**Original contribution**

# T1 high-grade bladder carcinoma outcome: the role of p16, topoisomerase-IIα, survivin, and E-cadherin ☆

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**Summary** High-grade papillary urothelial carcinoma with subepithelial connective tissue invasion (T1HG) is an aggressive disease at high risk of progression after transurethral resection/*Bacillus* Calmette-Guerin standardized therapy. The European Organization for Research and Treatment of Cancer has identified T1HG bladder carcinoma that is single and ≤3 cm in the largest dimension at first diagnosis as a category in which the prognosis cannot be further stratified based on conventional criteria. This category may benefit from biomarker analysis as a valuable tool to determine the patient's outcome. To further the issue of biomarkers in predicting aggressiveness in single T1HG bladder carcinoma ≤3 cm in greatest dimension at first diagnosis, we have conducted a validation study of the biomarker risk score set previously reported by our group. The study set included immunohistochemical detection of galectin-3, CD44, E-cadherin (E-CAD), CD138, p16, survivin, HYAL-1, and topoisomerase-IIα in 92 randomly selected specimens at participating institutions. Topoisomerase-IIα expression was identified as a predictor of disease-free survival. p16, survivin, and E-CAD expression predicted progression-free survival, but p16 and E-CAD also predicted

overall survival. The current study validates a panel of immunohistochemical markers with the potential of being implemented in practice and supports the use of biomarkers in predicting aggressiveness in patients with first diagnosis of single T1HG bladder carcinoma ≤3 cm in greatest dimension and therefore in identifying patients who need closer surveillance or earlier aggressive treatment.

## 1. Introduction

High-grade (HG) papillary urothelial carcinoma with subepithelial connective tissue invasion (T1 disease) represents about 20% of bladder urothelial carcinomas. After standardized therapy, up to 75% of patients with T1 disease will experience tumor recurrence within 2 years. Importantly, up to 25% of these tumors have a high risk of progression to invasive disease (T2-4) [1]. To identify the most aggressive subset of cases would be of great relevance in clinical practice [2,3].

The European Organization for Research and Treatment of Cancer (EORTC) risk tables may predict pTa and pT1 bladder tumor recurrence and progression including different variables such as tumor diameter, T stage, time to recurrence, association with urothelial carcinoma in situ (CIS), and grade. Furthermore, urothelial pT1HG bladder carcinoma less than 3 cm (greatest diameter) at first diagnosis is not amenable to stratification into prognostic categories because its behavior is not predictable [4]. No prognostic molecular markers, at this stage, have been included in clinical management [5]. The utilization of biomarkers can accurately predict progression in patients with non–muscle-invasive tumors and help identify patients who need closer surveillance or aggressive treatment. This might be very useful in the clinic [6-8].

Studies have shown independent prediction of recurrence and progression of non–muscle-invasive bladder carcinoma by Ki-67, p53, pRb, p21, and p27 [9,10].

We previously reported a biomarker-based risk score based on the immunohistochemical evaluation of galectin-3, CD44, E-cadherin (E-CAD), CD138, p16, survivin, HYAL-1, and topoisomerase-IIα (TOPO-2α) to predict tumor progression in patients with <3-cm single tumors [11]. The current study aimed to validate our biomarker-based risk score in a large series of T1 high-grade cases from different institutions and to assess their potential application in practice.

## 2. Materials and methods

### 2.1. Patients

The study series included 92 cases of T1 high-grade bladder cancer collected from participating institutions with the following histological and clinical inclusion criteria:

(1) histologic diagnosis of HG papillary urothelial carcinoma T1 with tumor size ≤3 cm and with presence of muscularis propria at the first transurethral resection of the bladder (TUR-B); (2) previous negative history of urothelial tumor; (3) repetitive transurethral resection of the bladder post–*Bacillus* Calmette-Guerin (BCG) therapy showing no residual disease; and (4) known follow-up.

We collected the materials from 6 departments of pathology of different European hospitals; all slides were reassessed by 2 specialized pathologists (A. L. B. and M. R. R.) to confirm the diagnosis of the tumor grade, the presence of muscularis propria, the presence of CIS, T1 substaging, and the presence or absence of tumor necrosis. Subsequently, appropriate immunohistochemical analysis was performed on selected representative samples.

