Tumor Budding: Prognostic Value in Muscle-invasive Bladder Cancer

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OBJETIVES	To assess if "tumor budding" (TB) behaves as a poor prognostic factor in muscle-invasive bladder carcinoma (MIBC). TB is the presence of tumor cells isolated or in small groups of fewer than 5 cells located at the tumor invasion front.
MATERIAL AND METHODS	Retrospective study of 106 patients with MIBC who underwent radical cystectomy. A cytokeratin AE1/AE3 immunostaining was applied to identify and quantify TB by the "hot-spot" method. The variables evaluated were: age, gender, Tumour, Node, Metastasis Classification (TNM) stage, associated Carcinoma *in situ*, differentiation degree, tumor size, tumor location, lymphatic, venous or perineural invasion, p53, Ki67, molecular subtype (basal/luminal) and chemotherapy. Main variables were overall and cancer-specific survival.
RESULTS	The mean follow-up time was 47 ± 46.45 months. The mean TB count was 32.3 ± 25.9 "buds." The ROC curve established 14 "buds" as the cut-off point: the median survival rate for the "low-grade TB" group (≤14 "buds") was 69.5 months, and for the "high-grade TB" group (>14 "buds") was 18.5 months (P= .003). In the multivariate analysis, independent predictive variables regarding mortality were: age, TB, and TNM stage. Patients with more than 14 "buds" had 2.27 times more risk of mortality, 95%CI:1.19-4.34, P = .013. In addition, the risk of mortality rises progressively as the number of "buds" increases, at a rate of 2% per "bud."
CONCLUSION	According to our results, TB becomes an independent predictor factor for cancer-specific mortality in MIBC, with a cut-off point of 14 "buds." UROLOGY 130: 93−98, 2019. © 2019 Elsevier Inc.

Bladder cancer is the 11th most common worldwide carcinoma and the second in the urinary tract after prostate carcinoma. It causes 150,000 deaths per year.[1] At diagnosis, 75% of cases of bladder cancer are confined to mucosa or submucosa (pTa, pT1 or Cis) and the remaining 25% are muscle-invasive bladder carcinomas (MIBCs) (pT2-T4), which have a worse prognosis and a different therapeutic management.[2] The standard treatment for MIBC is radical cystectomy. Nevertheless, approximately 50% of patients develop metastasis in the first 2-3 years after cystectomy.[3] Thus, MIBC remains as a high mortality disease despite recent improvements in its diagnosis and treatment. In addition to the prognostic factors already established for MIBC (tumor stage, lymphovascular invasion, tumor size, p53 status, Ki67 index, molecular subtype,[4] etc.), it is necessary to identify additional prognostic markers that would allow us to better stratify patients into risk groups and thereby develop personalized and more effective follow-up and adjuvant therapies.

With this aim, we considered the study of "tumor budding" (TB) status in MIBC. TB is an anatomopathologic concept consisting of the presence of isolated tumor cells or small groups of up to 4 tumor cells ("buds"), located in the stroma at the invasive tumor cell front.[5] Many authors have considered this phenomenon as the morphologic representation of the invasive ability acquisition by tumor cells and thus, the starting point for the development of future metastasis. These cells phenotype is characteristic leading to the emergence of migration (loss of epithelial markers such as E-cadherin and increase in mesenchymal markers such as vimentin or fibronectin). Therefore, TB has been associated with an epithelial-mesenchymal transition.[6,7]

TB has been widely studied in colorectal cancer (CRC) and established as an important prognostic factor.[8] Thus, patients with CRC and high-grade TB have a higher risk of local and distant recurrence and lower survival at 5 and 10 years[9-11] in accordance with other factors of poor prognosis, such as Tumour, Node, Metastasis Classification (TNM) stage,[12] tumor differentiation degree,[9] Dukes classification degree,[9,10] or lymphovascular and perineural invasion.[9-11]

Although much less studied than in CRC, TB has also been shown to behave as a prognostic factor in other types of carcinoma: oesophageal,[13] pancreatic,[14] breast,[15] head

and neck,[16,17] or lung.[18] But, it is virtually unexplored in bladder cancer, with only a single study published in a series of pT1 carcinomas.[19]

Therefore, the main objective of this study was to assess if TB is an independent survival prognostic factor in MIBC and work out a suitable cut-off point for significant worse prognosis.

