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ORIGINAL ARTICLE

Cell Cycle Markers in the Evaluation of Bladder Cancer

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Abstract

Bladder cancer (BC) is a heterogeneous neoplasia characterized by a high number of recurrences. Standardized clinical and morphological parameters are not always sufficient to predict individual tumor behavior. The aim of this study was to evaluate the expression of cell cycle regulators proteins as potential adjuvant in prognosis and monitoring of this disease. Block paraffin samples from patients with urothelial bladder carcinoma treated by transurethral resection (TUR) were collected to immunohistochemistry analysis for proteins p16, p21, p27, p53, pRb and Ki-67. Chisquare, logistic regression and Kaplan-Meier curve were used to analyze the prognostic value of these markers. Of the 93 patients included in the study, the main categories of staging observed were T1 (53%) and Ta (29%), and the distribution between tumor grades was 58% of patients with low grade to 42% of patients with high grade. The expressions of p16, p21, p27, p53, pRb and Ki-67 were altered in 31%, 42%, 60%, 91%, 27% and 56% of patients, respectively. The immunohistochemical expression of Ki-67 was associated with tumor histological grade ($p = 0.016$), and expression of pRb with recurrence-free survival ($p = 0.035$), but no isolated marker was significant associated with recurrence and progression in multivariate analysis. More than two markers abnormally expressed were associated with presence of recurrence ($p = 0.005$) and lower recurrence-free surviva ($p = 0.004$). Our panel marker has important prognostic value for BC, especially when more than two have altered expression predicting good clinical recurrence implication.

Keywords Bladder cancer · Cell cycle markers · Prognosis · Immunohistochemistry

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Introduction

Bladder cancer (BC) is a heterogeneous disease strongly associated with smoking. It is the most frequent urinary tract neoplasia, with approximately 430,000 new cases diagnosed in 2012 [1, 2]. Ninety percent of BC are urothelial carcinomas, classified according to TNM staging as non-muscle-invasive (Ta, Tcis, T1) or muscle-invasive (T2, T3, T4) [3, 4]. The majority of BC (75%) are non-muscle-invasive cancers. These tumours can recidivate in 60%–70% and progress in 20%–30% of cases [5, 6].

Cystoscopy, urinary cytology and transurethral resection (TUR) are utilized to establish diagnosis and follow-up in BC [6, 7]. Histological grade and TNM staging are important prognostic factors for aggressiveness in BC [6]. However, due to BC heterogeneity, there is no definitive parameter to predict the behaviour and prognosis of BC [8, 9].

For this reason, several markers have been investigated to complement standard cytopathology and histopathological examination. Cell cycle and proliferation proteins have been

investigated as important markers in diagnosis, prognosis and monitoring of bladder cancer patients [4, 9–13].

The aim of this study was to identify an immunohistochemical panel of cell cycle regulatory proteins (p16, p21, p27, p53, pRb and Ki67) in a retrospective cohort of BC patients and to predict its association with pathological parameters (grade and muscle invasion), recurrence and progression.

Materials and Methods

Ethical Considerations

This study meets The Ethics Code of the World Medical Association (Declaration of Helsinki) and was approved by the Ethical Committee of the Hospital. Patients received a clear explanation of the study aims, procedures and confidentiality. All patients signed voluntary informed consent forms.

Study Population

The study population was composed of a retrospective cohort of patients diagnosed with urothelial bladder carcinoma, after TUR. For stage confirmation, all our samples had to include bladder tissue and a muscle fragment in the specimen. During the period from December 2000 to May 2014, patients were selected from Santa Rita Hospital (ISCMPA/BR), who met the following criteria: older than 18 years and no evidence of associated cancer. The study population was selected based on histopathological reports from Laboratório de Patologia do Hospital Santa Rita.