*Recurrence* was defined as the finding of new non–muscle-invasive lesions after a complete resection and induction course with BCG, whereas *progression* was defined as the recurrence of a muscle-invasive lesion after TUR-B and BCG instillations.

### 2.2. Tissue specimens and immunohistochemistry

The specimens were fixed in 10% formalin before being processed in paraffin. Hematoxylin-eosin–stained sections from each histologic specimen were reviewed to confirm the histologic diagnosis of T1HG urothelial carcinoma or CIS. The slides and tissue blocks from first TUR-Bs were used for the immunohistochemical analysis.

The following immunohistochemical markers were evaluated: galectin-3, CD44, E-CAD, CD138, p16, survivin, HYAL-1, and TOPO-2α. The primary antibodies anti–galectin-3 (clone 9C4, ready to use), anti–E-CAD (clone EP700Y, ready to use), anti-CD138/syndecan-1 (clone B-A38, ready to use), and anti-p16INK4a CINtec histology (clone E6H4, ready to use, Ventana, Tucson, AZ) were placed on the slides and incubated on the Ventana automated stainer BenchMark ULTRA ICH system. For chromogenic detection, ultraView Universal DAB Detection Kit (Ventana Medical Systems, Tucson, AZ) was used. Additional serial sections were immunostained with rabbit monoclonal anti-survivin (clone EP2880Y, ready to use), anti–TOPO-2α rabbit monoclonal (cloneEP1102Y, ready to use) anti-CD44 (H-CAM, clone DF1485, ready to use, Biogenex, Fremont, CA), and anti–HYAL-1 rabbit polyclonal (dilution 1:100, Novus Biologicals, Littleton, CO). Antigen retrieval was carried out with Epitope Retrieval Solution Citrate buffer (10 mmol/L, pH 6; Dako, Glostrup, Denmark) in thermostatic bath (PT Link, Pre-Treatment Module; Dako). Anti–TOPO-2α and anti-survivin were incubated for 1 hour at room temperature, anti-CD44 for 2 hours at room temperature, and anti–HYAL-1 overnight at 4°C. The immunohistochemical analyses were
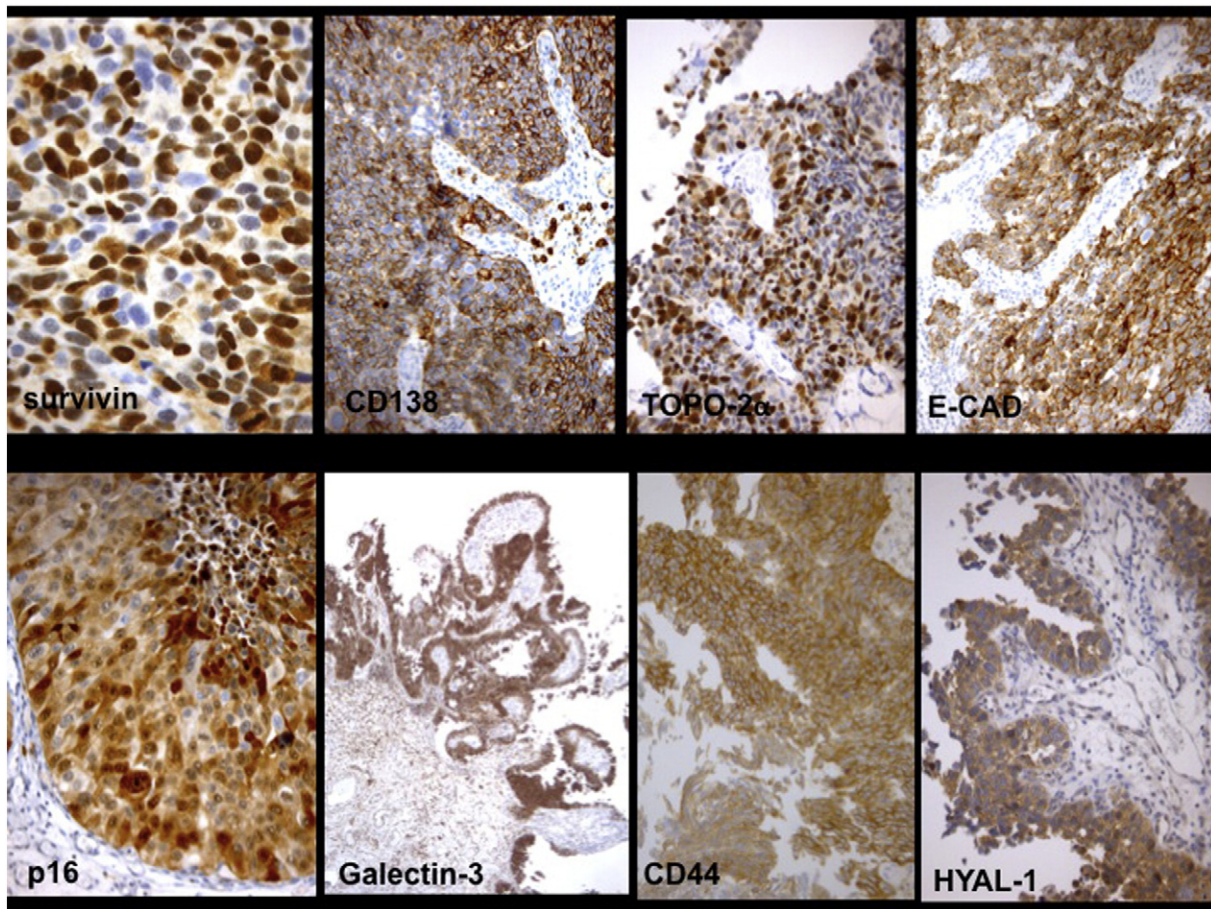
**Fig. 1** Examples of immunohistochemically determined biomarkers in the current study. Anti–galectin-3 (original magnification ×20), anti-CD44 (×10), anti–E-CAD (×10), anti-CD138 (×10), anti-p16 (×20), anti-survivin (×10), anti–HYAL-1 (×10), and anti–TOPO-2α (×10).

