% of superficial cases. Cytoplasmic bcl-2 immunopositivity was seen in 42.3% of low grade papillary urothelial carcinoma and 85.7% of high grade cases ($P = .001$) [11]. No statistically significant difference was found when the relationship of bcl-2 expression and histomorphological parameters were compared in our study.

Various proteins are active in the cell cycle control mechanism. Changes in the p16 protein controlled by G1-CDK activity and the Rb protein regulated by G1-CDK contribute to the development of many cancers [12]. Of the four cell regulators, p16 and pRB were found to be more significantly associated with bladder cancer than P53 and P21 in the study conducted by Sharkh *et al*., [6]. Hitchings *et al*., stated that P16 and P53 could be used as independent prognostic factors in terms of disease progression in a study they conducted on PTa and PT1 bladder carcinoma cases. They only found P16 changes to be significant in terms of progression when they evaluated P16, pRb and p53 [13]. İn our study the recurrence rate was significantly higher in cases with high immune expression compared to those with moderate immune expression of P16. P16 was thought to be an important factor for recurrence prediction. However, we found no statistically significant difference in P16 staining levels between PTa and PT1 cases ($p>0.05$).

Evaluating the effects of the CK20, bcl-2 and P16 parameters on recurrence logistic regression analysis showed that more than 1% staining with bcl-2 was found to have an explanatory power of 8.057 times on recurrence risk. These findings indicate that bcl-2 can help determine the possibility of recurrence.

In the normal urothelium the expression of Ki67 is low and limited to basal layers. In many studies authors agree that it varies considerably with histologic grade and clinical stage [14, 15].

Nevertheless, Rodríguez-Alonso *et al*., found that the correlation of relapse with Ki67 was not significant in their studies [16]. Moyano Calvo *et al*., found that there was an association of Ki67 with recurrence [17]. We found a marked statistically significant difference between Ki-67 staining levels and tumor grade. A statistically significant difference was also found between Ki-67 staining levels and presence of invasion ($p<0.05$). A normal Ki-67 staining rate was common in PTa patients while a Ki-67 staining rate of more than 50% was significantly more frequent in pT1 patients. When the grade and stage were evaluated together, a marked statistically significant difference was found between Ki-67 immune expression rates ($p<0.01$). Less than 10% Ki-67 immune expression was seen in cases diagnosed with PUCLG-PTa, PUCLG-PT1, PUNLMP, papilloma and inverted papilloma while a Ki-67 immune expression rate of more than 50% was found inPUCHG-PT1 cases and a Ki-67 immune expression rate of 10% to 50% in PUCHG-PTa cases. These findings indicate that Ki-67 immune expression can be beneficial as a supportive parameter in the evaluation of grade and presence of invasion.

P53 has been associated with prognostic parameters such as recurrence, tumor progression and metastasis-free interval. In some studies the expression of this antibody was associated with tumor progression [13, 16]. Stavropoulos *et al*., found that p53 did not contribute to predicting the recurrence and progression of superficial bladder tumors [18]. No statistically significant difference was found between the recurrence rates according to the immune expression level of p53 in our study. Tony T Wu *et al*., found no correlation between p53mutation and the stage and grade in their study on p53 expression and grade and invasion depth [19]. We also found no correlation between p53 status and the stage and grade ($p>0.05$).

Conclusion

It is quite important to determine which superficial bladder tumor cases will recur so that decisions can be made about patient follow-up. The histomorphological characteristics of the tissue alone are not adequate for predicting superficial bladder tumor recurrence. Our study showed that combined use of CK20, bcl–2, p16 could be useful in predicting tumor recurrence. We also believe that Ki-67 and CK20 immune expressions could be helpful in determining tumor grade and stage in superficial bladder carcinomas.

Authors' Contribution

ÖY: carried out the literature search, carried out the experiments and interpreted the results and prepared the draft manuscript.
YS: designed the study and performed the analysis
SÖ: carried out the literature search and prepared the draft manuscript.
NÖ: carried out the experiments and interpreted the results.
NM: conceived the study, participated in design and edited the final manuscript.
FE: conceived the study, participated in design and edited the final manuscript.

Conflict of Interests

The authors declare that there are no conflicts of interests.

Ethical Considerations

The study was approved by the institute ethics committee.

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