Data Collection

Demographic data were collected by using a sociodemographic questionnaire. We had access to patients' medical charts, including exams and histopathological reports. Paraffin blocks were collected and slide staining was performed for haematoxylin-eosin (H&E) and for immunohistochemistry. H&E slides were reviewed by a pathologist for diagnosis confirmation, following the International Union Against Cancer 2009 classification for TNM staging and the World Health Organization/International Society of Urological Pathology 2004 classification for grade.

Immunohistochemistry

Immunohistochemical staining was performed for p16, p21, p27, p53, pRb and Ki67 proteins, according to the peroxidase method. The slices, 4 μm in thickness, were deparaffinized and rehydrated according to standard protocols [14]. Then, antigen retrieval was performed using Tris-EDTA buffer (ethylenediaminetetraacetic acid; pH 9.0) for Ki-67 and p53 markers, and sodium citrate (pH 6.0) for p16, p21, p27 and pRb markers, for 40 min in a water bath at 95–98 °C. After heating, the slices were cooled at room temperature for 20 min, followed by blocking of the endogenous peroxidase activity through immersion in water with 5% H_2O_2 (3 times for 10 min each). In sequence, slides were washed with phosphate buffered saline (PBS) two times and incubated in a solution to block unspecific binding (bovine serum albumin 1%, for 1 h) [14].

The primary antibodies used were p16 (clone 6H12, Novocastra; Newcastle; UK; dilution 1:40), p21 (clone DCS-60.2, Neomarkers; Fremont; USA; dilution 1:100), p27 (clone 5X53G8, Dako; Cambridge; UK; dilution 1:100), p53 (clone DO-7, Dako; Cambridge; UK; dilution 1:100), Ki-67 (clone MIB-1, Dako; Cambridge; UK; dilution 1:200) and pRb (clone Rb1, Zymed; San Francisco; USA; dilution 1:200).

The primary antibodies were applied, and the slides were incubated in a dark, humid chamber for 60 min at room temperature then 4 °C overnight. After this procedure, the slides were left at room temperature for 1 h and then washed with PBS. Slides were incubated with secondary antibody (Dako advance TM HRP link) for 1 h, washed again with PBS and incubated with tertiary antibody (Dako Advance TM HRP enzyme) for 1 h. The antigen-antibody complex was visualized with diaminobenzidine tetrahydrochloride (DAB) and contrasted with haematoxylin [15].

As positive controls for staining, breast slides were used for p53, p21 and p27; hypophysis slides for p16 and tonsil slides for Ki-67 and pRb. As a negative control, the primary antibody was substituted with bovine serum albumin 1%.

Scoring

Five representative fields presenting good marker expression were captured on each slide with an Olympus BX51 optical microscope equipped with a DP72 camera and DP2-BSW software (Olympus™; Tokyo; Japan). Each hotspot underwent a manual count of nuclear positivity in 200 cells; two independent evaluators performed the count with Image-Pro Plus 6.3 software. Absence of expression was considered only when no immunohistochemical reaction could be observed. If discordance was greater than 20% between evaluators, a third researcher was consulted.

Nuclear immunoreactivity was considered altered when samples demonstrated expression ≥10% for p53 and Ki67; 0% or >50% for pRb and p16; <10% for p21; and <30% for p27, according to the literature [8, 16, 17].

Statistical Analysis

Data were plotted, processed and analysed with SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Chi-square and Fisher's exact test were performed to verify associations between clinical parameters and protein expression and smoking. Logistic regression was used for the multivariate analysis. The Kaplan-Meyer method was performed to evaluate patients' time free of recurrence. Differences were considered statistically significant at $p < 0.05$.

Results

Sample Characterization

A total of 108 patients were selected to participate in the study; however, 15 were excluded because the paraffin block was not available. The final study sample consisted of 93 patients whose clinical-pathological characteristics are summarized in Table 1.

