ELSEVIER

Urologic Oncology: Seminars and Original Investigations 37 (2019) 158–165

UROLOGIC ONCOLOGY

Original Article

Immunohistochemical expression of Ki-67, Cyclin D1, p16INK4a, and Survivin as a predictive tool for recurrence and progression-free survival in papillary urothelial bladder cancer pTa / pT1 G2 (WHO 1973)

José Antonio March-Villalba, Ph.D., M.D.[a,*], David Ramos-Soler, Ph.D., M.D.[b], Pilar Soriano-Sarrió, Ph.D., M.D.[b], David Hervás-Marín[c], Laura Martínez-García[b], José María Martínez-Jabaloyas, Ph.D., M.D.[d]

[a] *Department of Urology, University Clinic Hospital of Valencia, Universitat de València, Valencia, Spain*
[b] *Department of Pathology, Universitat de València, Valencia, Spain*
[c] *Biostatistic Department, IIS La Fe, Valencia, Spain*
[d] *Department of Urology, University Clinic Hospital of Valencia. Surgery Department, Universitat de València, Valencia, Spain*

Received 22 March 2018; received in revised form 11 August 2018; accepted 1 October 2018

Abstract

Objectives: To investigate the expression of several immunohistochemical (IHC) markers and their predictive ability for the recurrence-free and progression-free survival of papillary urothelial bladder cancer (UBC) pTa/pT1 G2 (WHO 1973) compared to classical anatomo-clinical variables using a multidimensional analysis.

Materials and Methods: A population-based cohort of 213 primary stage UBC (pTa/pT1) G2 (WHO 1973) was evaluated by classic anatomopathological variables and characterized by immunohistochemistry (23 IHC markers, representative of different oncogenic pathways). The most important variables as a predictor of recurrence-free and progression-free survival were selected using multidimensional statistical models, such as random survival forests and least absolute shrinkage and selection operator (. Recurrence and progression-free survival of the previously selected variables were also calculated.

Results: Mean follow-up was 58 ± 33.5 months. Recurrence and progression rates were 54.5% ($n = 116$) and 17,4% ($n = 37$), respectively. The most influential variables in the low recurrence-free survival were in order: number of resected tumors, high expression of Ki67 (> 10%), Cyclin D1 (> 10%), and low cytoplasmic staining of p16INK4a. Regarding low progression-free survival, the most important variables were Ki67 (> 15%), multicentric tumor arrangement and Survivin nuclear expression (> 20%). Kaplan-Meier and cox-regression model analyses showed that the variables selected by multidimensional models were able to discriminate the clinical outcome.

Conclusions: Ki67 index is the most useful IHC marker, since it can improve the prediction of both recurrence and progression-free survival in papillary UBC pTa/pT1 G2 (WHO 1973). There are other markers, whose utility is specific to recurrence-free survival, such as Cyclin D1 and p16INK4a or in progression-free survival, such as Survivin.

Keywords: Bladder cancer; Immunohistochemistry; Clinical outcome; Stage Ta/T1; Multidimensional analysis

1. Introduction

The incidence of urothelial bladder cancer (UBC) has increased during the last decades; in western countries, it is the fourth most common tumor type, after prostate, lung, and colorectal cancer [1]. UBC has a worldwide incidence of 294,345, and 88,315 cases per year in men and women (European prevalence of 112,819, and 30,736 cases, respectively).

Papillary UBC pTa/pT1 is the most frequent diagnosis since it occurs in 75% to 85% of patients with primary bladder tumor [1–3].

*Corresponding author. Tel.: +34648131545; fax: +34963447070
E-mail addresses: joseantoniomarch@gmail.com, joseantoniomarch@hotmail.com (J.A. March-Villalba).

https://doi.org/10.1016/j.urolonc.2018.10.005
1078-1439/

An important aspect of these tumors is the heterogeneity in their clinical evolution. Five-year series report a recurrence rate ranging from 30% to 80% and also a considerable percentage of progression to muscle-invasive disease of 1% to 45% [3]. This correlates with the diagnostic uncertainty that can be observed throughout the studies published, especially in grade 2 tumors (WHO 1973), this latter subgroup containing the highest percentage of diagnosed tumors. Although, the 2004 WHO classification have been reassigned the grade 2 tumors group, it has not proven itself to be much better than the 1973 WHO classification so far [1,4–10].

Immunohistochemical (IHC) markers are defined as characteristic and representative molecules of various tumor properties frequently involved in several biological pathways [11,12].

There have been a large number of IHC markers studied so far, but none of them has been used systematically in clinical practice. Moreover, previous studies were based on heterogeneous samples including tumors of low and high potential for malignancy. Heterogeneity in the different methodologies was also encountered. Therefore, these studies could be considered to be partially inconsistent [13,14].

Currently, there are statistical tools to analyze the influence of a large number of variables on a biological event and even determine the importance of each one in it. Multidimensional analysis (MD) like random survival forest (RSF) and the least absolute shrinkage and selection operator (LASSO) (logistic regression of penalized coefficients) models were suitable [15,16].

The main objective of the present study was the analysis of the clinical usefulness with respect to the recurrence-free and progression-free survival of a varied group of IHC markers representative of the different oncogenic pathways, as well as of anathomoclinical variables currently used in the clinical management of the UBC pTa/pT1 papillary grade 2 (G2) (WHO 1973) using a MD. As far as we know, some of them have not been previously studied.