performed using EnVision FLEX Systems (Dako) and 3,3′-diaminobenzidine (Dako) as chromogen in Dako Autostainer Link48 Instrument.

Sections of strongly positive tonsil (CD138), papillary carcinoma of thyroid (galectin-3), lung adenocarcinoma (E-CAD), colorectal adenoma (CD44), urinary bladder carcinoma (survivin), endometrial glands (HYAL-1), and uterine cervix carcinoma (TOPO-2α) were used as positive controls. Negative control was performed with FLEX Negative Control Mouse and FLEX Negative Control Rabbit (ready to use, Dako). The control sections were treated in parallel with the samples. The sections were lightly counterstained with Mayer hematoxylin.

## 2.3. Biomarker expression and evaluation of immunostaining

The stains for HYAL-1 and galectin-3 were considered positive if any cytoplasmic staining was seen in the neoplastic tissue. A cytoplasm staining with a membranous-reinforced staining pattern was required for considering CD138 positivity. A membrane staining was required for E-CAD and CD44. The stains for survivin and TOPO-2α were considered positive if

nuclear staining was seen in the neoplastic tissue. For survivin, we evaluated only the nuclear-labeling index because previous studies demonstrated the nuclear positivity of this marker to be a prognostic tool for Ta/T1 urothelial carcinoma of the bladder [12].

The stain for p16 was considered positive if nucleus and cytoplasm or only nucleus staining was seen in the tumor cells. For galectin-3, CD44, and E-CAD, we used the von Brunn nests as positive internal control, whereas for the other markers, we used the von Brunn nests as negative internal control. Fig. 1 shows positive staining of the studied markers.

All slides were evaluated by 2 uropathologists (A. L. B. and M. R. R.) in a blind evaluation. For semiquantitative assessment of the immunohistochemical data, the mean percentage of positive tumor cells was determined at ×400 magnification for each section. Sections were graded on the basis of the percentage of tumor cells. The following were considered negative: no tumor cells with positive nuclei for survivin, TOPO-2α, and p16; no tumor cells with cytoplasm staining with a membranous-reinforced staining pattern for CD138; no tumor cells with cytoplasmatic staining for HYAL-1 and galectin-3, or no tumor cells with membrane staining for E-CAD and CD44.

