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ORIGINAL ARTICLE

Ki-67 and Cell Cycle Regulators p53, p63 and cyclinD1 as Prognostic Markers for Recurrence/ Progression of Bladder Urothelial Carcinoma

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Abstract Deregulation of the cell cycle regulating genes is common in urothelial bladder carcinoma (UBC). We aimed to examine the prognostic significance of ki-67, p53, p63 and cyclinD1expression in UBC and to identify optimal cut-off points to help identifying patients at high risk of tumor recurrence. We evaluated the immunohistochemical expression of ki-67, p53, p63 and cyclinD1 in 100 UBCs. The conventional and the classification and regression trees-guided (CART-guided) methods were utilized to determine the independent predictors of tumor recurrence. The p53 and Ki-67 expression didn't associate significantly with tumor recurrence.p63 and cyclinD1 exhibited significant hazard ratios. Using CART, no recurrence was observed when p63 was ≥87.5%. The recurrence incidence increased and the disease free survival (DFS) time shortened as the p63 decreased. CyclinD1 associated significantly with tumor recurrence only if p63 was <35%. Using the CART cut-off values¬, cases were categorized into three groups; (groups I: p63 ≥ 35%, II: p63 < 35% and cyclinD1 < 10% and III: p63 < 35% and cyclinD1 ≥ 10%). Group I patients revealed the least incidence of recurrence at the longest DFS. Group III had the worst prognosis followed by Group II. p63 represents a surrogant biomarker to predict UBC recurrence.CyclinD1 can be used only when p63 is <35%. CART proved helpful with data among which the number of cases with positive outcomes is too small relative to the number of studied predictors. Large cohort studies for ki-67 and p53 are recommended to be performed with standardized criteria as regards patients' characteristics, cut-off values, and follow-up time.

Keywords Ki-67,cell cycle regulators · Bladder carcinoma · CART · Survival

Introduction

Urothelial bladder carcinoma is the fifth most common cancer worldwide [1, 2]. More than 70% of cases present as superficial urothelial carcinomas that are confined to the mucosal layer (pTa) or invade the lamina propria without muscularis propria invasion (pT1) [3]. For non-muscle invasive bladder carcinoma (NMIBC) histological tumor grade is considered an important prognostic factor [4]. Muscle-invasive bladder carcinoma (MIBC) comprise the remaining 30% of cases. Pathologic tumour stage and nodal involvement have been proven to be the only independent predictors of MIBC survival [5].

Up to 70% of NMIBC patients experience tumour recurrence, and up to 15% progress to a muscle-invasive subtype [6]. Thus, identifying the potential markers that could predict recurrence of NMIBC might help selecting patients with high-risk tumours who require close monitoring [7].

Mutations and deregulation of genes involved in the regulation of normal cell cycle progression are frequent events in human urothelial carcinomas [8]. p53 gene mutation is common in urothelial bladder carcinoma. Some studies reported the overexpression of p53 in higher stages and grades of

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urothelial carcinoma and the overexpression of the p53 gene product has been reported as a marker of progression in urothelial carcinoma [9].

p63, a member of the p53 gene family, encodes multiple proteins that may either transactivate p53 responsive genes or act as a dominant-negative factor toward p53 [10]. It is suggested to play a critical role in the normal development and maintenance of the human urothelium [11]. p63 is deregulated in bladder carcinogenesis [10]. Some studies showed a down-regulation in muscle-invasive tumours [12], while others demonstrated maintained (retained) expression with biological aggressiveness, suggesting a role in tumour progression [13].

CyclinD1, a key regulator of the cell cycle, forms complexes with cyclin-dependent kinase 4 or 6 in the cytoplasm. These complexes then enter the nucleus [14] and inactivate the cell-cycle suppressive retinoblastoma protein, thereby promoting progression from G1 to the S-phase [15]. Aberrant expression of CyclinD1 and alterations in its coding gene CCND1 are frequent in human cancers [14] and may therefore harbor prognostic [16–19] and predictive [20, 21] information. Recently, overexpression [22] and polymorphic variability of cyclinD1 gene have been implicated in human bladder cancer [23]. However, the clinical evidence implicating cyclinD1 in urothelial bladder carcinoma is conflicting.

Moreover, the expression of the cell proliferation marker, Ki-67 (MIB-1) [24], revealed variability in the mitotic activity between NMIBC and the more aggressive muscle-invasive tumours, thereby reflecting its prognostic significance [25].

In a trial to identify urothelial carcinoma patients who are at high risk of tumour recurrence, and are thereby in need of closer surveillance and aggressive treatment, in an attempt to initiate individualized therapy strategy, this study was undertaken. We aimed to examine the prognostic significance of expression of the proliferation marker ki-67 and the cell cycle regulators p53, p63 and cyclinD1 in urothelial bladder carcinoma. We used the classification and regression trees (CART) - a non-parametric regression approach- to identify the optimal cut-off points for the studied immunohistochemical markers, and to find interactions among multiple predictors to help in the prediction of recurrence among urothelial carcinoma cases. We also aimed to evaluate the association between the expression of those biomarkers and the clinicopathological features of urothelial bladder carcinoma.

Material and Methods

This retrospective study was carried out on 100 consecutive cases of transuretheral resections of bladder urothelial carcinoma specimens submitted to the Pathology Department, Faculty of Medicine, Alexandria University, during the period from July 2012 to December 2014. The study was approved by the Alexandria University, Faculty of Medicine Research Ethics Committee and REMARK criteria [26] were applied. All cases were for Egyptian patients (92 males and 8 females), aged between 40 and 77 years (M = 59, SD = 7). The clinicopathological characteristics of the studied cases are summarized in Table 1.

Clinical data of all cases were obtained from the patients' files at the Urosurgery Department including the disease free survival time (time to event occurrence, whether recurrence, or progression). The follow up period (measured from the date of primary diagnosis to the time of last follow-up visit) ranged from 4 to 50 months (Mdn = 37, M = 29.2, SD = 16.2). During this period, out of the study sample, 26 patients (26%) experienced the event of tumor recurrence. The median disease free survival (DFS) time was 39.5 (SE = 1.8; 95% CI: 36, 43) months. Out of the 26 cases that experienced recurrence, 12 cases were non-muscle invasive tumours at initial diagnosis. Only two out of those 12 cases recurred as non-muscle invasive tumours (i.e. non-progressed) with a disease free survival time of 9 months, while the remaining ten cases progressed to muscle invasive tumours in the recurrence with a median progression free survival of 7 months.