2. Materials and methods

2.1. Cohort description

A population-based cohort ($n = 213$) of stage (pTa/pT1) primary UBC G2 (WHO 1973) diagnosed between January 1999 and December 2012 was randomly selected from the database of Pathology Department from the Clinic Hospital of Valencia, Spain. The study adhered to the ethical principles for medical research in human beings set forth in the Helsinki Declaration (and its subsequent updates) of the World Medical Association and was approved by the Regional Ethics Committee (Ref. No. F-CE-GEva v1.1 2015).

2.2. Pathologic evaluation of tumor sections

Sections from primary transurethral resection of the bladder tumor and resection base after transurethral resection of the bladder samples were re-evaluated by 2 uropathologists to confirm WHO 1973 G2 and reassing the samples to WHO grade 2004 according to diagnostic criteria, lacking concomitant urothelial carcinoma in situ [4]. Tumor stages were also revised to confirm Ta/T1 avoiding T2 samples.

Anatomopathological variables (tumor diameter, number of samples, Ta/T1 tumor stage, 2004 WHO grading system low-grade/high-grade) were collected, with respect to the time from surgery. Multicentricity (different than tumor number) is defined such as more than 2 tumors located in several bladder areas.

2.3. Tissue microarray construction and IHC evaluation

Twenty-three molecular markers, representative of different oncogenic pathways, were finally selected. The clinical utility of all these markers was previously revised along the literature. These markers were categorized as follows: cell cycle regulators/promoters of cell proliferation (P53, P14, topoisomerase-IIα, Ki-67, p16INK4a, P27, P21, PRb, Cyclin G, Cyclin E, and Cyclin D1); modulators of apoptosis (Bcl2, Survivin, nuclear and nucleolar human Telomerase [hTERT], CtIP, c-kit [CD117], and Wwox); angiogenic regulation (VEGFR2); extracellular matrix modulation (cytokeratin 20 [CK-20]); and signal transduction and cellular metabolism factors (GAPDH, COX-2, and racemase).

Tissue microarrays were constructed by means of 3 cylinders of 1mm-diameter for each tumor sample and 2 cylinders from the control samples. All donated cylinders were extracted from the most representative areas of the tumor within the paraffin block, according to anatomophatologic criteria. Immunostaining, fixation, and processing were then performed according to a previously published protocol [17]. The information about the respective antibodies used is given in the Supplementary Figure.

Diagnostic evaluation of tissue microarrays was performed by 2 expert uropathologists using the 2004 WHO guideline [4]. The pathologists did not previously know the clinical variables from each patient.

Two scoring criteria were assessed: first, a quantitative scale of the percentage of stained nuclei (0%–100%) for nuclear expression markers; and second, in cytoplasmic expression markers a qualitative scale referred to cytoplasmic staining intensity (ranging from 0–6) [15].

Topographical localization of the immunostaining (absent-diffuse-basal-apical) was also evaluated for Bcl-2 and CK 20 [11].

2.4. Clinical follow-up

Clinical data (age, sex, occupational risk of bladder cancer, smoking, alcohol consumption, hypertension, and diabetes mellitus) were collected, with respect to the time from surgery. All patients were, subsequently, treated with the same intravesical chemotherapy regimen (Mitomycin C). None of the patients received BGC treatment.

The new presence (histopathological confirmation after transurethral resection) of a UBC was defined as a tumor recurrence up to 3 months from the resection of the primary tumor. The presence of a real tumor recurrence in the bladder whose pathological analysis revealed muscle infiltration (T2) was considered a progression of the disease, as well as the presence of lymphadenopathy or metastasis in distant organs with biopsy and further histological confirmation. Another criterion for tumor progression was the existence of a superior urothelium carcinoma of at least high-grade T1 carcinoma.

2.5. Statistical analysis

To analyze a large number of variables regarding not a large cohort, selection of predictive variables by MD used the RSF and the LASSO (logistic regression of penalized coefficients) models were performed [16,17]. The variable must be chosen by both analytical methods. For RSF analysis, variable must have been obtained a coefficient of importance greater than 0.005. Cut off point to define high expression of selected markers were defined according RSF analysis.

The statistical program R was used for the calculation of RSF and LASSO [version 3.0.2.R Core Team (2013)], R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria, URL http://www.R-project.org/).

Kaplan-Meier curves survival analysis and multivariate cox regresion model were used with respect to recurrence-free and progression-free time, stratifying the sample according to the selected IHC markers by MD previously performed and well-know pronosticators variables like: stage, grade (OMS 2004), tumor number, diameter and gender [1−3]. Log Rank (Mantel-Cox) was the statistical tool used. C-Index was calculated to evaluate the clinical impact of the model [18]. The statistical significance considered was 0.05. The software used for analysis was IBM SPSS Statistics v. 20.0 (IBM, Armonk, New York).

3. Results

3.1. General description and clinical follow-up

The mean age was 69.5 (10.4) years, male/female ratio 3:1. Clinical and pathologic characteristics in the entire follow-up cohort are shown in Table 1.

The mean follow-up was 58 ± 33.5 months. Recurrence rate was 54.5% ($n = 116$). The median recurrence-free survival was 37.8 (25.6−50) months. The recurrence-free

Table 1
Clinical and pathologic characteristics in the entire follow-up cohort

Clinical variables	(n) %	Anatomopathological variables	(n) %
Age (69.5 ± 10.52[a]/71.4 (63.1−77.5)[b])		Tumor number (2.4 ± 2.04[a]/1 (1−3)[b])	
< 70 years old	98 (46%)	Solitary tumor	111 (52.1%)
≥ 70 years old	115 (54%)	2 or more tumors	102 (47.9%)
Sex		Tumor diameter (2.6 ± 2.01[a]/2 (1−3.2)[b])	
Male	191 (89.7%)	< 20 mm	118 (55.4%)
Female	22 (10.3%)	≥ 20 mm	95 (44.6%)
Smoker		Stage	
Yes	109 (51.2%)	pTa	83 (39%)
No	50 (23.5%)	pT1	130 (61%)
Former smoker	54 (25.4%)		
Alcohol		WHO 2004 grade	
Non-consumer/occasional	191 (89.7%)	Low	62 (29.1%)
Moderate/high	22 (10.3%)	High	151 (70.9%)
Occupation risk (bladder cancer)		Multicentricity	
Yes	61 (28.6%)	Yes	78 (36.6%)
No	152 (71.4%)	No	135 (63.4%)
Diabetes mellitus			
Yes	42 (19.7%)		
No	171 (80.3%)		
Hypertension			
Yes	88 (41.3%)		
No	125 (58.7%)		

[a] mean (standard deviation).
[b] median and interquartile range.