**Table 1** Demographic and pathologic characteristics of patients with T1 high-grade bladder cancer included in the current study

| Characteristics | n (%) |
| --- | --- |
| Age (y), median (range) | 72.2 (70.2-74.2) |
| Sex | |
|   Female | 12 (13.0%) |
|   Male | 80 (87.0%) |
| T category substaging | |
|   T1 focal | 42 (45.7) |
|   T1 extensive | 50 (54.3) |
| Concomitant CIS | |
|   No | 66 (71.7) |
|   Yes | 26 (28.3) |
| Focal tumor necrosis | |
|   No | 77 (83.7) |
|   Yes | 15 (16.3) |
| Tumor recurrence | |
|   No | 65 (70.7) |
|   Yes | 27 (29.3) |
| Tumor progression | |
|   No | 75 (81.5) |
|   Yes | 17 (18.5) |
| Overall survival | |
|   Alive | 65 (70.7) |
|   Dead of bladder cancer | 14 (15.2) |
|   Dead, other cause | 13 (14.1) |
| p16, median (range) | 39.4 (30.5-48.2) |
| CD138, median (range) | 85.16 ± 17.62 |
| TOPO-2$\alpha$, median (range) | 36.7 (31.9-41.4) |
| Survivin, median (range) | 37 (32-42) |
| Galectin-3, median (range) | 56 (50-62.5) |
| CD44, median (range) | 13.8 (7.9-19.6) |
| HYAL-1, median (range) | 31.6 (24.9-38.4) |
| E-CAD, median (range) | 78.7 (73.4-84.1) |

## 2.4. Statistical analysis

Statistical analysis was performed using SPSS (Chicago, IL) 21.0 software at the Jaén Hospital Research Unit, Spain (R. J. L.). A descriptive analysis was performed, and then receiver operating curve analyses were performed for each marker to find reliable cutoff values, selecting those with a greater sensitivity and specificity for recurrence, progression, and disease-related mortality. After determination of cutoff points, association between variables was tested with Fisher exact test. Univariate survival analysis was conducted using the Kaplan-Meier method, with differences among groups being tested for significance using the log-rank test. Multivariate analysis of probable prognostic factors for survival was performed with Cox proportional hazard regression analysis, and the relative risk with 95% confidence interval was calculated. A $P$ less than .05 was considered statistically significant.

## 3. Results

We evaluated the specimens from transurethral resection of 92 patients with T1HG bladder carcinoma that was single and $\leq$3 cm in greatest dimension at first diagnosis. Main demographic and clinicopathological data of the patients as well as the mean expression of biomarkers in the study are summarized in Table 1. Most patients in the study were male (87%), and 28% of patients had concomitant CIS. On follow-up ranging 13-170 months, 29%, 18.5%, and 15% of patients recurred, progressed, or died of disease, respectively.

Table 2 presents univariate survival analysis of the patients in this series. The current study identified TOPO-2$\alpha$ expression (cutoff at 30%) as predictor of disease-free survival ($P$ = .021) (Fig. 2). p16 (cutoff at 60%) ($P$ = .04), survivin (cutoff at 50%) ($P$ = .012), and E-CAD (cutoff at 90%) ($P$ = .007) immunoexpressions predicted progression-free survival (Fig. 3). In addition, p16 (cutoff at 60%) ($P$ = .009) and survivin (cutoff at 50%) ($P$ = .000) predicted overall survival (Fig. 4).

In the multivariate analysis (Table 3), TOPO-2$\alpha$ was selected as an independent predictor of disease-free survival ($P$ = .029), and survivin was selected as an independent predictor of progression-free survival ($P$ = .020). However, survivin ($P$ = .006) and E-CAD ($P$ = .030) were selected as independent predictors of overall survival in the current study.

None of the other investigated biomarkers (galectin-3, CD44, CD138, HYAL-1) was found to be correlated to survival outcome in the current study.

## 4. Discussion

In this study, we confirmed that some biomarkers can support risk assessment in high-grade bladder cancer with early stromal invasion. In fact, there is a subset of these tumors in which predictors of aggressiveness are very limited as demonstrated by the EORTC studies. Currently, conventional predictors cannot predict aggressiveness in primarily diagnosed single T1HG urothelial carcinoma less than or equal to 3 cm in diameter; likewise, available data show that these patients have 35% probability of recurrence and 10% probability of progression at 24 months of initial diagnosis. Therefore, our group has suggested that using biomarker expression could potentially provide additional information of value in predicting aggressiveness in this particular group of tumors.