Histopathological Examination

The haematoxylin and eosin (H&E) stained slides of all cases were reviewed to determine the histological type and grade of the tumour, presence/absence of muscularis propria invasion, presence/absence of squamous or glandular differentiation, and presence/absence of lymphovascular invasion. Tumour grading and staging were carried out according to the WHO/ISUP (2004) and UICC-TNM (2002) respectively [27, 28].

Tissue Microarray Construction

Tissue microarray (TMA) construction was done following the method described by Kononen et al. [29]. The H&E-stained tumour tissue sections were reviewed to select morphologically representative regions from each tumour for TMA study. Two tumour spots were chosen under microscopy for each case and the corresponding spots were marked on the tissue block. A manual tissue arrayer punch (Beecher Instruments Inc., Sun Prairie, Wisconsin, USA) was used to remove tissue cores 1 mm in diameter in the marked area on the donor block. The retrieved tissue cores were transferred to corresponding receiver pores in the recipient paraffin block, that were arranged in a precisely spaced array pattern in order to subsequently construct a TMA block according to a predetermined scheme. The block was then heated for 15 min at 40 °C and the surface was flattened. From each TMA block, a 4 μm thick section was cut and H&E-stained to confirm the adequacy of sampling. Then, other sections were cut and mounted on Superfrost/Plus slides (Thermo Scientific, USA) for immunohistochemical staining. Two hundred tumour spots

Table 1 Clinico-pathological characteristics of the 100 studied urothelial carcinomas and their relation to p63 and cyclinD1 immunohistochemical scores

Clinico-pathological parameter	No.	(%)	p63		*U*	Cyclin D1		*U*
			Mdn	*(IQR)*	*(p-value)*	*Mdn*	*(IQR)*	*(p-value)*
Sex								
Male	92	(92)	50	(75)	358	0.00	(10)	297
Female	8	(8)	58	(68)	(.898)	5.00	(44)	.296
Urothelial carcinoma type								
Papillary	68	(68)	50	(75)	1025	0.00	(10)	1053
Non papillary	32	(32)	65	(55)	.638	0.00	(0)	.766
Tumor stage								
Non muscle invasive	46	(46)	60	(60)	986	0.00	(10)	1168
Muscle invasive	54	(54)	50	(75)	.073	0.00	(10)	.556
Tumor grade								
Low	44	(44)	65	(78)	1003	0.00	(10)	1200
High	56	(56)	50	(75)	.108	0.00	(10)	.798
Associated Bilharziasis								
Absent	92	(92)	55	(73)	304	0.00	(10)	291
Present	8	(8)	30	(75)	.412	15.00	(44)	.260
Squamous differentiation								
Absent	68	(68)	60	(75)	1067	0.00	(10)	737
Present	32	(32)	45	(55)	.875	10.00	(40)	.003
Glandular differentiation								
Absent	90	(90)	60	(68)	296	0.00	(10)	160
Present	10	(10)	40	(43)	.074	10.00	(36)	<.001

U stands for Mann-Whitney test

representing the 100 studied bladder urothelial carcinoma cases were performed (two spots per case). The results were interpreted in correspondence with the predetermined scheme of arrangenment of the TMA block.

Immunohistochemical Staining

Immunohistochemical staining was performed following the streptavidin-biotin-immunoenzymatic antigen detection method, performed according to the manufacturer's protocol on 4 μm thick sections cut from the tumour TMA block. After deparafinization in xylene, and rehydration in descending grades of alcohol, the TMA paraffin sections were subjected to heat induced antigen retrieval in 0.01 M citrate buffer (pH 6.0) in a 700 W microwave for 20 min. Endogenous peroxidase activity was blocked using 3% H2O2. Primary antibodies were then applied overnight (all purchased from Lab Vision, Fremont, California, USA); p53 Ab-8 (Clone DO-7 + BP53–12, mouse monoclonal), diluted at 1:100; p63 Ab-1 (Clone 4A4) mouse monoclonal, diluted at 1:200, Ki-67 (RM-9106-S0, rabbit monoclonal), diluted at 1:200, and cyclinD1 (Clone SP4, rabbit monoclonal), diluted at 1:50. Antigen visualization was performed using the Thermo Scientific UltraVision LP Detection System (USA). Immunohistochemical reactions were developed with diaminobenzidine and sections were counterstained with Harrris hematoxylin. All immunostains were manually processed. Appropriate positive (colon carcinoma for p53 and ki-67, normal skin for p63, and breast carcinoma for cyclinD1) and negative (omission of the primary antibody) controls were included.

Scoring of Immunostained Slides

Evaluation of the immunostained slides was performed blindly and independently without knowledge of the clinical outcome or other clinicopathologic variables. Only nuclear immunoreactivity was semiquantitatively assessed for all four antibodies. To quantify Ki-67 immunostaining, a proliferation index (PI) was done by evaluating the percentage of nuclear staining of neoplastic cells and the total number of cells counted on 40× microscopic field. Ki-67 staining of 10% or more was considered as positive [3, 24, 30]. Regarding p53, tumours with nuclear immunoreactivity of 10% or more were considered positive according to previous studies [24, 31].

The fraction of tumour cells stained with antibodies to p63 and cyclinD1 were counted as a percentage and scored. The percentage of reactive nuclei for cyclinD1 ranged from 0 to 70% with a median value of zero. The median p63 value was 50%, ranging from 5 to 98%.

Statistical Methodology

Statistical analyses were performed using IBM SPSS® Version 21.0 and "rpart" package [32] in R [33]. Quantitative data were described using median (Mdn), minimum and maximum or mean (M) and standard deviation (SD). Qualitative data were described using number and percentage. Correlations between quantitative variables were tested using Pearson's correlation coefficient. Agreement between qualitative markers was tested using Cohen's kappa coefficient. Mann-Whitney U test was used to compare quantitative variables between two groups, as the assumption of normality was violated.

To determine the independent predictors of tumour recurrence among the study sample, we used two statistical methodologies; the conventional and the classification and regression trees-guided (CART-guided) methods.

Conventional Method

In the conventional statistical method, we conducted bivariate analysis first to determine the potential predictors, then, multivariate analysis to determine the independent predictors among the potential predictors. In bivariate analysis, we used log-rank test to compare the probability of recurrence between the different groups (using the test for trend for ordered categories with more than two groups) and univariate Cox regression to test the association between continuous covariates and patients' outcome. In multivariate analysis, we used multivariate Cox regression model.