MATERIAL AND METHODS

Patient Selection
A retrospective study was carried out, in which we evaluated patients that had undergone radical cystectomy at our center, between January 2004 and December 2014. Our inclusion criteria were: patients diagnosed with MIBC (pT2, pT3, or pT4) and with urothelial cell histology. The exclusion criteria were: death due to postoperative complications (within 30 days following cystectomy), history of other oncological pathology that could condition survival (including upper urinary tract tumor), histologic type other than urothelial (glandular or squamous differentiation, micropapilar variant, sarcomatoid carcinomas…, due to the worse prognosis of these histologic variants), immunosuppressed patients (those patients receiving immunosuppresive therapy, eg, transplants or patients with inflammatory/autoimmune diseases, since typically the oncological pathology behaves more aggressively in these cases), cases with invalid histologic material, loss of patient follow-up and doubtful or unknown cause of death. The study was approved by the Biomedical Research Ethics Committee of the Hospital with registration number: 2016/0558.

Therapeutic Approach
The surgery applied in our series involved open radical cystectomy, associated with bilateral iliobturator lymphadenectomy and Bricker urinary diversion in most cases. No patient in our series received neoadjuvant chemotherapy. The cases with a high risk of recurrence (positive margins in the cystectomy piece, positive lymph nodes, lymphovascular invasion…) received adjuvant chemotherapy. After cystectomy, follow-up involved performing computer tomography every 4 months during the first year, every 6 months during the second and third years and annually afterwards. Deaths during follow-up were evaluated in order to establish cancer-specific mortality.

Immunohistochemistry
For patients who met the selection criteria, all tissue blocks stored in the Pathological Anatomy Service (samples fixed in 10% buffered formalin and included in paraffin) were recovered. The purpose was to select within each patient the slide with the highest density of TB, so that we achieved the most representative tissue section. Thus, for each case, all tumor tissue was analyzed (the slides of both transurethral resection (TUR) and cystectomy specimens), with hematoxylin-eosin by an experienced uropathologist. Therefore, we selected one slide (obtained from TUR or cystectomy specimen). In fact, we subjectively selected the section with the highest TB density (without further quantification of TB). Only in this selected slide we carried out cytokeratin (CK) AE1/3 immunostaining for a more accurate account of TB. In addition to CK AE1/3, the following immunohistochemical techniques were performed on the selected slide in each case: CD34 (venous invasion study), D2.40 (lymphatic invasion study), P53 (p53 index), Ki67 (Ki67 proliferative index) and CK20-CK5/6-CD44 (basal or luminal molecular subtypes of invasive bladder carcinoma).

CK AE1/3 immunostaining was performed to facilitate the identification and the count of TB (Fig. 1). Three-micron-thick sections were made on paraffin embedded blocks, which after passing through an oven at 37°C, were subject to antigen recovery (*Target Retrieval Solution High pH (50×)*) for 20 minutes at 95°C. Deparaffinization, hydration and antigenic recovery were carried out automatically. All this was followed by endogenous peroxidase blockade (*Peroxidase-blocking Reagent*), washing with Wash Buffer (20×) commercial buffer and incubation with primary monoclonal antibodies (AE1/3, QBEnd10, D2.40, DO-7, MIB-1, Ks20.8, D5/16BA, and DF1485 (*Ready-to-use*) by Dako®). Immunodetection was performed with *EnVision Flex* kit by Dako® and the antigen-antibody reaction was visualized with a chromogen (*Substrate buffer with DAB + Chromogen*). Finally, counter-staining with hematoxylin and assembly for its microscope visualization were carried out.

Assessment of Anatomopathologic Parameters
TB was defined as the presence of isolated tumor cells or small groups of up to 4 cancer cells ("buds"), located at the invasive
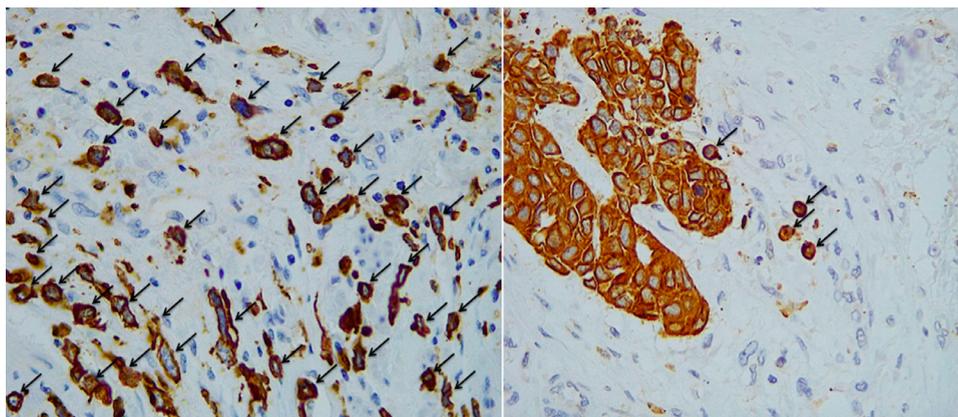