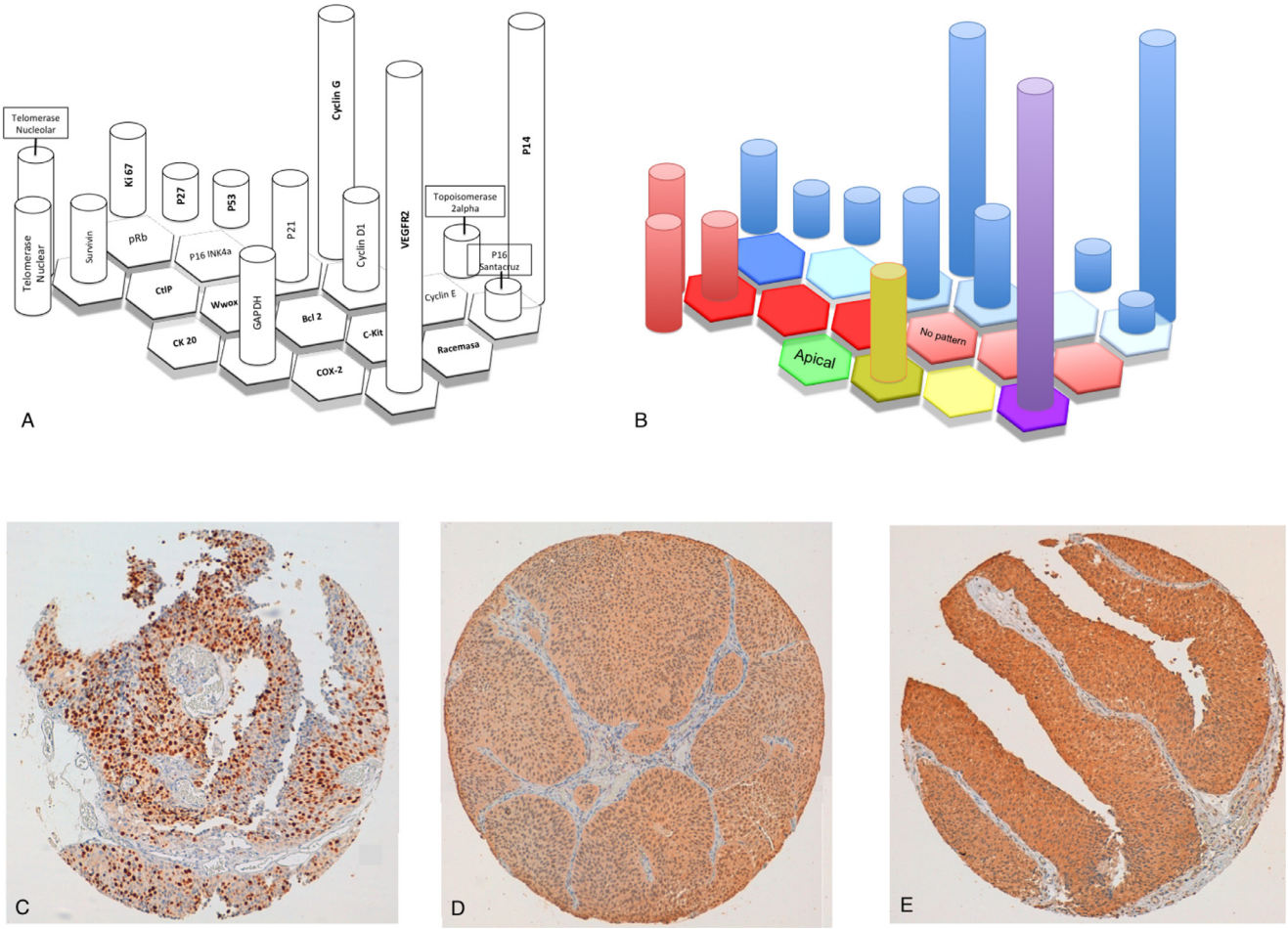

Fig. 1. IHC expression of urothelial pTa/pT1 G2 (WHO 1973) markers. Three-dimensional map of the IHC expression pattern. (A) Scheme of the distribution of markers. (B) Final result. The markers are colored according to their family/oncogenic route: cell cycle regulators/cell proliferation promoters (blue), apoptosis modulators (red), extracellular matrix modulator molecules (green), angiogenesis regulators (purple) and signal transduction factors and cellular metabolism (yellow). The hexagons correspond to the expression of the cytoplasmic markers. The columns correspond to the nuclear markers. The most frequent topographic distribution is named above the corresponding marker (Bcl-2 and CK 20). There is a predominance in the expression of the markers: cyclin G, P14, and pRb (cell cycle), survivin, CtIP, Wwox (apoptosis), VEGFR2 (angiogenesis) and GAPDH (metabolism and signal transduction). Representative IHC staining expression in cores of Ki-67 (C), survivin (D) nuclear and cytoplasmatic expression, and p16INK4a (E) cytoplasmic expression. Areas shown are 0.6 mm × 0.6 mm.

survival rate for 1 year was 78% and for 5 years, 40%. The mean and median number of recurrences was 1.2 (1.5) and 1 (0–2) times, respectively.