CART-Guided Method

We used Olshen survival, a variant of classification and regression trees (CART) specialized to deal with survival data, then, we used parametric statistical tests to examine the hypotheses generated by the CART.

CART is a non-parametric regression approach in which the data are sequentially split into dichotomous groups, such that each resulting group contains observations which have similar outcome [34, 35]. However, it is not appropriate to use this method without modification to deal with survival analysis as some sizable fraction of the data is "censored", i.e. the time to the event is not known. Richard Olshen, one of the four creators of CART, developed the Olshen survival tree which is a specialized variant of CART to deal with survival data [35]. We used the Olshen survival to find the important predictors of tumour recurrence among the study sample, to determine the optimal cut-off values for continuous variables and to detect the potential interactions between the important predictors. All the demographic, clinicopatholgoical parameters as well as the immunohistochemical expression results of the four studied markers were entered into the CART model.

The end product of a typical CART analysis is a tree that starts with a "root node" which contains the observations from which the tree will be grown. The observations are then partitioned into two "child nodes" -each containing a subset of the observations- according to the value of one of the predictors. Each child node may be further divided, again according to the value of one of the predictors. This process continues until any further splitting would not improve the overall R-squared (overall fitting of the model) by 1%. The final child nodes are named terminal nodes and they form a complete partition of the observations in the root node.

Then, the decisions developed by the CART were further tested by parametric statistical tests to find out if they can be generalized to the whole population or not. If the p-value was small (≤ 0.05), the decision made by the CART was accepted and the null hypothesis was rejected. If the p-value was large (>0.05), the decision made by the CART was considered specific to the current sample, thus it was ignored and the null hypothesis was not rejected.

Significance of the test results were quoted as two-tailed probabilities, and judged at the 5% level. Kaplan-Meier curves and CART were used for the presentation of the results.

Results

The current study included 100 consecutive cases of transuretheral resections of bladder urothelial carcinomas, the clinicopathological features of which are summarized in (Table 1).

Immunohistochemical Expression of the Proliferation Marker Ki-67 and the Cell Cycle Regulators p53, p63 and cyclinD1

Among the 100 studied cases, only positive brown nuclear staining in the tumour cells was considered positive for all four antibodies. Some cases showed substantial cytoplasmic cyclinD1 staining, which was not included in the scoring. While 28 cases showed p53 overexpression (p53 score $\geq$ 10%), 48 cases showed high proliferation index (ki-67 score $\geq$ 10%). The median p63 score was 50, ranging from 5 to 98 (M = 52, SD = 33), and the median cyclinD1 score was zero, ranging from 0 to 70 (M = 11, SD = 19).

The Relation between the Clinicopatholgoical Characteristics and the four Studied Markers

Patients' age showed no significant association with any of the four studied markers, (t = .3, p = .804 for ki-67; t = .415, p = .679 for P53; r = −.05, p = .646 for p63; and r = −.001, p = .990 for cyclinD1;). (Table 1) highlights the relation between p63, cyclinD1 and the clinicopathologic parameters,

while (Table 2) summarizes the association between p53, ki-67 and the clinicopathologic characteristics.

The Relation Between the Four Studied Immunohistochemical Markers

Among the four studied markers, only p53 and Ki-67 showed a moderate agreement (k=.4, $p < .001$). In 72% of the cases, both markers showed the same result (Table 3). Both markers did not show a significant association with the other two markers, namely p63 and cyclinD1. Moreover, the correlation between p63 and cyclinD1 was not statistically significant ($r = .05$, $p = .646$).

Immunohistochemical Expression of the Proliferation Marker Ki-67 and the Cell Cycle Regulators p53, p63 and cyclinD1 in the Recurrent/Progressed Cases

A larger proportion of patients who experienced tumour recurrence exhibited high proliferation index and showed p53 overexpression compared to cases who did not experience the event of recurrence; [14 (54%) vs 34 (46%)] and [8 (31%) vs 20 (27%)] respectively. (Figs. 1, 2) Moreover, among the recurrence group, the median p63 was much lower and the cyclinD1 showed more dispersion [(Mdn = 10%, IQR = 23%) vs (Mdn = 67.5%, IQR = 48%)] and [(Mdn = 0, IQR = 40) vs (Mdn = 0, IQR = 10)] respectively. (Figs. 3, 4).

Among the 10 cases that experienced tumor progression, 6 cases exhibited a high proliferation index. The median cylcinD1 was 0 (IQR = 23) and the median p63 was 15 (IQR = 20) -which was not different from the two recurrent non-progressed cases as cyclinD1 was zero in both cases, and the p63 was 5% in one case and 10% in the other. The p53 was less than 10% among all 12 cases.

Prognostic Significance of the Studied Markers

A. Conventional Analysis

Bivariate Analysis

Table 4 shows the association between the recurrence of urothelial carcinoma and the different clinicopatholgoical factors. Apart from bilharzial infestation that associated with higher incidence of tumour recurrence and shorter disease free survival, none of the other clinicopatholgoical factors,

Table 2 Clinico-pathological characteristics of the 100 studied urothelial carcinomas and their relation to Ki-67 and p53 immunohistochemical scores

Clinico-pathological parameter	Ki-67				X^2	P53				X^2
	<10%		≥10%		(p-value)	<10%		≥10%		(p-value)
	n	(%)	n	(%)		n	(%)	n	(%)	
Sex										
Male	46	(50)	46	(50)	FET	67	(73)	25	(27)	FET
Female	6	(75)	2	(25)	(.272)	5	(63)	3	(38)	(.683)
Urothelial carcinoma type										
Papillary	44	(65)	24	(35)	13.7	60	(88)	8	(12)	27.8
Non papillary	8	(25)	24	(75)	(<.001)	12	(38)	20	(63)	(<.001)
Tumor stage										
Non muscle invasive	30	(65)	16	(35)	5.6	44	(96)	2	(4)	*23.6*
Muscle invasive	22	(41)	32	(59)	(.015)	28	(52)	26	(48)	(<.001)
Tumor grade										
Low	28	(64)	16	(36)	4.3	42	(95)	2	(5)	21.4
High	24	(43)	32	(57)	(.039)	30	(54)	26	(46)	(<.001)
Associated Bilharziasis										
Absent	50	(54)	42	(46)	FET	68	(74)	24	(26)	FET
Present	2	(25)	6	(75)	(.149)	4	(50)	4	(50)	(.215)
Squamous differentiation										
Absent	40	(59)	28	(41)	4.0	52	(76)	16	(24)	2.1
Present	12	(38)	20	(63)	(.046)	20	(63)	12	(38)	(.160)
Glandular differentiation										
Absent	50	(56)	40	(44)	4.6	70	(78)	20	(22)	14.9
Present	2	(20)	8	(80)	(.033)	2	(20)	8	(80)	(<.001)