Figure 1. Invasive tumor front in 2 of our cases of MIBC with CK AE1/AE3, large scale (×40). In the case on the left, there is a high density of "tumor budding"; and in the case on the right, a very low density (color version available online). MIBC, muscle-invasive bladder carcinoma.

front of the tumor.[5] Quantification of TB by the "hot-spot" method was performed only in the selected slide after CK AE1/3 immunostaining was carried out. In this methodology, we visualized first the entire tumor invasion front at low magnification (×4 or ×10), and within the area with highest TB density we further counted the number of "buds" present in one high-power field (×40: 22 mm lens and 0.55 mm^2 area). Quantification was carried out by 2 independent blinded pathologists. The "hot-spot" method has the advantage of requiring only 1 power-field to assess TB. In other words, we consider this method to be a suitable method in cases with limited size of tissue or with fragmented specimen in the TUR.

For p53 and Ki67, the percentage of positive tumor cells was assessed. For CD34 and D2.40, it was assessed whether or not venous and lymphatic invasion were observed, respectively. And for CK20, CK5/6, and CD44, depending on their positivity or negativity, the molecular subtype of the tumor (basal or luminal) was determined (Supplementary Fig. 1). Each case was evaluated "blindly"—without knowing the evolution of the patient—by 2 examiners (DR and LL), independently and jointly discussing the discordant cases.

Evaluated Variables

The variables evaluated in each case were: age, gender, TNM stage at diagnosis, degree of differentiation (1973 WHO grading), associated Carcinoma in situ tumor size, location (trigonal involvement or not), "buds" count, p53 (%), Ki67 (%), presence of venous, lymphatic or perineural invasion, molecular subtype (basal or luminal), follow-up time, adjuvant chemotherapy, local or distant recurrence, and overall, cancer-specific and progression-free survival.

Statistical Analysis

The statistical analysis was made with STATA (version 13.1.). Quantitative variables were defined with mean and standard deviation, and qualitative variables were defined with frequencies and percentages. Kaplan-Meier curves were used to estimate survivals, and a ROC curve was set to establish a suitable cut-off point in the TB count. To assess associations between TB and other prognostic factors, chi-square (χ^2), Fisher's exact and Mann-Whitney U tests were used. Finally, a survival multivariate analysis (Fine-Gray competing risk regression) was conducted to assess cancer-specific mortality above other death causes. All statistical tests were carried out with a significance level of $P<.05$ and estimates with a confidence interval of 95%.

RESULTS

Clinical and Pathologic Characteristics

During the studied period, 209 cystectomies were performed, out of which 106 met the selection criteria and were included in the study. The main descriptive characteristics of our series are described in Table 1. Regarding the quantification of TB, the mean count was 32.3 ± 25.9 "buds." Regarding the presence of tumor invasion, 58 patients (54.7%) did not present venous, lymphatic, or perineural invasion. Of the remaining 45.3% (48 patients), 29 had venous invasion (isolated or not), 32 had lymphatic invasion (isolated or not), and 21 had perineural invasion (isolated or not). Forty-two patients (40%) received adjuvant chemotherapy.

The mean follow-up time was 47 ± 46.5 months (range: 0-170 months). During this period, 66 patients (62.3%) developed local or distant recurrence and 68 patients (64.2%) died, of which 66 (97%) due to oncological cause and 2 (3%) for another reason. At the end of the follow-up, 4 (3.8%) patients were alive with stable disease and 34 (32.1%) disease-free patients.

Regarding of overall survival, the median survival was 22 months (median 95% CI: 14.2-29.8). And for progression-free survival, the median survival was 15 months (median 95% CI: 10.8-19.2).