Regarding progression, the rate obtained at the end of the study was 17.4% ($n = 37$). A progression-free survival rate was observed for 1 year and for 5 years for 98% and 85%, respectively.

3.2. IHC expression

In general, 4 activated oncogenic routes have been observed in urothelial pTa/pT1 G2 (WHO 1973) bladder carcinoma as follows: the family of cell cycle regulators (P14, Ki-67, Cyclin G and PRb); apoptosis regulators (Survivin, Wwox and CtIP); GAPDH within signal modulators and metabolism; and VEGFR2 within angiogenesis markers (Fig. 1).

3.3. Predictive variables of clinical behavior

In addition to recurrence-free survival, the LASSO and RSF models selected cases with nuclear expression markers (Ki-67, Cyclin D1), and p16INK4a cytoplasmic expression as the most important variables. The graphs showed an increase in the probability of presenting a short recurrence time as the number of resected tumors increased and the percentage for nuclear expression of Ki-67 and Cyclin D1 increased. Less recurrence-free survival, however, is associated with a low cytoplasmic expression of p16INK4a (Fig. 2).

Regarding progression-free survival, both models agreed on the selection of the strongest variables, multicentric arrangement, Ki-67 index and Survivin nuclear immunoexpression. There was a strong association between increased Ki-67 labelling index and Survivin and the likelihood of a shorter period of progression (Fig. 3).

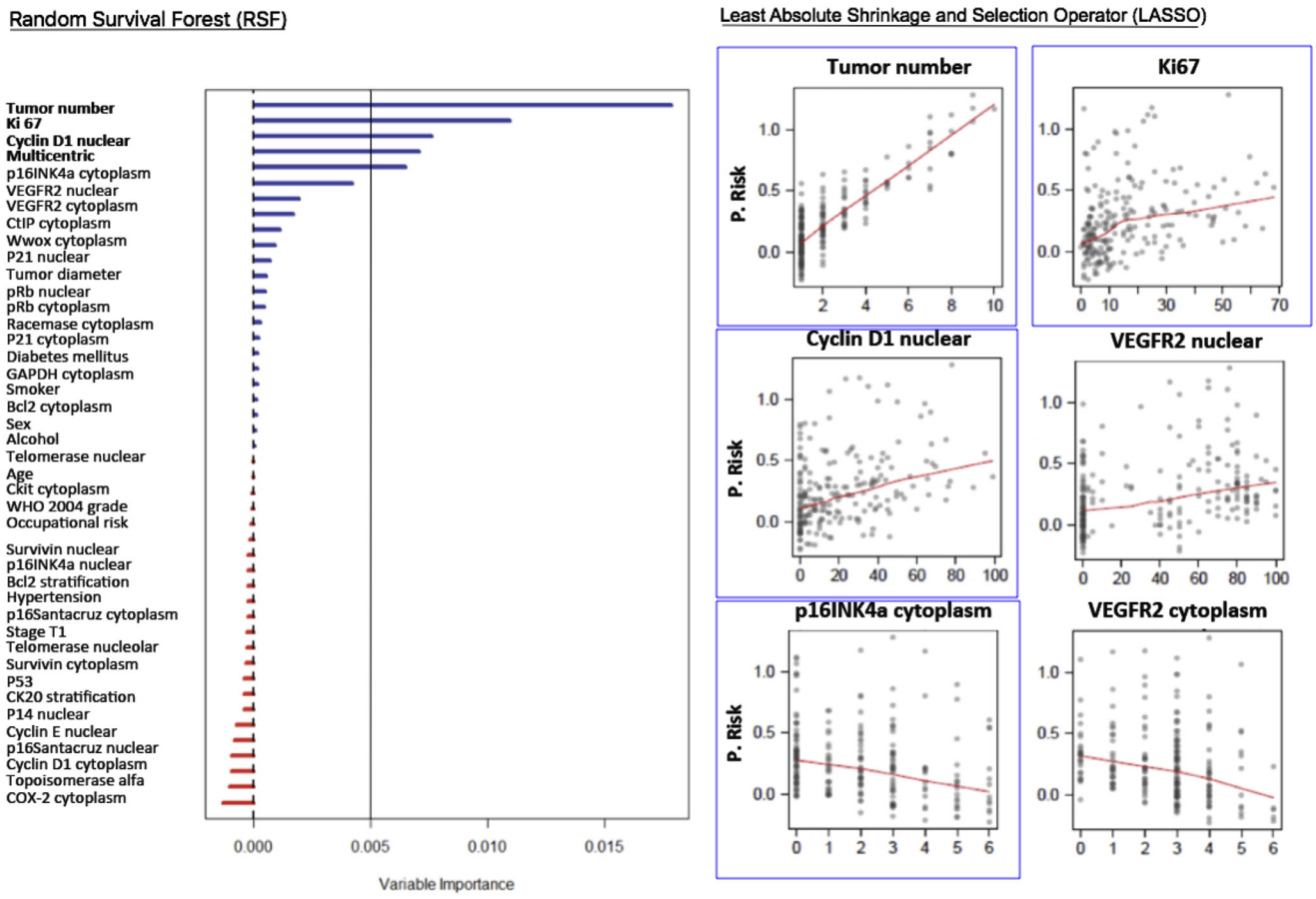

Fig. 2. Classification chart of the variables according to the predictive capacity of a short recurrence-free time after the RSF analysis and graphs of the predictive variables selected by LASSO and RSF or of the trend of the event. On the abscissas for Ki-67 and cyclin D1 nuclear, the percentage of expressed nuclei is represented. On the abscissas for p16INK4a cytoplasm, cytoplasmic expression intensity (0–6) is plotted. On the ordinate of all graphs, both the probability percentage of the event (LASSO graph), as well as the coefficients generated by RSF are shown. LASSO = Least Absolute Shrinkage and Selection Operator regression; P Risk= Risk Probability; RSF= random survival forest.