FET stands for Fischer's exact test

Table 3 The relation between the immunohistochemical expression of Ki-67, and the cell cycle regulators p53, p63, and cyclinD1 among the studied bladder carcinoma cases

Marker	Ki-67					p63			Cyclin D1		
	<10		≥10		*k*	*Mdn*	*(IQ)*	*U*	*Mdn*	*(IQ)*	*U*
	n	*(%)*	*n*	*(%)*	(*p*-value)			*(p-value)*			*(p-value)*
P53											
< 10%	48	(48)	4	(4)	.430	45	(75)	1005	0	(10)	915
10 or more	4	(4)	24	(24)	(<.001)	65	(70)	(.981)	0	(38)	(.411)
Ki-67											
< 10%						45	(75)	1112	0	(10)	1203
10 or more						68	(73)	(.342)	0	(25)	(.721)

U stands for Mann-Whitney test

k stands for Kappa statistic for agreement

including the patients' sex, and age (HR = .979, 95% CI = (.924, 1.04), $p = .466$) showed any significant association with tumour recurrence.

The immunohistochemical expression of both the proliferation index (Ki-67 score) and p53 did not show a significant association with tumour recurrence, **(**Table 4**)**. Conversely, p63 and cyclinD1 showed statistically significant hazard ratios, (HR = .97, 95% CI = .957,.986, p = <.001) and (HR = 1.02, 95% CI = 1.01, 1.04, $p = .008$), respectively. Every p63 unit increase was associated with 3% reduction in the risk of recurrence, and every cylcinD1 unit increase was associated with 2% increase in the risk of recurrence.

Multivariate Analysis

The Cox regression model included p63, cyclinD1 and associated bilharzial infestation, the variables that were significantly associated with tumour recurrence by bivariate analysis, as well as the interaction term between p63 and cyclinD1.Tumour grade and tumour stage were also added to adjust for disease severity, **(**Table 5**)**.

Cox regression showed that the immunohistochemical expression of both p63 and cyclinD1 were significantly associated with urothelial carcinoma recurrence while controlling for the other predictors included in the model. Neither the interaction between p63 and cyclinD1, nor the associated bilharzial infestation were significantly associated with recurrence while controlling for the effects of p63 and cyclinD1.

B. Classification and Regression Trees (CART) Guided Methodology

All of the tested predictors including; demographic data, clinicopatholgoical characteristics and the four immunohistochemical markers were included in the CART model.

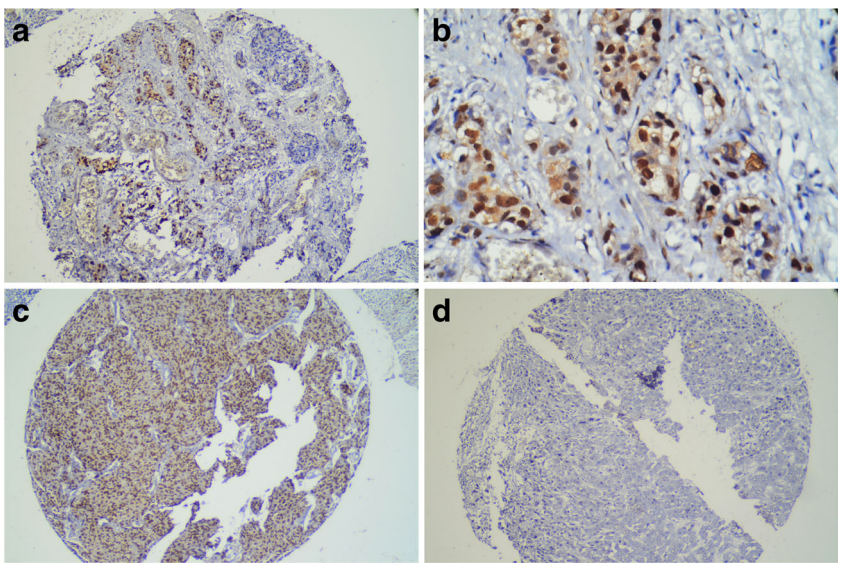

Fig. 1 p53 immunostaining: **a** A recurrent case of UBC showing p53 overexpression, (X100). **b** Higher power view (of Fig. 1a) showing dark brown immunostaining in >10% of tumor cell nuclei, (X400). **c** A non recurrent case featuring p53 overexpression, (X100). **d** A non recurrent case negative for p53 immunostaining, (X 100)