Table 1 Clinical and pathologic characteristics of patients

	$n = 93$
Median follow-up period, mo, median (IQR)	40 (5–120)
Age at diagnosis, yr., median (IQR)	63 (56–69)
Gender, no. (%)	
Male	67 (72)
Female	26 (28)
Histological grade, no. (%)	
Low	54 (58)
High	39 (42)
Tumor stage, no. (%)	
pTa	27 (29)
pTis	0
pT1	49 (53)
pT2	13 (14)
pT3	3 (3)
pT4	1 (1)
Recurrence, no. (%)	
Yes	23 (25)
No	70 (75)
Progression, no. (%)	
Yes	3 (3)
No	90 (97)
Smoking status, no. (%)	
Active	82 (88)
Passive	4 (4)
No	7 (8)

IQR = Interquartile range

Marker Expression and Clinical-Pathological Parameters

As shown in Table 2, Ki-67 expression was significantly associated with high grade BC ($p = 0.016$) but not with muscle invasion. Association with clinical-pathological parameters was not observed for any other protein. Figure 1 shows the expression pattern of markers in high and low histological grade of BC.

No isolated marker showed a significant association with recurrence (Table 3). However, when we evaluated the number of altered markers, a relation was observed ($p = 0.023$). Multivariate analysis demonstrated an association with recurrence cases and altered expression in two or more markers ($p = 0.005$).

Recurrence-Free Survival

Estimation of 10-year recurrence-free survival related to marker expression is demonstrated in Fig. 2. The isolated proteins Ki-67, p16, p21, p27 and p53 did not show significant relations with recurrence-free survival, but the altered expression in Ki-67presented a tendency towards lower recurrence-free survival in BC patients ($p = 0.059$). In the multivariate analysis by logistic regression, no protein was revealed as a predictor of significant recurrence.

Abnormal expression of pRb was clearly related to lower recurrence-free survival compared with normal pRB expression in BC patients ($p = 0.035$). Patients who had one or two markers altered had higher recurrence-free survival than those with more than two abnormally expressed markers ($p = 0.004$).

Smoking as a Risk Factor

Smoking status was positive in 92% of the study population. Therefore, patients who had contact with cigarettes, whether from direct smoking or second-hand smoke, did not display significant differences from non-smokers in terms of grade, invasion, recurrence or disease progression in our study.

Treatment

A total of 21 patients were treated with cystectomy during the follow-up period. All the muscle invasive cases underwent cystectomy, except for one patient who refused to undergo the procedure. Only five cases of non-muscle-invasive bladder cancer also underwent cystectomy due to medical criteria.

Table 2 Molecular characteristics related to tumor grade and invasiveness

	Total	Grade			Invasiveness		
		Low	High	*P* value	Non-invasive	Invasive	*P* value
p53, no. (%)							
Normal	8 (8.6)	6 (11.1)	2 (5.1)	0.461	6 (7.9)	2 (11.8)	0.635
Abnormal	85 (91.4)	48 (88.9)	37 (94.9)		70 (92.1)	15 (88.2)	
p16, no. (%)							
Normal	64 (68.8)	37 (68.5)	27(69.2)	1,000	53 (69.7)	11 (64.7)	0.908
Abnormal	29 (31.2)	17 (31.5)	12 (30.2)		23 (30.3)	6 (35.3)	
p21, no. (%)							
Normal	54 (58.1)	33 (61.1)	21 (53.8)	0.626	46 (60.5)	8 (47.0)	0.456
Abnormal	39 (41.9)	21 (38.9)	18 (46.2)		30 (39.5)	9 (53.0)	
p27, no. (%)							
Normal	37 (39.8)	22 (40.7)	15 (38.5)	0.994	29 (38.1)	8 (47.0)	0.686
Abnormal	56 (60.2)	32 (59.3)	24 (61.5)		47 (61.9)	9 (53.0)	
pRb, no. (%)							
Normal	68 (73.1)	40 (74.0)	28 (71.8)	0.994	58 (76.3)	10 (58.8)	0.243
Abnormal	25 (26.9)	14 (36.0)	11 (28.2)		18 (23.7)	7 (41.2)	
Ki-67, no. (%)							
Normal	41 (44.1)	30 (55.6)	11 (28.2)	0.016	34 (44.7)	7 (41.2)	1.000
Abnormal	52 (55.9)	24 (44.4)	28 (71.8)		42 (55.3)	10 (58.8)	