3.3.1. Recurrence

According to the variables selected by the MD, RSF determined a poor prognosis profile that was defined by the presence of up to 2 resected tumors, a nuclear expression of Ki-67 ($>10\%$), Cyclin D1 ($>10\%$) or/and cytoplasmic staining greater than or equal to 2 for the marker P16INK4a (Fig. 4).

Regarding multivariate analysis, cox regression showed that Ki-67, Cyclin D1, and P16INK4a were included in the recurrence-free model with tumor stage, number of tumors, and grade (OMS 2004). The c-index calculated of the model was 0.74 (0.67-0.80) ($P < 0.001$) (Table 2).

3.3.2. Progression

The MD-RSF high-risk profile was characterized by a multicentric arrangement of tumors, a Ki-67 expression $>15\%$ and/or a nuclear expression of Survivin $>20\%$ (Fig. 4).

Cox regression showed that Ki-67 and Survivin were included in the model with tumor stage and tumor diameter. The c-index calculated of the progression model was 0.80 (0.72–0.87) ($P < 0.001$) (Table 2).

Kaplan-Meier analyses showed that the IHC markers selected by multidimensional models were able to discriminate recurrence/progresion clinical outcome (Fig. 4).

4. Discussion

The major finding of the current population-based study is that IHC markers such as Ki-67, Cyclin D1, P16IK4a, and Survivin in papillary UBC pTa/pT1 G2 (WHO 1973) may contribute to improve the prediction of clinical behavior with respect to recurrence and tumor progression-free survival that classical variables such as grade (OMS 2004), stage, tumor number, or multicentricity give.

Statistical tools for the MD like RSF and LASSO, have shown that they can be used in the selection of variables, such as IHC markers, pondering their participation in the behavior of this specific type of bladder tumor.

With respect to the IHC markers selected, there was a prognostic utility of Cyclin D1 nuclear expression and