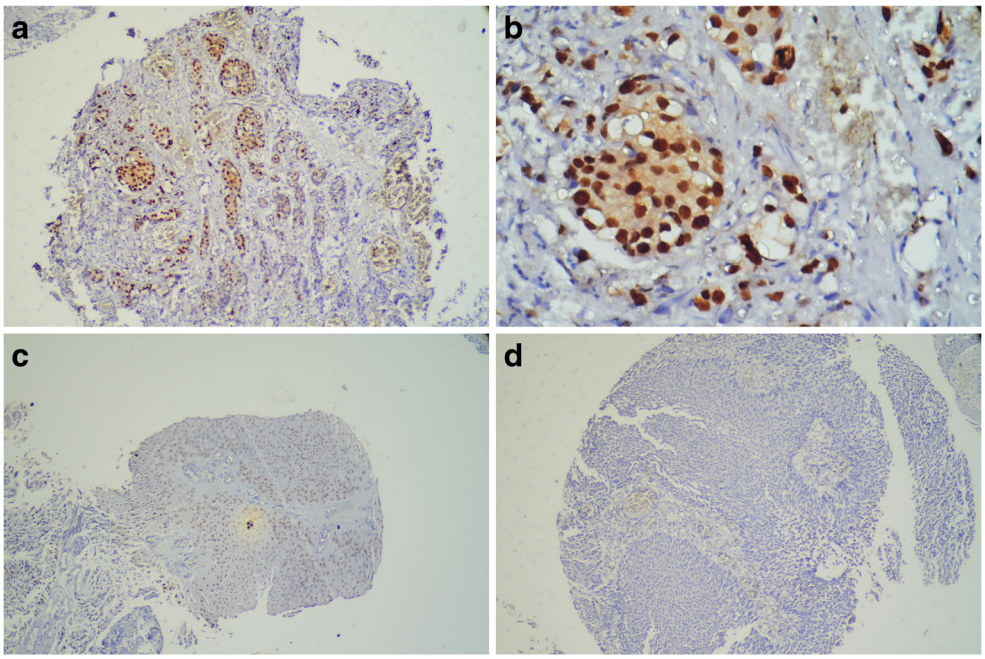

Fig. 2 Ki-67 immunostaining: **a** A recurrent UBC case showing high proliferation index, (X100). **b** Higher power view (of Fig. 2a) showing the positive nuclear staining in >10% of tumor cell nuclei, (X400). **c** A non recurrent UBC case showing Ki-67 index >10%, (X100). **d** A non recurrent UBC case featuring low proliferation index (ki-67 index = 0%), (X100)

CART classified the study sample according to the immunohistochemical expression level of p63 into two nodes; node 2 and node 7. Node 2 included all the cases whose p63 score was 35% or more; and it was further subdivided into three nodes; node 3, node 5 and node 6, according to the level of p63. Node 3 included cases whose p63 was 87.5% or more ($n = 16$). Node 5 included cases whose p63 ranged from 35 to less than 72.5% ($n = 21$). Cases with p63 of 72.5% to less than 87.5% ($n = 25$) were included in node 6. On the other hand, node 7, included all urothelial carcinoma cases with p63 scores less than 35%, and was further subdivided, according to the cyclinD1 immunohistochemical score into two nodes; node 8 and node 9. Node 8 included cases with cyclinD1 score below 10%, while node 9 included the remaining cases. No recurrence was observed among patients in node 3 (cases with p63 ≥ 87.5%). The incidence of recurrence got higher and the disease free survival time got shorter as we moved from node 5, to node 6, to node 8 to node 9. **(**Fig. 5**).**

The aforementioned hypotheses that were generated by the CART model were further tested by parametric statistical tests.

- Among the whole sample, low level of p63 immunohistochemical expression associated with increasing the hazard of recurrence. This association was found to be statistically significant, (HR = .97, 95% CI = .957, .986, p = <.001)
- Among patients with p63 ≥ 35% (at node 2), the higher the level of p63, the lower the hazard of recurrence. This hypothesis was tested and no sufficient statistical evidence supported it (HR = .998, 95% CI: .96,1.04, $p = .923$)
- Among patients with p63 higher than or equal to 35% and less than 87.5% (at node 4), the higher the level of p63, the higher the hazard of recurrence. Again, this hypothesis was tested and no sufficient evidence supported it, (HR = 1.03, 95% CI: .97, 1.09), $p = .357$).

Among patients with p63 score below 35% (node 7), the higher the cyclinD1, the higher the hazard of recurrence. This

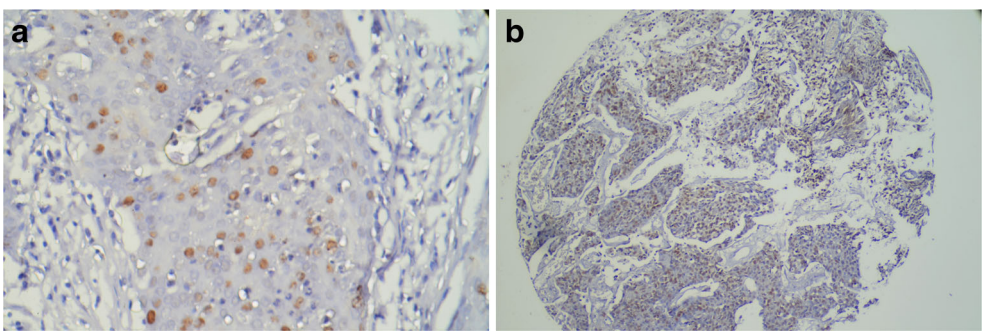

Fig. 3 p63 immunostaining: **a** A recurrent case of UBC featuring positive nuclear staining in >35% of tumor cell nuclei, (X400). **b** A non recurrent UBC case featuring diffuse positive nuclear staining in >87.5% of tumor cell nuclei, (X100)

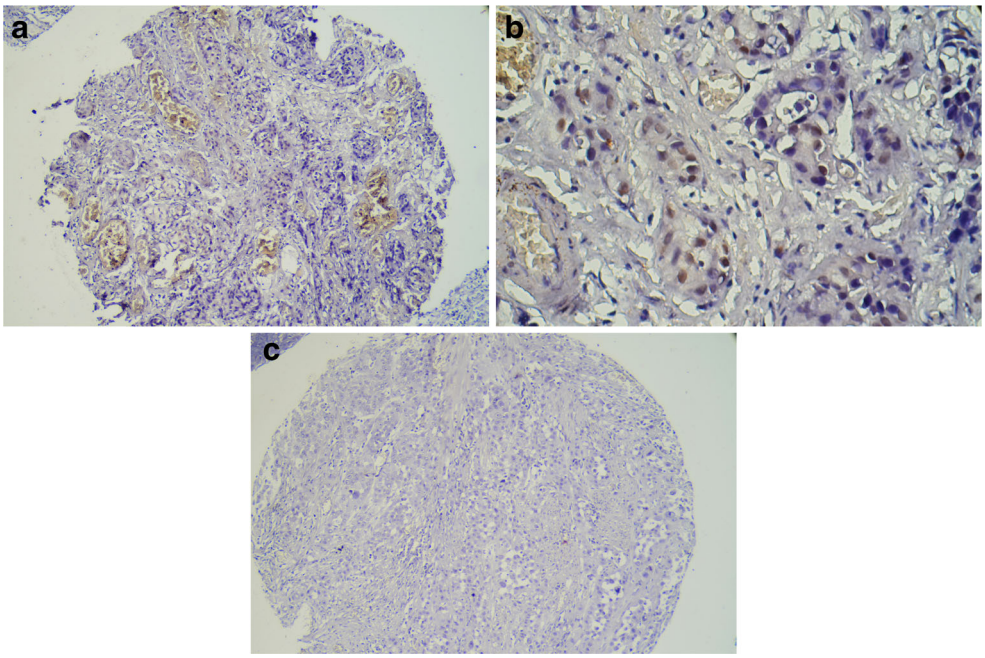

Fig. 4 CyclinD1 immunostaining: **a** A recurrent case of UBC featuring cyclinD1 nuclear staining, (X100). **b** Higher power view (of Fig. 4a) showing the brown nuclear staining in >10% of tumor cells, (X400). **c** A non recurrent case totally negative for cyclinD1, (X100)