Discussion

BC is a heterogeneous neoplasia that presents high probability of recurrence and progression, showing different rates of metastasis and mortality, depending on tumour grade and staging. As reported by many authors and guidelines, BC occurs mostly in men, affecting three times the number of men as women [3, 6, 18, 19]. Our study sample had a ratio of 2.57 men for each woman, very close to the population estimate. In agreement with pre-existing data, the mean age at diagnosis was 63 years (60–70 years) [8, 16, 20].

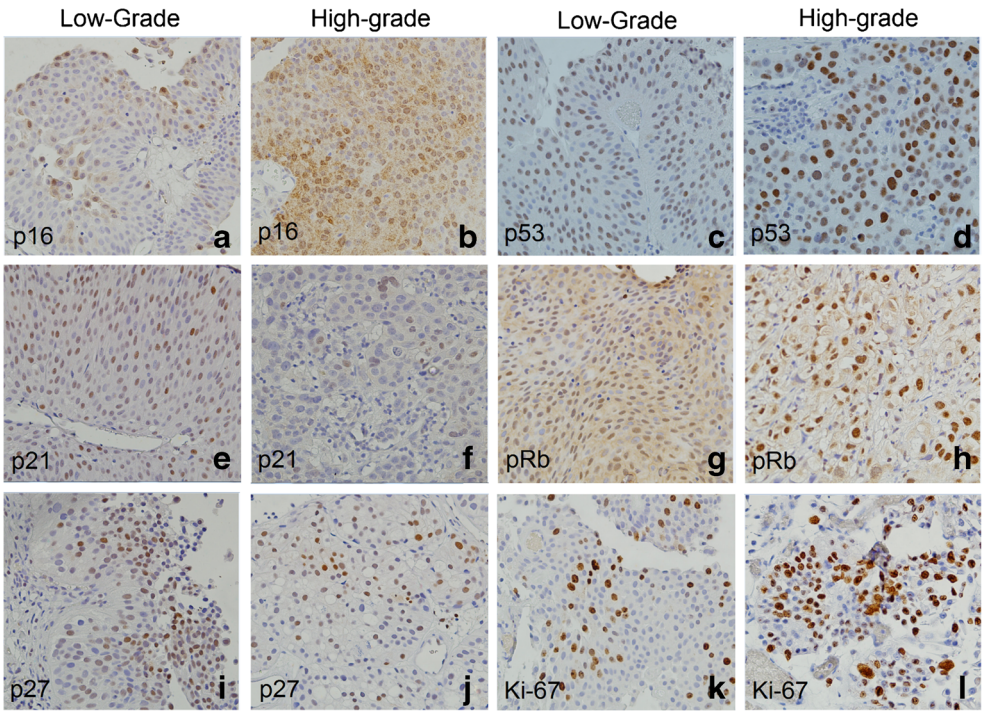

Fig. 1 Immunohistochemical expression. Low and High-grade for p16 (A,B), p53 (C,D), p21 (E,F), pRb (G,H), p27 (I,J) and Ki-67 (K,L). (×200)