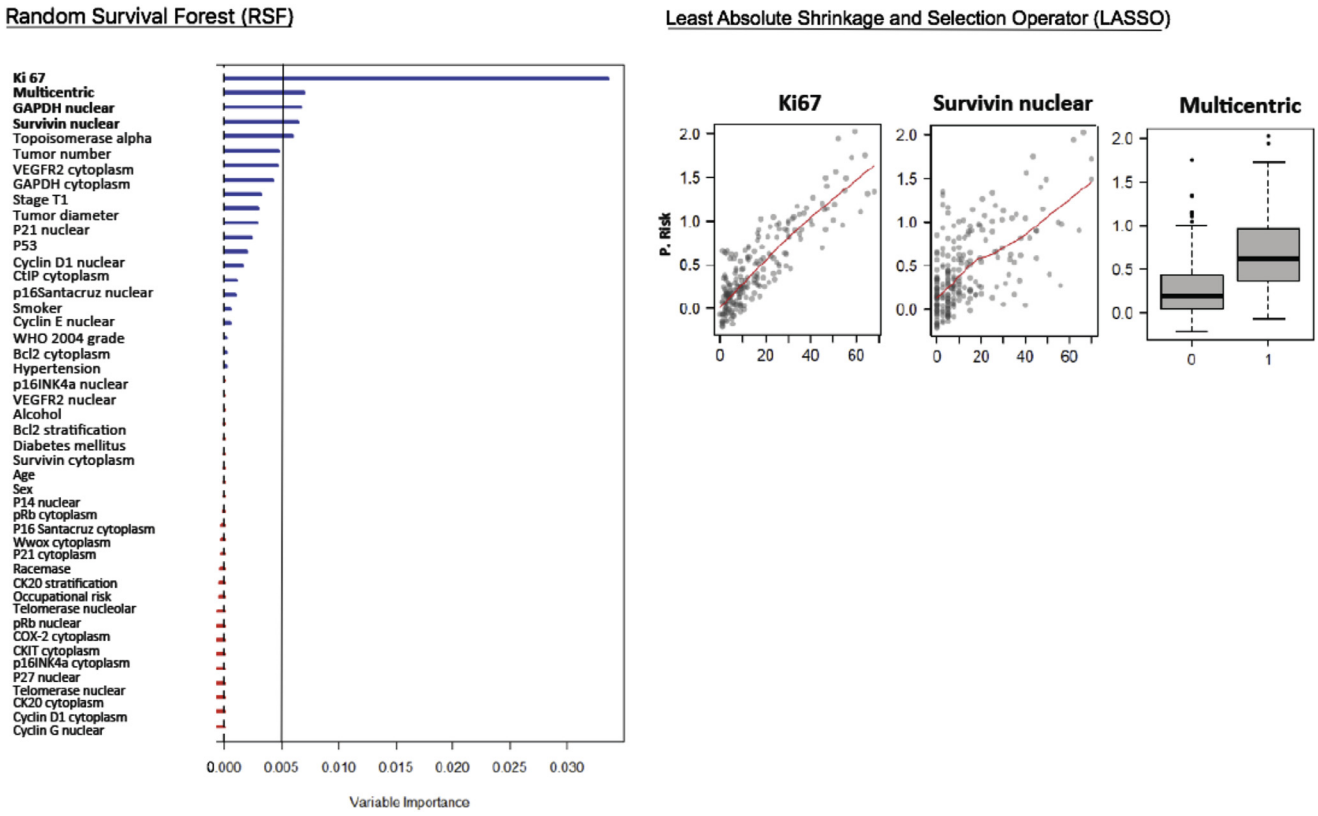

Fig. 3. Classification chart of the variables according to the predictive capacity of a short progression-free period after the RSF analysis and charts of the predictive variables selected by LASSO and RSF or of the trend of the event. On the abscissas of the Ki-67 and Survivin graphs, the percentage of expressed nuclei is represented. The multicentric variable is shown in the box diagram. On the ordinates of all graphs, the probability percentage of the event (LASSO chart) are plotted. RSF = random survival forest; LASSO = Least Absolute Shrinkage and Selection Operator regression.

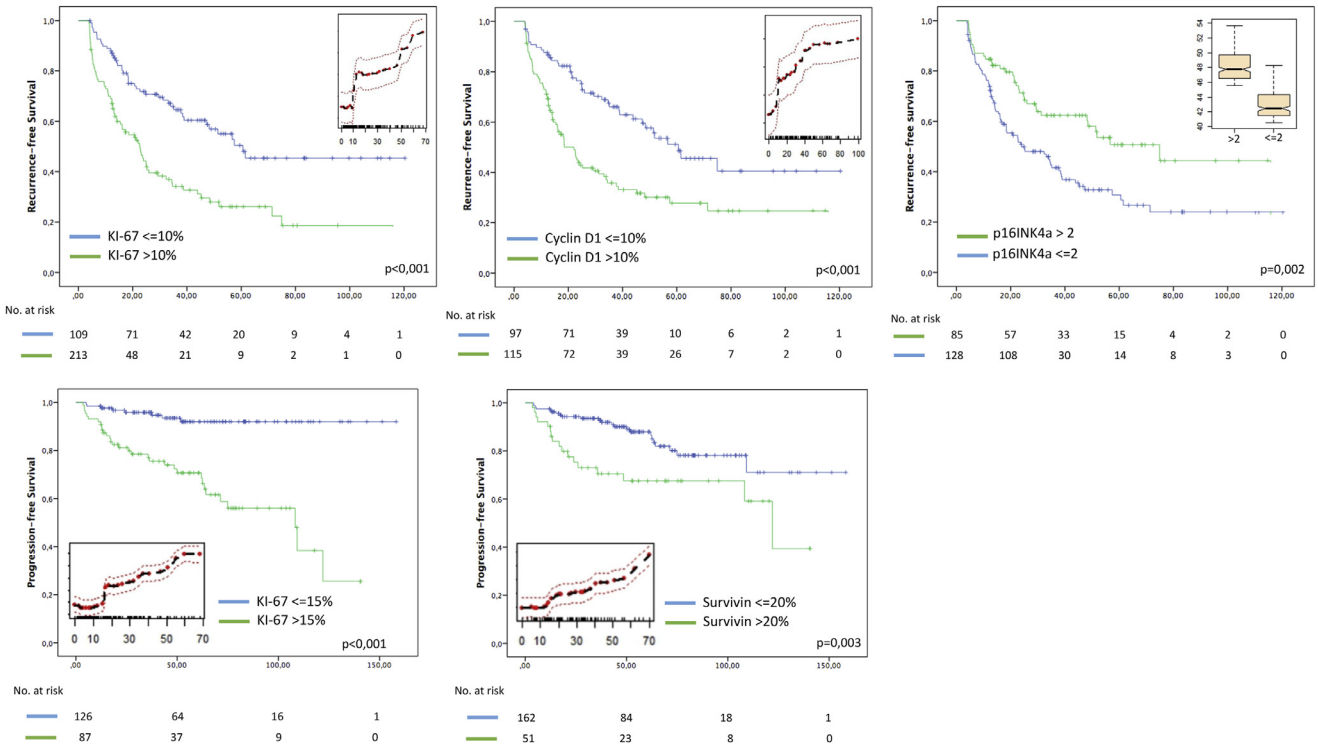

Fig. 4. Kaplan-Meier curves depicting recurrence-free and progression-free survival in urothelial pTa/pT1 G2 (WHO 1973) tumors according to cut off point of selected IHC markers performed by RSF (Graphs inside Kaplan-Meier plots). Statisticians used Log Rank (Mantel-Cox).

Table 2
Cox-regression analysis including well-known prognosticators and the IHC markers selected by RSF and LASSO

Characteristics	Disease recurrence			Disease progression		
	HR	95% CI	*P value*	HR	95% CI	*P value*
Stage T1	1.65	(1.09−2.49)	0.017	2.42	(1.04−5.62)	0.039
High grade OMS 2004	1.58	(1.10−2.46)	0.043	0.61	(0.25−1.51)	0.285
Tumor number	1.22	(1.08−1.39)	0.002	1.05	(0.87−1.28)	0.604
Tumor diameter	0.98	(0.89−1.09)	0.807	1.17	(1.03−1.34)	0.016
Multicentric	0.92	(0.52−1.60)	0.759	0.55	(0.22−1.35)	0.189
Age	0.99	(0.97−1.01)	0.991	1.02	(0.99−1.06)	0.209
Gender (male)	0.82	(0.15−1.51)	0.528	0.74	(0.24−2.26)	0.599
Smoker	1.03	(0.58−1.84)	0.912	0.48	(0.15−1.50)	0.207
p16INK4a (> 3)	0.837	(0.745−0.941)	0.003		————————	
Cyclin D1	1.011	(1.003−1.019)	0.006		————————	
Ki-67	1.016	(1,004−1.029)	0.010	1.033	(1.013−1.053)	0.001
Survivin		————————		1.031	(1.014−1.048)	0.001

The c-index calculated of the regression and progression model were 0.74 (0.67−0.80) ($P < 0.001$) and 0.80 (0.72−0.87) ($P < 0.001$), respectively.

p16INK4a citoplasmatic expression in terms of recurrence-free survival [19,20]. Our results confirm this association specifically in the UBC pTa/pT1 G2 (WHO 1973).