Table 4 The association between sex of the patient, other clinicopatholgoical characteristics, p53 and Ki-67 with tumor recurrence among the 100 urothelial carcinoma patients

Predictors	Total N	Events		Disease free survival time		Log rank test	
			(%)	*M*	95% CI	X^2	(*p*-value)
Sex							
Male	92	23	(25)	40.0	(36.3, 43.6)	0.8	.820
Female	8	3	(38)	32.1	(18.0, 46.2)		
Clinicopatholgoical characteristics							
Urothelial carcinoma type							
Papillary	68	18	(26)	39.0	(34.7, 43.4)	0.0	.999
Non papillary	32	8	(25)	38.9	(33.2, 44.6)		
Tumor stage							
Non muscle invasive	46	12	(26)	39.1	(33.8, 44.4)	0.0	.925
Muscle invasive	54	14	(26)	38.2	(33.6, 42.7)		
Grade							
Low	44	12	(27)	38.6	(33.1, 44.1)	0.0	.831
High	56	14	(25)	38.7	(34.3, 43.1)		
Associated Bilharziasis							
Absent	92	22	(24)	40.5	(37.0, 44.0)	4.0	.046
Present	8	4	(50)	26.8	(12.0, 41.5)		
Squamous differentiation							
Absent	68	18	(26)	39.0	(34.6, 43.3)	0.0	.895
Present	32	8	(25)	39.2	(33.6, 44.9)		
Glandular differentiation							
Absent	90	22	(24)	39.7	(35.9, 43.4)	0.9	.342
Present	10	4	(40)	35.6	(25.6, 45.6)		
Tumor Markers							
P53							
< 10%	72	18	(25)	39.8	(35.7, 43.9)	0.4	.533
10 or more	28	8	(29)	37.5	(30.9, 45.0)		
Ki-67							
< 10%	52	12	(23)	39.0	(34.5, 43.4)	0.7	.420
10 or more	48	14	(29)	38.3	(33.0, 43.7)		

Table 5 Cox Regression model to determine the independent predictors of tumor recurrence among the 100 urothelial carcinoma cases

Predictor	*b*	*(p-value)*	*HR*	95.0% CI for *HR*	
				Lower	Upper
Stage	−.189	.802	.828	.190	3.601
Grade	−.650	.385	.522	.120	2.264
p63	−.025	.006	.975	.958	.993
Cyclin D1 index	.036	.006	1.036	1.010	1.063
Bilharziasis	.283	.655	1.328	.382	4.613
Interaction €	.000	.309	1.000	.999	1.000

€The interaction is calculated as by multiplying p63 and cyclinD1 after they were centered by their means to avoid potential multicollinearity among the interaction and the main markers

association was statistically significant. Every cyclinD1 score unit increase associated with 3% increase in the risk of recurrence, (HR = 1.03, 95%CI = 1.01, 1.04, p = .002). On the other hand, the cyclinD1 score showed no significant association with recurrence among cases whose p63 score was 35% or more (node 2), (HR = .996, 95%CI = .944, 1.05, p = .879).

Finally, we categorized our 100 studied urothelial carcinomas into three groups by the cut-off values recommended by the CART and examined for statistical significance using Cox Hazard regression. As shown in (Table 6), the recurrence distribution was statistically significant between Group I vs Group II and Group I vs Group III, but not between Group II and Group III (p = .057). Group I (n = 62) included patients with p63 ≥ 35% and with the least incidence of recurrence at the longest disease free survival. Group II (n = 23) and group III (n = 15) included cases with p63 < 35%, but with cyclinD1 score < 10% and ≥10% respectively. Group III cases had the worst prognosis (the estimated risk of recurrence among them was 11 times as that of Group I); followed by Group II (the estimated risk of recurrence was 5 times as that of Group I). (Fig. 6).

Discussion

At presentation, 70–80% of bladder tumors are non-muscle invasive papillary tumours pTa or pT1 [3]. The accurate prediction of high risk patients who will experience recurrence or progression is still very difficult [36], thereby mandating the need for new and reliable prognostic factors [37].

In a trial to identify urothelial carcinoma patients who are at high risk of tumour recurrence, and are thereby in need of closer surveillance and aggressive treatment, this study was undertaken. We aimed to examine the prognostic significance of expression of the proliferation marker ki-67 and the cell cycle regulators p53, p63 and cyclinD1 in urothelial bladder carcinoma. We used the classification and regression trees (CART) - a non-parametric regression approach- to identify the optimal cut-off points for the studied immunohistochemical markers, and to find interactions among multiple