In the current biomarker study, we have seen that a panel including TOPO-2$\alpha$ expression, p16, survivin, and E-CAD might predict survival in single pT1HG less than or equal to 3 cm in diameter. Immunohistochemical expression of p16, survivin, and E-CAD at given cutoff points correlated with progression-free survival, and it correlated with increasingly aggressive outcome and increased risk of tumor progression. Similarly, immunohistochemical expression of p16 and survivin turned out to be predictors of overall survival in the univariate analysis performed in the current study. Interestingly, survivin

**Table 2**  Univariate survival analysis according to Kaplan–Meier method and the log-rank test

|  | Overall | Disease-free survival | Log-rank | P | Progression-free survival | Log-rank | P | Overall survival | Log-rank | P |
|---|---|---|---|---|---|---|---|---|---|---|
| p16 |  |  | 2.022 | .155 |  | 3.773 | .049 |  | 6.917 | .009 |
| <60% | 56 | 20 |  |  | 7 |  |  | 4 |  |  |
| >60% | 36 | 7 |  |  | 10 |  |  | 10 |  |  |
| CD138 |  |  | 0.155 | .694 |  | 0.985 | .321 |  | 0.545 | .460 |
| <70% | 13 | 3 |  |  | 1 |  |  | 3 |  |  |
| >70% | 79 | 24 |  |  | 16 |  |  | 11 |  |  |
| Topoisomerase |  |  | 5.302 | .021 |  | 0.093 | .761 |  |  |  |
| <30% | 30 | 13 |  |  | 6 |  |  | 6 | 1.436 | .231 |
| >30% | 62 | 14 |  |  | 11 |  |  | 8 |  |  |
| Survivin |  |  | 0.233 | .629 |  | 6.337 | .012 |  | 16.632 | .000 |
| <50% | 65 | 19 |  |  | 8 |  |  | 4 |  |  |
| >50% | 27 | 8 |  |  | 9 |  |  | 10 |  |  |
| Galectin |  |  | 1.089 | .297 |  | 0.334 | .563 |  | 0.128 | .721 |
| <50% | 37 | 14 |  |  | 8 |  |  | 7 |  |  |
| >50% | 55 | 13 |  |  | 9 |  |  | 7 |  |  |
| CD44 |  |  | 0.445 | .505 |  | 1.775 | .183 |  | 0.746 | .388 |
| Negative | 65 | 18 |  |  | 10 |  |  | 9 |  |  |
| Positive | 27 | 9 |  |  | 7 |  |  | 5 |  |  |
| Hyaluronidase |  |  | 0.345 | .557 |  | 0.072 | .788 |  | 0.278 | .598 |
| <70% | 72 | 22 |  |  | 13 |  |  | 10 |  |  |
| >70% | 20 | 5 |  |  | 4 |  |  | 4 |  |  |
| E-CAD |  |  | 2.266 | .132 |  | 7.270 | .007 |  | 2.645 | .104 |
| <90% | 46 | 16 |  |  | 3 |  |  | 3 |  |  |
| >90% | 46 | 11 |  |  | 14 |  |  | 11 |  |  |

remained as an independent predictor of overall survival, together with E-CAD, which became independent with a minor decreasing of expression, as low as 10% reduction. We also observed that the immunohistochemical expression of TOPO-2α when seen in more than 30% of cancer cells is a positive predictor of disease-free survival.
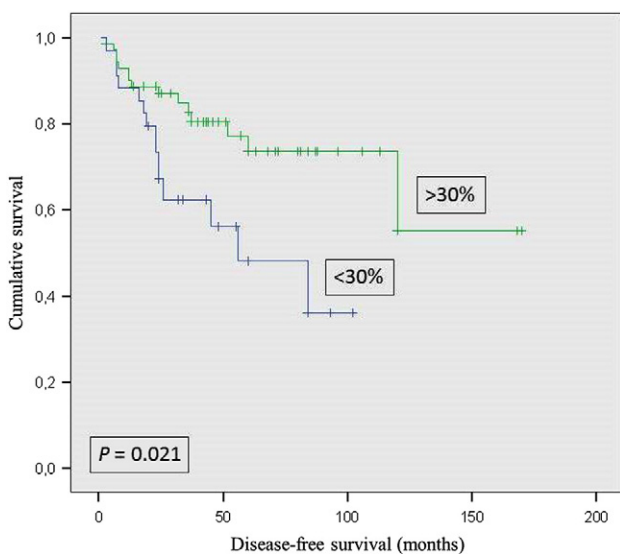