Cut-off Point in the TB Count

According to the ROC curve (Fig. 2), the cut-off point with the highest sensitivity (74.6%) and specificity (64.1%) for mortality, with an area under the curve of 0.76, was 14 "buds." Based on this cut-off point, we stratified the patients: 28 (26.4%) had ≤14 "buds" (referred to as "low-grade TB") and 78 (73.6%) had >14 "buds" (referred to as "high-grade TB"). The survival curves of both groups showed a statistically significant difference between them ($P = .003$): the median survival for the "low-grade TB" group was 69.5 months, whereas for the "high-grade TB" group was 18.5 months (Fig. 2).

Association Between TB and Other Prognostic Factors

We found statistically significant association between "high-grade TB" and the classical variables: age ($P= .043$), pT (tumor stage) ($P= .046$) and pN (lymph node involvement) ($P = .013$) (Table 1). Patients with "high-grade TB" had a lower mean age, a higher pT stage (69.2% of these had pT3 or pT4), and a higher score of pN (53.8% of these had lymph node involvement).

Prognostic Value of TB in the Multivariate Analysis

We applied the "Fine-Gray model for competitive risks" since 2 patients died due to a nontumor cause. The dependent variable was cancer-specific mortality. We included in the analysis all prognostic variables studied, including adjuvant chemotherapy.

First of all, TB was defined as a qualitative dichotomous variable (more or less than 14 "buds"). The significant independent predictive mortality risk variables were: pT (tumor stage), pN (lymph node involvement), pM (distant metastasis), TB and age (Table 2). Thus, those patients with a TB count above 14 "buds" had a hazard risk of mortality between 1.19 and 4.34 times higher than those under 14 "buds" report.

In addition we repeated the multivariate analysis using TB as a continuous variable. All previous variables remained significant, and again TB, raised the risk of mortality progressively as the number of "buds" increased: with each extra "bud," the risk of mortality increased approximately by 2% (hazard ratio [HR] 1.02 [1.01-1.03]) (Table 2).

All other variables assessed in the study, did not statistically fit the model.

DISCUSSION

The prognostic value of TB has been clearly established in CRC.[5,8] Likewise, TB has been studied in other types of carcinomas where it has also shown to behave as a poor prognostic factor.[13-18] To our knowledge, reviewing urothelial carcinoma it has only been evaluated in a single article focused on the study of TB as a predictive factor of recurrence and progression in non—muscle-invasive bladder cancer.[19] The singularity of our work is that it

Table 1. Clinicopathologic characteristics of our cases and association between TB and MIBC prognostic factors.

Risk Factor	Total (%) N = 106	"Tumor budding" (%)		P value
		Low grade (≤14 "buds") N = 28, 26.4%	High grade (>14 "buds") N = 78, 73.6%	
Age (y)				
Mean ± SD	64.6 ± 10.6	68.5 ± 11.4	63.2 ± 9.9	**0.043**
Sex				
Man	93 (87.8)	25 (89.3)	68 (87.2)	0.771
Woman	13 (12.2)	3 (10.7)	10 (12.8)	
pT				
pT2	40 (37.7)	16 (57.1)	24 (30.8)	
pT3	36 (34)	7 (25)	29 (37.2)	**0.046**
pT4	30 (28.3)	5 (17.9)	25 (32)	
pN				
pN0	58 (54.7)	22 (78.6)	36 (46.2)	
pN1	10 (9.4)	2 (7.1)	8 (10.2)	**0.013**
pN2	25 (23.6)	1 (3.6)	24 (30.8)	
pN3	13 (12.3)	3 (10.7)	10 (12.8)	
pM				
pM0	95 (89.6)	27 (96.4)	68 (87.2)	0.169
pM1	11 (10.4)	1 (3.6)	10 (12.8)	
Associated CIS				
Present	14 (13.2)	4 (14.3)	10 (12.8)	0.844
Absent	92 (86.8)	24 (85.7)	68 (87.2)	
Differ. grade				
G1-G2	3 (2.8)	1 (3.6)	2 (2.6)	0.627
G3	103 (97.2)	27 (96.4)	76 (97.4)	
Tumor size (cm)				
Mean ± SD	8 ± 3	4.5 ± 1.8	4.8 ± 2.3	0.167
Tumor location				
Trigone	50 (49)	11 (39.3)	43 (55.1)	0.150
No trigone	56 (51)	17 (60.7)	35 (44.9)	
Invasion (vasc. or perineur.)				
Positive	48 (45.3)	10 (35.7)	38 (48.7)	0.236
Negative	58 (54.7)	18 (64.3)	40 (51.3)	
p53				
Positive (≥20%)	74 (69.8)	20 (71.4)	54 (69.2)	0.828
Negative (<20%)	32 (20.2)	8 (28.6)	24 (30.8)	
Ki67				
High (≥10%)	100 (94.3)	29 (100)	71 (92.2)	0.260
Low (<10%)	6 (5.7)	0 (0)	6 (7.8)	
Molecular subtype				
Basal	47 (44.3)	9 (32.1)	38 (48.7)	0.130
Luminal	59 (55.7)	19 (67.9)	40 (51.3)	