In reference Survivin immunoexpression, some studies indicated a significant association of its nuclear expression with a worse stage, grade, and prognosis of Ta/T1 [21,22]. A meta-analysis by Jeon et al. confirmed that Survivin overexpression predicted cancer-specific relapse, progression, and survival in bladder tumors. In that study, the authors drew attention to the diversity of published cutoff points to define Survivin overexpression [22]. There have been no studies published evaluating the clinical utility of survivin in Ta/T1 G2 or highlighting its importance with respect to other markers. Our study shows a clinical utility of surviving in UBC pTa/pT1 G2 (WHO 1973).

Regarding to Ki-67 labeling index, many studies have described its utility as a predictor marker of tumor relapse and progression in Ta/T1 [23−25]. Jeon et al. observed the predictive utility of Ki-67 in the clinical evolution of Ta/T1 patients with respect to recurrence and progression [22]. Seo et al. observed that the risk of progression of adjuvant mitomycin-c treatment was 3-fold higher in those who significantly expressed Ki-67 (cut-off value 15%), suggesting that this marker might be a useful tool in the therapeutic decision between BCG and Mitomycin-c [24]. However, both studies included a few series of patients and tumors from all grades G1 to G3 (WHO 1973). The current study has corroborated these results to a certain degree, and it has analyzed a large number of IHC markers. With regard to the simultaneous utilization of Ki-67 and other classic clinical variables, our study has shown its prognostic utility in terms of recurrence and progression-free period by stratifying the sample according to risk profiles with respect to the recurrence and progression-free intervals, as Ding et al. have argued. They studied Ki-67 in conjunction with the risk score of the EORTC (classical variables) and found that their joint use improved the prediction of recurrence and progression [25].

Hence, IHC markers such as Ki-67, Survivin, cyclin D1, and p16INK4a, could help in the stratification of the "intermediate" papillary UBC pTa/pT1 G2, as evidenced by the risk groups for relapse and progression from the LASSO/RSF analysis [26]. In addition, it would be very useful to use markers with greater evidence, such as Ki-67 index and Survivin, in daily clinical practice by implementing the EORTC risk tables [25,26].

Regarding the elimination of heterogeneous moderately differenciated (G2) category of the 1973 system, the 2004/2016 classification was expected to provide a more reproductible stratification of patients with differing prognoses and improving treatment and follow-up. Nowadays several studies have shown interobserver variability and its prognostic value is still a matter of debate [27,28]. In our study, WHO classification 2004 of grade only predicts disease recurrence, as it occurs in other studies [28]. For this reason, it would be useful to implement the classification of this tumor with IHC markers as selected in our study.

The limitations of the present study lie first in its mainly retrospective nature and the need for an external validation of results.

With respect to the analysis of the data, we have not divided the samples according to stage TNM variable in Ta/T1, because we wanted to analyze its relationship with the rest variables. The stage variable has behaved according to other studies results [7,8,28].

All patients were treated with intravesical mytomicin C and none with BCG, because being a retrospective sample; patients were managed in the period in which there was a global BCG shortage (2012−2014) [29]. Treatment indications were modified and their use was restricted mainly to T1G3. We also wanted to manage a homogeneous sample in terms of treatment.

5. Conclusions

The results presented herein demonstrate Ki-67 index is the most useful IHC marker, since it can improve the prediction of both recurrence and progression-free survival in papillary UBC pTa/pT1 G2 (WHO 1973). There are other markers whose utility is specific to recurrence-free survival, such as Cyclin D1, and p16INK4a or in progression-free survival, such as Survivin.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.1016/j.urolonc.2018.10.005.

References

[1] Kirkali Z, Chan T, Manoharan M, Algaba F, Busch L, Cheng L, et al. Bladder cancer: epidemiology, staging and grading, and diagnosis. Urology 2005;66:4–34.

[2] Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer J Int du Cancer 2015;136:E359–86.

[3] Van Rhijn BW, Burger M, Lotan Y, Solsona E, Stief CG, Sylvester RJ, et al. Recurrence and progression of disease in non-muscle-invasive bladder cancer: from epidemiology to treatment strategy. Eur Urol 2009;56:430–42.

[4] Sauter G, Algaba F, Amin MB, et al. Tumours of the urinary system: non-invasive urotelial tumours. In: Eble JN, Sauter G, Epstein JI, Sesterhenn IAP, eds. Pathology and genetics of tumours of the urinary system and male genital organs, Lyon: WHO-International Agency for Research on Cancer IARC; 2004:110–23.

[5] May M, Brookman-Amissah S, Roigas J, Hartmann A, Störkel S, Kristiansen G, et al. Prognostic accuracy of individual uropathologists in noninvasive urinary bladder carcinoma: a multicentre study comparing the 1973 and 2004 World Health Organisation classifications. Eur Urol 2010;57:850–8.

[6] Pellucchi F, Freschi M, Ibrahim B, Rocchini L, Maccagnano C, Briganti A, et al. Clinical reliability of the 2004 WHO histological classification system compared with the 1973 WHO system for Ta primary bladder tumors. J Urol 2011;186:2194–9.