**Fig. 2**  Kaplan-Meier plots of disease-free survival according to immunoexpression of TOPO-2α.

These results are not surprising because p16 is a protein encoded by *CDKN2A* oncosuppressor gene, which belongs to the CDKI family; its function is to restrict the activity of kinases. This protein is able to stop the cellular cycle and to restrict mitotic activity to assist in the process of DNA repair [13]. The gene encoding p16 is mutated or downregulated in several cancer cells. Breast carcinoma progression has been related to overexpression of p16 [14], and this has been reported in head and neck squamous carcinoma [15], in prostate carcinoma [16], and also in aggressive subtypes of bladder carcinoma [17-20].

Survivin is a member of the inhibitors of the apoptosis family. The survivin protein limits caspase activation, leading to a negative regulation of apoptosis [21]. Cases of highly aggressive pT1 bladder carcinoma can be identified by high survivin expression [22-24].

TOPO-2α, a DNA gyrase isoform, plays an important role in the cell cycle and catalyzes the isomerization of DNA by facilitating the passage of 1 strand of DNA through a reversible break in the second strand of DNA. Deregulation or qualitative alterations of the TOP-2α expression during the cell cycle have been reported in both normal tissue and various human neoplasms [25]. Enhanced expression of TOPO-2α is positively associated with a high rate of recurrence and progression in primary non–muscle-invasive bladder cancer [26].

Finally, among the genetic alterations, the different expression of the cadherin family seems to have a link with bladder cancer [27]. The cadherin family includes N-, P-, and E-CAD. Only
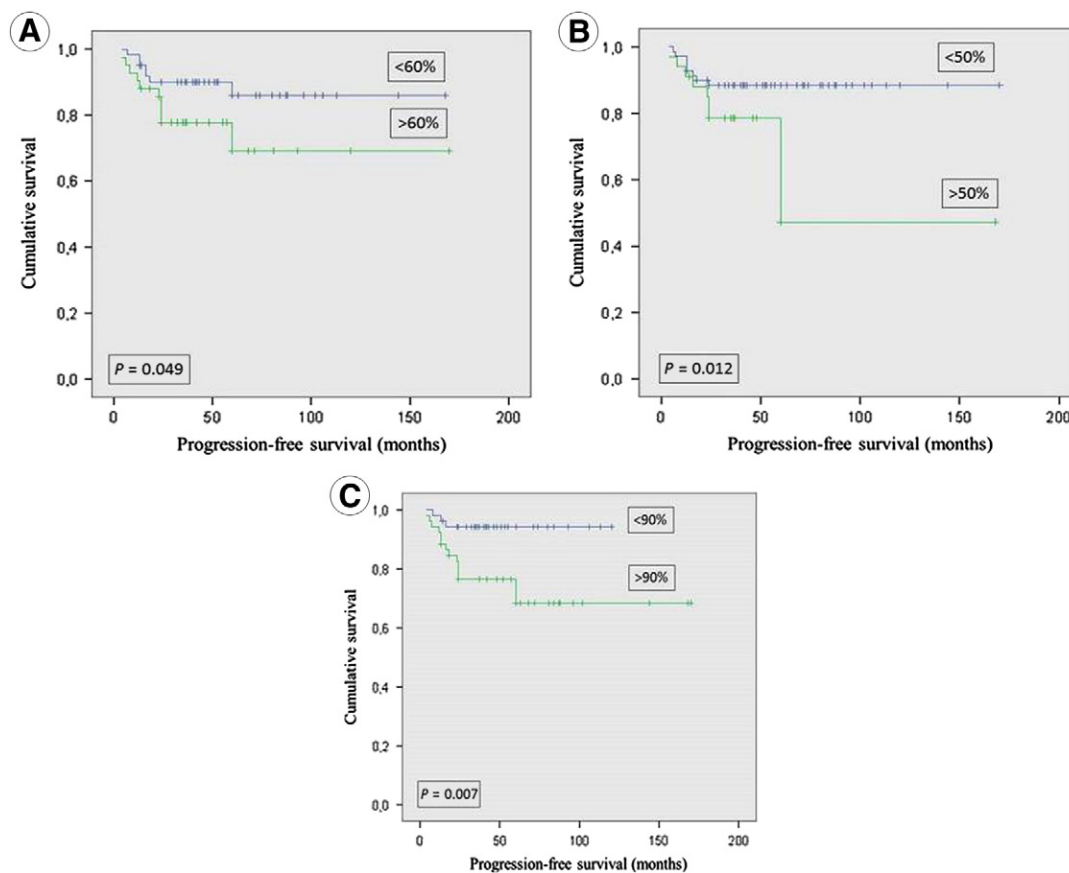
**Fig. 3** Kaplan-Meier plots of progression-free survival according to immunoexpression of p16 (A), survivin (B), and E-CAD (C).