Statistical significance (P < 0.05).
Chi-square (χ^2), Fisher's exact, and Mann-Whitney U tests were used for bivariate analysis.

represents the first study focused in the presence of TB in MIBC and its prognostic value.

According to our results, TB might become an independent predictor of mortality in MIBC. Based on the cut-off point established by the ROC curve, we classified patients with a count above 14 "buds" as patients with "high-grade TB" and we found that in these cases the risk of mortality was higher than in those with a count below 14 "buds" or "low-grade TB" (HR 2.27 (1.19-4.34), P= .013). Actually, most published literature describes TB as a dichotomous variable.[20,21] On the other hand some authors have stated the use of "buds" quantification as a continuous variable.[22,23] In our study, we would rather take TB as continuous data; with each extra TB unit, the risk of mortality increased approximately by 2% (HR 1.02 (1.01-1.03), P <.001). Thus, comparing 2 patients—with

the same age and the same TNM stage at diagnosis—one with 15 "buds" and the other with 60 "buds," the hazard risk differs remarkably (15.3 and 61.2, respectively), while considering a qualitative cut-off point of 14 "buds," both cases would be considered as a "high-grade TB" and assumed to have approximately the same risk.

In relation to the methodology used to evaluate TB, there is a great heterogeneity in literature.[20,21] In our series, we reviewed all slides in each case (TUR and cystectomy), and consequently, we are convinced that we have properly selected the optimal tissue sample for the study: the most representative slide with the highest density of TB. Subsequently, we applied the CK AE1/3 immunostaining in the selected slide, since it greatly facilitates the identification of "buds" with respect to hematoxylin & eosin staining, thereby avoiding erroneous
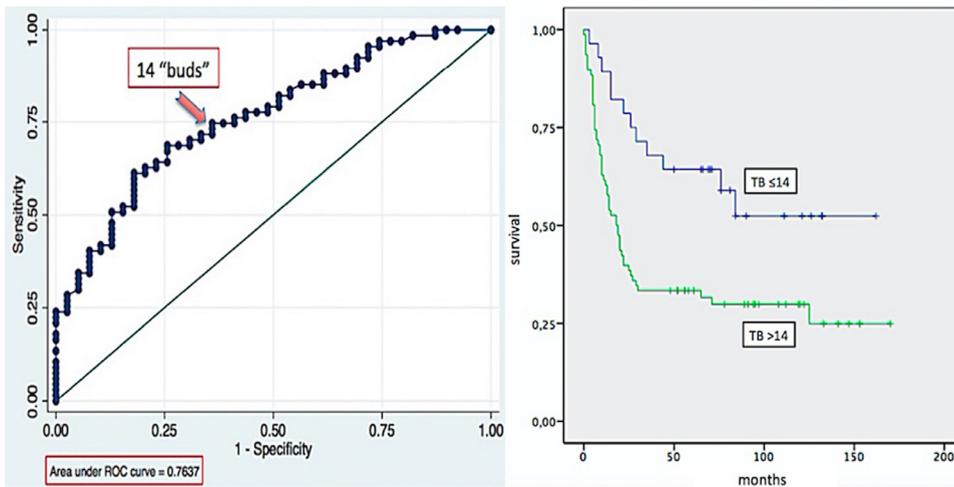
Figure 2. ROC curve established our cut-off point at 14 "buds" (AUC = 0.7637) (left). Survival curves for "low-grade TB" group (TB ≤14 "buds") and "high-grade TB" group (TB >14 "buds"), log-Rank test: *P*= .003 (right) (color version available online). AUC, area under curve; TB, tumor budding.