[7] Cao D, Vollmer RT, Luly J, Jain S, Roytman TM, Ferris CW, et al. Comparison of 2004 and 1973 World Health Organization grading systems and their relationship to pathologic staging for predicting long-term prognosis in patients with urothelial carcinoma. Urology 2010;76:593–9.

[8] Sylvester RJ, van der Meijden AP, Oosterlinck W, Witjes JA, Bouffioux C, Denis L, et al. Predicting recurrence and progression in individual patients with stage Ta T1 bladder cancer using EORTC risk tables: a combined analysis of 2596 patients from seven EORTC trials. Eur Urol 2006;49:466-5; discussion 475-7.

[9] Hernández V, De La Peña E, Martin MD, Blázquez C, Diaz FJ, Llorente C, et al. External validation and applicability of the EORTC risk tables for non-muscle-invasive bladder cancer. World J Urol 2011;29:409–14.

[10] Pillai R, Wang D, Mayer EK, Abel P, et al. Do standardised prognostic algorithms reflect local practice? Application of EORTC risk tables for non-muscle invasive (pTa/pT1) bladder cancer recurrence and progression in a local cohort. ScientificWorldJournal 2011;11:751–9.

[11] Malmström PU. Bladder tumours: time for a paradigm shift? BJU Int 2011;107:1543–5.

[12] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646–74.

[13] Cheng L, Zhang S, MacLennan GT, Williamson SR, Lopez-Beltran A, Montironi R. Bladder cancer: translating molecular genetic insights into clinical practice. Hum Pathol 2011;42(4):455–81.

[14] Sanguedolce F, Bufo P, Carrieri G, Cormio L. Predictive markers in bladder cancer: do we have molecular markers ready for clinical use? Crit Rev Clin Lab Sci 2014;51:291–304.

[15] Ishwaran H, Kogalur UB. Consistency of random survival forests. Stat Probab Lett 2010;80:1056–64.

[16] Simon N, Friedman J, Hastie T, Tibshirani R. Regularization paths for Cox's Proportional hazards model via Coordinate Descent. J Stat Softw 2011;39:1–13. URL http://www.jstatsoft.org/v39/i05/.

[17] Ramos D, Ruiz A, Morell L, Navarro S, Villamón R, Gil-Salom M, et al. Prognostic markers in low-grade papillary urothelial neoplasms of the urinary bladder. Curr Diang Pat 2005;11:141–50.

[18] Kim Y, Kong L. Estimation of C-index for cox proportional hazards model with censored biomarker covariate subject to limits of detection. J Biopharm Stat 2015;25:459–73. https://doi.org/10.1080/10543406.2014.920859.

[19] Lin HH, Ke HL, Wu WJ, Lee YH, Chang LL. Hypermethylation of E-cadherin, p16, p14, and RASSF1A genes in pathologically normal urothelium predict bladder recurrence of bladder cancer after transurethral resection. Urol Oncol 2012;30:177–1781.

[20] Behnsawy HM, Miyake H, Abdalla MA, Sayed MA, Ahmed Ael-F, Fujisawa M. Expression of cell cycle-associated proteins in non-muscle-invasive bladder cancer: correlation with intravesical recurrence following transurethral resection. Urol Oncol 2011;29:495–501.

[21] Yin W, Chen N, Zhang Y, Zeng H, Chen X, He Y, et al. Survivin nuclear labeling index: a superior biomarker in superficial urothelial carcinoma of human urinary bladder. Mod Pathol 2006;19:1487–97.

[22] Jeon C, Kim M, Kwak C, Kim HH, Ku JH. Prognostic role of survivin in bladder cancer: a systematic review and meta-analysis. PLoS One 2013;8:e76719.

[23] Quintero A, Alvarez-Kindelan J, Luque RJ, Gonzalez-Campora R, Requena MJ, Montironi R, et al. Ki-67 MIB1 labelling index and the prognosis of primary TaT1 urothelial cell carcinoma of the bladder. J Clin Pathol 2006;59:83–8.

[24] Seo HK, Cho KS, Chung J, Joung JY, Park WS, Chung MK, et al. Prognostic value of p53 and Ki-67 expression in intermediate-risk patients with nonmuscle-invasive bladder cancer receiving adjuvant intravesical mitomycin C therapy. Urology 2010;76:512.

[25] Ding W, Gou Y, Sun C, Xia G, Wang H, Chen Z, et al. Ki-67 is an independent indicator in non-muscle invasive bladder cancer (NMIBC); combination of EORTC risk scores and Ki-67 expression could improve the risk stratification of NMIBC. Urol Oncol 2014;32:42.

[26] van Rhijn BW, Musquera M, Liu L, Vis AN, Zuiverloon TC, van Leenders GJ, et al. Molecular and clinical support for a four-tiered grading system for bladder cancer based on the WHO 1973 and 2004 classifications. Mod Pathol 2015;28:695–705.

[27] Bosschieter J, et al. Reproducibility and prognostic performance of the 1973 and 2004 World Health Organization Classifications for grade in non-muscle-invasive bladder cancer: a multicenter study in 328 bladder tumors. Clin Genitourin Cancer 2018:pii: S1558-7673(18) 30196-4.

[28] van de Putte EEF, et al. The World Health Organization 1973 classification system for grade is an important prognosticator in T1 non-muscle-invasive bladder cancer. BJU Int 2018. https://doi.org/10.1111/bju.14238.

[29] Perera M, et al. The impact of the global bacille Calmette-Guérin shortage on treatment patterns: population-based data. BJU Int 2018;121:169–72.