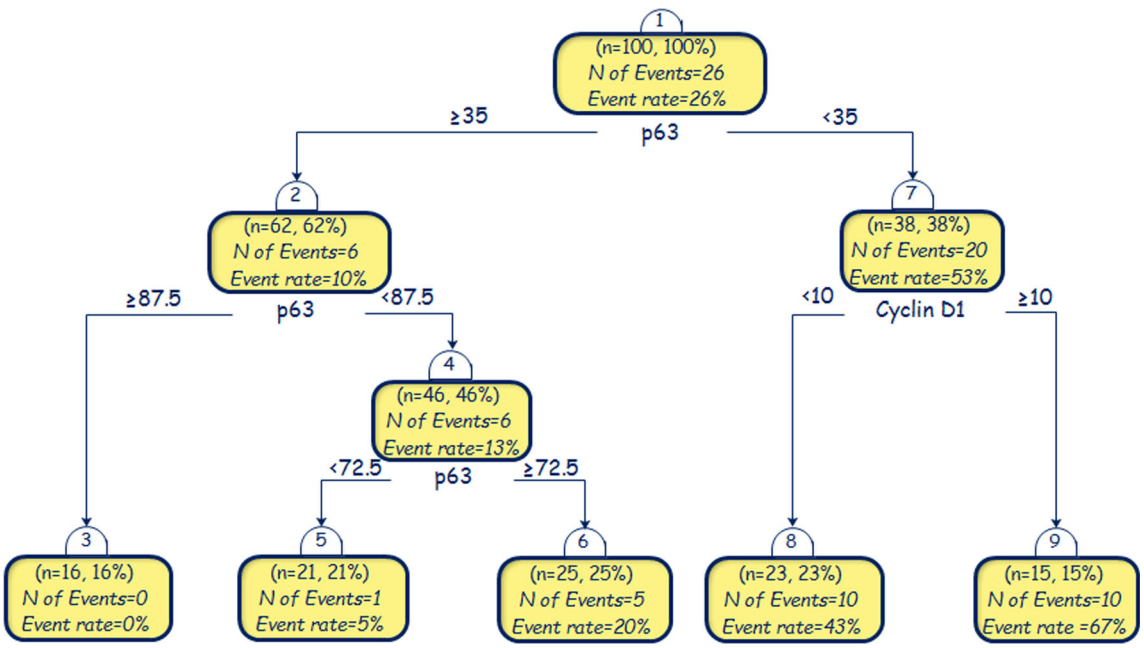

Fig. 5 The dendogram produced by the Olshen's survival CART to predict recurrence among the 100 studied bladder carcinoma patients. Node 1 is the root node, containing the whole study sample. As shown 26 patients experienced the event of tumour recurrence. It is split into two child nodes according to the p63 level, nodes 2 and 6, which are further split according to the p63 and cyclinD1 levels respectively. The terminal nodes are nodes 3, 5, 6, 8, and 9. The incidence of recurrence was zero in node 3, and got higher as we moved from node 5, to node 6, node 8 and became the highest at node 9 (67%)

Table 6 The recurrence distribution among the 100 studied urothelial carcinomas grouped by the best cut-offs of p63 and cyclinD1 according to the CART

Patient Group	Group I	Group II	Group III
Recurrence			
n/total N (%)	6/62 (10)	10/23 (43)	10/15(67)
Disease Free survival (months)			
M (95%CI)	46 (43,49)	31 (24,39)	22 (12,32)
Pairwise Comparisons	*Log rank X^2(p-value)*	*Log rank X^2(p-value)*	
Group II	14 (<.001)		
Group III	28 (<.001)	3.6 (<.057)	
Hazard ratio	*HR (95% CI)*	*HR (95% CI)*	
Group II	5 (2,15)		
Group III	11 (4,31)	2 (1,5)	

Group I are urothelial carcinoma cases whose p63 was ≥35%

Group II are urothelial carcinoma cases whose p63 was <35% and cyclinD1 score < 10%

Group III areurothelialcarcinoma cases whose p63 was <35% and cyclinD1 score ≥ 10%

predictors to help in the prediction of tumour recurrence. We presented how the results and conclusions would have been different if we had only used the conventional statistical tests (log rank test, univariate and multivariate Cox regression). We also aimed to evaluate the association between the expression of those biomarkers and the clinicopathological features of urothelial bladder carcinoma.

In agreement with others, [38, 39] we confirmed that among bladder carcinoma patients the overexpression of p53, which is the most commonly inactivated tumour suppressor gene [40], is associated with higher tumour stage and grade as well as non-papillary tumor morphology. Furthermore, similar to others [37, 39] we demonstrated that p53 overexpression is correlated with the mitotic index. Moreover, the high agreement between ki-67 and p53 among our cases, suggested that one of them could substitute the other, so we can use the more convenient or the less expensive one.

In the current study, in agreement with others [3] the expression of both p63 and cyclinD1–in opposition to ki-67 and p53- did not significantly associate with the tumour histologic type, tumour grade or pathologic stage; i.e. no significant differences in p63 and cyclinD1 expression were noted in papillary vs non-papillary, non-muscle invasive vs muscle invasive, and low vs high grade urothelial carcinomas. Both p63 and cyclinD1 did not significantly associate with the other two

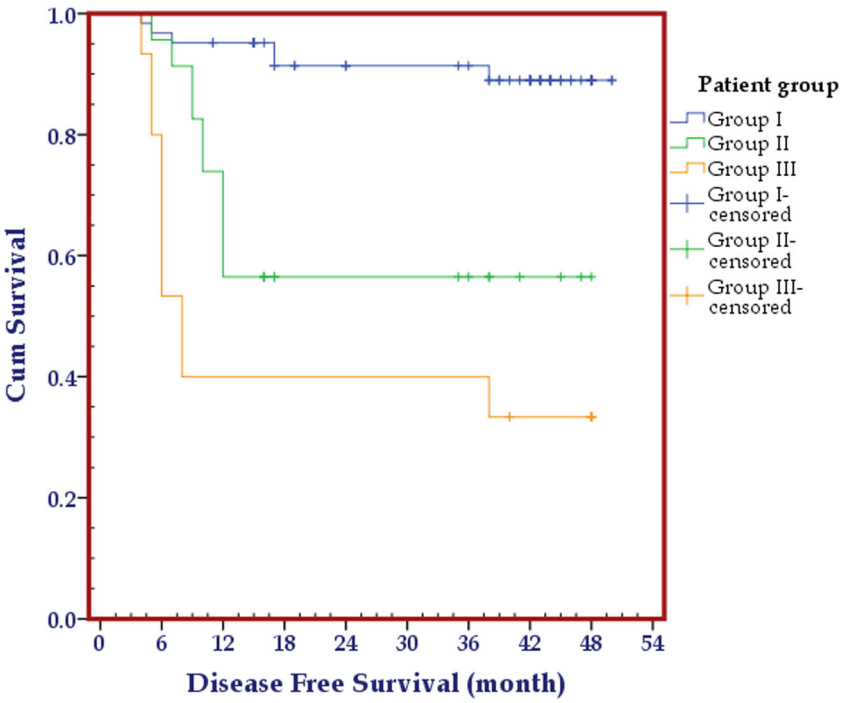

Fig. 6 Kaplan-Mier plot showing the recurrence distribution among the urothelial carcinoma patient groups. Group I included patients whose p63 ≥ 35%, group II patients whose p63 < 35% and cyclinD1 < 10% and group III patients whose p63 < 35% and cyclinD1 ≥ 10%. Among the first two groups, most of the recurrence incidents occurred within the first year and half (18 months). In the third group, all the incidents occurred within 8 months. Note that while the number of patients in the first group remained above 30 till the 42nd month, the number of patients in the other two groups was less than 30 patients at the beginning of the period of follow-up and decreased afterwards

markers, namely ki-67 and p53. Moreover, the correlation between p63 and cyclinD1 was not statistically significant ($r = -.0$, $p = .646$). These findings are in agreement with previous studies [3, 41, 42] that reported that cyclinD1 overexpression is not related with the Ki67 labeling index. This observation suggested that cyclinD1 overexpression is not sufficient per se to induce an increased proliferative activity of tumour cells in vivo compared to cyclinD1-negative tumours.