quantification, especially in cases where this may be difficult, as in the presence of abundant inflammatory infiltrate at the invasive front of the tumor.[12,20,23] Regarding the TB assessment method, we used a quantitative technique, as these have been shown to be more reproducible and have a better correlation with cancer-specific survival.[12,22] Furthermore, the technique used in our case ("hot-spot") has the additional advantage of being highly reproducible, easy, and quick to perform.[5,23]

MIBC is a prevalent and poor prognostic disease. At diagnosis, many patients have reduced survival despite improvements in postoperative care, surgical techniques, and chemotherapy protocols.[24,25] In addition, although performing curative radical surgery, some patients still have a high mortality rate, especially in the first 2-3 years.[3] Conversely, another small subgroup of patients is characterized by a favorable clinical outcome, with complete recovery and prolonged survival. For this reason, it would be of great interest to identify since the beginning of the disease, which patients are going to show an unfavorable outcome in order to consider more aggressive therapies at an earlier stage and a closer follow-up. In this way, the identification of new parameters with prognostic

relevance in MIBC that could play a role in its carcinogenesis or tumor progression would be very useful to offer a more personalized medicine, adjusting the therapeutic and follow-up scheme to each patient, thereby improving the results in terms of survival. Based on this, establishing TB count as a histopathologic phenomenon that represents the process of invasion-metastasis in its earliest phase and that provides valuable prognostic and specific information for each case of MIBC, would be more than justified.

In relation to this, we consider useful to know whether the value of TB in the tissue of the TUR specimen could provide valuable prognostic information. Thus, in those patients with the selected slide from the TUR specimen and CK AE1/3 immunostaining (48 patients), we compared the Kaplan-Meier survival curves between those with a count of TB higher than 14 "buds" (32 patients) and less than 14 "buds" (16 patients). We observed that the survival was significantly lower (*P*= .048) in those patients with a count of TB higher than 14 "buds" in the TUR. Therefore, in our opinion, TB count in the TUR specimen could be very useful in clinical practice, for instance, to facilitate the

Table 2. Results of the multivariate analysis for cancer-specific mortality with TB as a dichotomous variable (*P*value model <.001; AUC 0.854) and with TB as a continuous variable (*P*value model <.001; AUC 0.875).

		TB: Dichotomous Variable			TB: Continuous Variable	
		HR (95% CI)	*P* value		HR (95% CI)	*P* value
pT	pT3	3.11 (1.58-6.14)	.001	pT3	3.07 (1.55-6.09)	.001
	pT4	4.06 (1.88-8.89)	<.001	pT4	2.50 (1.08-5.82)	.033
pN	pN1	4.30 (2.14-8.64)	<.001	pN1	4.80 (2.25-10.23)	<.001
	pN2	2.18 (1.09-4.35)	.028	pN2	2.57 (1.40-4.72)	.002
	pN3	3.15 (1.65-5.99)	<.001	pN3	3.19 (1.65-6.17)	.001
pM	pM1	3.52 (1.52-8.17)	.003	pM1	5.34 (2.55-11.21)	<.001
TB	TB >14	2.27 (1.19-4.34)	.013		1.02 (1.01-1.03)	<.001
Age		1.07 (1.04-1.10)	<.001		1.07 (1.04-1.10)	<.001

CI, confidence interval; HR, hazard ratio; TB, tumor budding.

selection of patients that could benefit from neoadjuvant chemotherapy before cystectomy. However, this hypothesis should be demonstrated in further studies with a larger series of patients.

With regard to the limitations of our study, the first is the fact of being a retrospective study, with the drawbacks inherent in this design. Nevertheless, the vast majority of studies published on TB are also well-designed retrospective studies. Second, our series consisted of a relatively small sample, due to the application of highly strict selection criteria in order to avoid confusing prognostic factors. Finally, we believe that the results of the current study should generate multicenter large prospective projects providing better external validation in order to consolidate stronger evidence on TB in bladder cancer and to achieve the implementation of a standardized methodology to report it.

CONCLUSION

According to our results, TB has strongly shown to fit as an important independent predictive factor of mortality in MIBC. Thus, those patients with a count above 14 "buds" have an increased risk of mortality and are associated with a higher tumor stage. In other words, the risk of mortality rises hand in hand with the TB count, in such a way that with each "bud" more, the risk of cancer-specific mortality increases approximately by 2%. Finally, the implementation of the TB assessment in daily practice would provide relevant prognostic information.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.urology.2019.04.006.

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