E-CAD, a 120-kDa glycoprotein, the gene of which is located on chromosome 16, is associated with intracellular proteins called *catenins* that mediate between extracellular signals and cytoskeleton microfilaments. The loss of E-CAD expression is associated with a loss of cellular differentiation and increased cellular invasiveness [28,29], and it may correlate with muscle involvement and tumor recurrence. Loss of E-CAD is also described in some aggressive subtypes of urothelial carcinoma of the bladder [30].

In conclusion, our study shows that we were able to identify a series of easily applied immunohistochemical biomarkers to offer further predictive information in addition to conventional prognostic parameters used in the EORTC scoring system; and this may be useful in identifying patients at high risk of
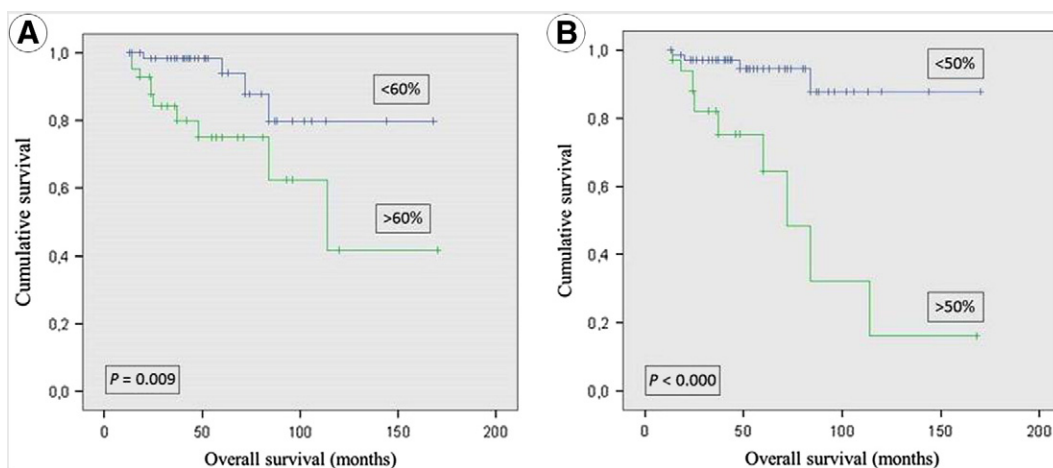


**Fig. 4** Kaplan-Meier plots of overall survival according to immunoexpression of p16 (A) and survivin (B).

**Table 3** Cox multivariate statistical analysis showing independent variables related to disease-free, progression-free, and overall survival of patients with T1 high-grade bladder cancer

|  | $P$ | RR |
|---|---|---|
| Disease-free survival |  |  |
| TOPO-2$\alpha$ ≤30% *vs* >30% | .029 | .978 |
| Progression-free survival |  |  |
| Survivin ≤50% *vs* >50% | .020 | 1.024 |
| Overall survival |  |  |
| Survivin ≤50% *vs* >50% | .006 | 6.175 |
| E-CAD ≤90% *vs* >90% | .030 | 10.958 |

Abbreviation: RR, relative risk.

progression or of death from disease. This particular set of biomarkers includes TOPO-2$\alpha$, p16, survivin, and E-CAD, and if properly investigated may assist to accurately predict survival probability in single T1 high-grade urothelial carcinoma less than 3 cm in diameter.

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