Table 3 Alterations of markers according to recurrence

	Total	Recurrence		
		Yes	No	*P* value
p53, no. (%)				
Normal	8 (8.6)	2 (8.7)	6 (8.6)	1,000
Abnormal	85 (91.4)	21 (91.3)	64 (91.4)	
p16, no. (%)				
Normal	64 (68.8)	14 (60.9)	50 (71.4)	0.491
Abnormal	29 (31.2)	9 (39.1)	20 (28.6)	
p21, no. (%)				
Normal	54 (58.1)	11 (47.8)	43 (61.4)	0.366
Abnormal	39 (41.9)	12 (52.2)	27 (38.6)	
p27, no. (%)				
Normal	37 (39.8)	8 (34.8)	29 (41.4)	0.749
Abnormal	56 (60.2)	15 (65.2)	41 (58.6)	
pRb, no. (%)				
Normal	68 (73.1)	14 (60.9)	54 (77.1)	0.209
Abnormal	25 (26.9)	9 (39.1)	16 (22.9)	
Ki-67, no. (%)				
Normal	41 (44.1)	8 (34.8)	33 (47.1)	0.427
Abnormal	52 (55.9)	15 (65.2)	37 (52.9)	
Altered markers no. (%)				
0	0	0	0	0.023
1	5 (5.4)	0	5 (7.1)	
2	25 (26.9)	2 (8.7)	23 (32.9)	
3	35 (37.6)	13 (56.5)	22 (31.4)	
4	18 (19.4)	3 (13.1)	15 (21.4)	
5	7 (7.5)	4 (17.4)	3 (4.3)	
6	3 (3.2)	1 (4.3)	2 (2.9)	
Altered markers no. (%)				
≤2	30 (32.3)	2 (8.7)	28 (40.0)	0.005
>2	63 (67.7)	21 (91.3)	42 (60.0)	

Regarding bladder tumour grade and TNM classification, it is known that low grade and pT1 cases are more common than other categories [6]. In this study, we observed that 58% of patients had a low-grade diagnosis, and the most prevalent staging was pT1 (53% of patients). BC grade and stage are the main prognostic indicators used in medical clinical practice [6]. However, due to the high biological heterogeneity of BC, sometimes these parameters become insufficient to safely predict aggressive tumour behaviour [8, 11, 16].

In this context, several studies [8, 16, 17, 21–24] have been performed associating immunohistochemical markers with grade, staging and recurrence. These markers include proteins responsible for cell cycle control and proliferation, corroborating the hypothesis that their expression levels can be explored as prognostic factors, either alone or in immunohistochemical panels [17].