Among our cases, both the proliferation index and p53 overexpression did not show any significant association with tumour recurrence. These results oppose previous reports [43–45] that showed that the proliferation marker Ki-67 associated with a worse course of urothelial carcinomas, and that p53 expression was able to identify patients with high risk for recurrence in pTa or pT1 urothelial tumours [46]. This could be explained by differences in the characteristics of the patients, cut-off values, and follow-up time between the different studies; facts that were previously highlighted through two meta-analysis studies that summarized the results of the published data on p53 and urothelial bladder carcinomas [37, 47]. Those meta-analyses concluded that based on the available information, p53 overexpression in NMIBC patients treated with BCG may be associated with poor relapse free survival (RFS), especially in Asian populations, and recommended large cohort studies to be performed in the future with uniform criteria for high p53 expression.

On the other hand, p63- a member of the p53 family, that has been shown to play a role in urothelial differentiation [48] - associated significantly with recurrence among our studied cohort of UBCs. The incidence of recurrence got higher and the disease free survival time got shorter as the p63 score decreased. No recurrence was observed among patients with p63 scores of 87.5% or more. A possible explanation is that p63 acts by a pathway distinct from p53 [11, 49]. CART, in this study, determined 35% as the optimal cut-off value for p63. After classifying the patients into two groups according to their p63 (at cut off 35%), the change of the level of p63 did not significantly associate with recurrence any more.

The prognostic impact for cyclinD1 with regard to recurrence- and progression-free survival has primarily been evaluated in non-muscle invasive bladder cancer. However, data were inconsistent showing cyclinD1 expression as a marker of adverse [50–52] or favorable [22, 53–57] prognosis or without prognostic impact [58]. This inconsistency could be explained by previous reports of a dual role of cyclinD1 in the regulation of cell growth; whereby a moderate increase in the expression of cyclinD1 was suggested to enhance cell growth, whereas a high level of expression can have an inhibitory effect [3]. In addition, our findings further explain this debate as we found that the association between cyclinD1 expression and urothelial carcinoma recurrence differed according to patients' p63 level. The cyclinD1 score exhibited a statistically significant association with tumour recurrence only among patients with p63 score below 35%, (i.e. cyclinD1 expression showed no significant association with recurrence among cases whose p63 score was 35% or more), whereby patients with cyclinD1 ≥ 10% had a higher incidence of recurrence. This is in accordance with a recent report by Fristrup and colleagues [54] that showed that high nuclear cyclinD1 expression is associated with poor patient outcome in superficial urothelial bladder carcinomas.

An interesting finding among our results was that, although p63 and cyclinD1 showed significant association with tumour recurrence, there was a lack of a linear correlation between them. Accordingly, every one of them could explain a unique side of the recurrence, and, thus, the prediction of recurrence among UBCs would improve when they are used together. This was also supported by our findings; as we showed that patients with p63 of less than 35% had poor prognosis; and among them, those with higher cyclinD1 (10% or more) had worse prognosis.

While testing the prognostic significance of p63 and cylcinD1, we included the stage and the grade of the tumour in the multivariate analyses (in both methodologies; i.e., the conventional and the CART- guided). The grade and stage of the tumour did not show statistical significance in the multivariate Cox regression and were not selected by the CART as important classifiers. Thus, we can conclude that the predictive performance of p63 and cyclinD1, reached in the current study, can be generalized to all urothelial bladder carcinoma irrespective of the tumour grade or stage.

In this paper we described how Olshen survival, a variant of CART specialized to deal with survival data, can be used as an alternative method for identifying interactions among multiple predictors. Our work, to the best of our knowledge, is the first to utilize the "Olshen survival trees" to generate hypotheses about the interaction effect and to find optimal cut-off points for the studied immunohistochemical markers in a way serving tumour recurrence prediction.

We presented the results of Cox-proportional hazard regression analysis to show how our conclusions would have been limited if we were committed to the conventional approach in statistical analysis. In the conventional analysis, the interaction effect was not significantly contributing to the model. This may be explained by the structural multicollinearity between the interaction effect— product of multiplication of p63 by cyclinD1— and the corresponding main effects (p63 and cyclinD1). We tried to reduce multicollinearity by centering the markers (subtracting the mean from every value) before modeling, but the interaction terms deemed to be insignificant.

On the other hand, the CART approach offers some potential advantages over the conventional approach. CART makes no assumption of a monotonic or parametric relationship with the outcome and can handle datasets where the number of

predictors is high relative to the number of observations. It was able to identify complex interactions among the predictor variables without a prior specification of the interaction terms.

One of the drawbacks of the CART algorithms is the inability to determine the estimates of hazard ratio [59]. Thus, we could not estimate the hazards associated with change of p63 and cyclinD1 using CART alone. Moreover, the algorithm generated by the CART may be just specific to the current study sample. We dealt with those two limitations by testing the CART algorithm using parametric statistical tests to estimate the hazard ratio and to guarantee the generalizability of the algorithm from the study sample to the whole UBC population.

A limitation in the current study was the small number of patients in the second and third groups. Moreover, the study sample was not sufficient to validate the algorithm of prediction as the number of cases with the event was too small.

Conclusion

According to the results of the current study, p63 represents a surrogate biomarker to predict recurrence among urothelial bladder carcinoma patients, whereas, the use of cyclinD1 can be limited to patients whose p63 score is less than 35%. The application of data mining techniques, such as CART, to deal with data among which the number of cases with positive outcomes is too small relative to the number of studied predictors can help revealing important findings. Future large cohort studies investigating the prognostic significance of ki-67 and p53 overexpression in UBC are recommended to be performed with standardized criteria as regards characteristics of the patients, cut-off values, and follow-up time.

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Authors' Contribution S.E.: Design of the study, collection of samples, immunohistochemical analysis, and elaboration of the manuscript.

GA.: Design of the study, statistical analysis, and elaboration of the manuscript.

Both authors critically reviewed the manuscript, and approved the final version of the manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare no conflict of interest.

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