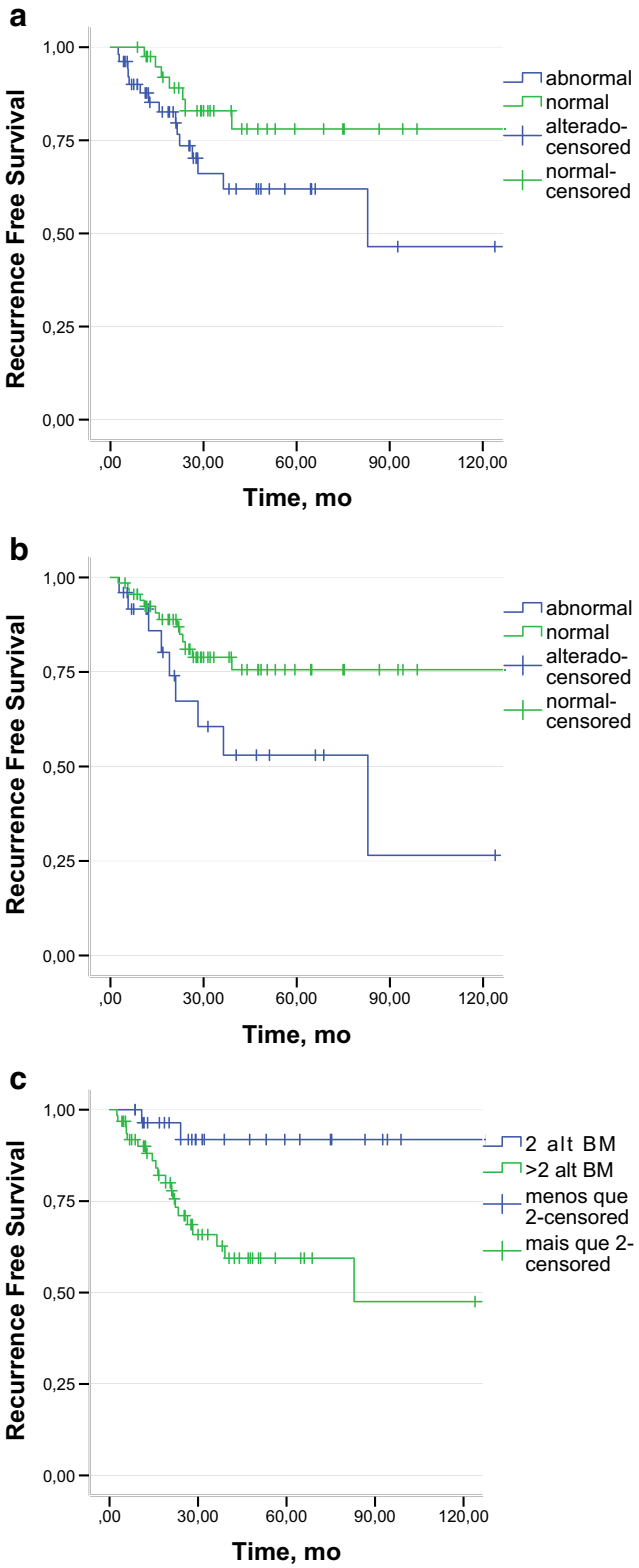

Fig. 2 Kaplan-Meier analysis of recurrence-free survival for Ki-67 (p = 0,059) (**a**), pRb (p = 0,035) (**b**) and number of altered biomarkers (p = 0,004) (**c**). alt. = altered; BM = biomarker

In our study, the isolated analysis of p53, p16, p21, p27 and pRb proteins did not show significant associations with grade,

invasion, recurrence or progression. These results agree with Olsson et al. [17], who also did not reveal associations between these parameters and the proteins p16, p53, p21 and pRb. A study by Lee et al. [25] obtained a significant association between BC invasiveness and the altered expression of pRb and p53. An explanation for the divergence of data can be the heterogeneity of the populations studied, as well as the criteria used for marker classification. For p16, Olsson et al. [17] used a cut-off of 0% or >50%, while Lee et al. [25] and Kruger et al. [26] considered 0% or >76% and <10%, respectively.

Studies with the Ki-67 proliferation index have shown a significant association with grade, staging and recurrence, demonstrating its strong relation with tumour aggressiveness [13, 21, 22, 27]. Our results confirm these findings, presenting significant association with high tumour grade and altered expression of Ki-67 ($p = 0.016$). The association of this protein with recurrence remains in conflict between studies. Weihong et al. [27] reported a significant association between altered Ki-67 and recurrence in both univariate and multivariate analyses. Other studies only obtained correlations in univariate analysis [21, 22]. Most authors, including us, used a cut-off criteria for determination of altered proliferation equal to ≥10% [16]; however, others considered a cut-off of ≥20% [8, 22].

With the Kaplan-Meier curve, we demonstrated an association of pRb protein with lower 10-year recurrence-free survival ($p = 0.035$ and $p = 0.059$, respectively), and a further tendency towards an association for Ki-67 ($p = 0.059$). Weihong et al. [27] found an association between Ki-67 expression and recurrence-free survival ($p < 0.0001$), when analysing only non-muscle-invasive BC.

As important finding in our study was that more than two altered cell cycle markers were associated with recurrence and lower recurrence-free survival in BC patients. These data confirm previous findings by Lotan et al. [16], who demonstrated significance in the number of altered biomarkers and predicted disease recurrence ($p = 0.004$). These results corroborate the hypothesis that the combination of markers has greater prognostic power than the isolated markers.

Cell cycle and proliferation regulatory proteins are potentially able to improve the prognostic ability of currently used clinical-pathological parameters; however, due to the high biological and clinical heterogeneity of BC, it is unlikely that a single marker can predict precise prognostic categories. Therefore, an important finding for determination of recurrence using our marker panel seems to be how many cell cycle markers have altered expression, specifically, more than two. However, more studies are needed to increase the reproducibility of these results, given its important implications in the clinical management of patients with BC.

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