Expression of E-cadherin, Ki-67, and p53 in urinary bladder cancer in relation to progression, survival, and recurrence

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ABSTRACT

Although the incidence varies with age and gender, urothelial bladder cancer is a relatively frequently occurring malignancy with variable clinical behavior that often has high recurrence rates. In this study, we analyzed the tumor tissues of 224 patients with pTa, pT1, and pT2 urinary bladder cancer. We performed a histomorphologic analysis and immunohistochemistry for p53, Ki-67, and E-cadherin, which were selected as markers of the malignant process. For pTa and pT1, univariate analyses of cancer-specific survival (CSS), progression-free survival (PFS), and recurrence-free survival (RFS) were calculated using the Kaplan-Meier method, the log-rank test and Cox regression. Multivariate analysis was performed by the Cox regression analysis. Ki-67 ($P<0.001$) was significantly associated with CSS, but the highest association was shown for E-cadherin ($P<0.001$). For pT1 and pTa, the Kaplan-Meier analysis and the log-rank test revealed significantly worse PFS for patients with higher levels of Ki-67 ($P<0.001$) and lower levels of E-cadherin ($P<0.001$). Based on these obtained results, it can be clearly stated that Ki-67 and E-cadherin expression levels are associated with CSS, PFS and RFS. The clinical utility of these markers is valuable for pTa and pT1 urinary bladder cancer and should be further verified with prospective multi-center trials.

Key words: Urothelium; bladder; cancer; E-cadherin; Ki-67; p53.

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Introduction

Urothelial bladder cancer (UBC) is a frequently occurring malignant disease. In the European Union, the age-standardized incidence rate (ASR - per 100,000) is 30.9 for men and 6.5 for women.[1] UBC and its clinical behavior vary substantially. Currently, tumor stage, grade, and metastasis are regarded as the major prognostic factors for UBC. Although these factors are well defined, there are subgroups within the stages that exhibit different clinical behaviors and prognoses.[2] Non-muscle invasive bladder cancer (NMIBC), is defined as pTa, pT1 and carcinoma *in situ* (CIS) UBC.

A pTa tumor is a heterogeneous disease with a large variation in reported recurrence rates;[3] nevertheless, guidelines for stratification and follow up have been established.[4] Within pT1 tumors, there is significant prognostic variability. In particular, for pT1G3 tumors, the natural course of the disease varies from no recurrence after resection to rapid progression and muscle-invasive and metastatic stages. This variability may compromise standard clinical management and requires individual risk stratification.[5] In muscle-invasive UBC (T2–T4), all cases are high-grade urothelial carcinomas. For this reason, no prognostic information can be provided by grading.[6] However, the identification of certain morphological subtypes may be important for prognosis and treatment decisions.[7] Markers that can stratify patients with an unfavorable prognosis and those who may benefit from early systemic therapy are greatly needed. The currently used system is unable to accurately predict the prognosis of UBC patients with diverse and complicated tumor backgrounds. A search for novel markers to use in combination with the standard systems (stage, grade) is therefore warranted to precisely guide clinical decisions.[8] The biomarkers that have been most extensively studied are E-cadherin, Ki-67, and p53.

E-cadherin is a calcium-dependent adhesion molecule, which is associated with histogenesis, differentiation and stabilization of epithelial cells.[9] It belongs to the cadherin superfamily of adhesive molecules that are utilized in the structure of adherens junctions, which mediate cell-to-cell adhesion and help in maintaining the functional and structural integrity of epithelial tissues.[10] Aberrations and reduction in E-cadherin expression cause ruptures in cell to cell contacts, which allow cells to migrate.[11] Downregulation of E-cadherin expression causes these ruptures and leads to epithelial-to-mesenchymal transition (EMT). EMT speeds up the progression and metastasization of many epithelium-derived carcinomas (including UBC), it also increases the mesenchymal characteristics of tumor cells and subsequently promotes invasive properties and motility of these cells.[12,13] These processes accelerate the progression of tumors. Aberrant E-cadherin expression was reported to accelerate the infiltration and metastasization of malignant cells.[14] Furthermore, chemoresistance and radioresistance of tumor cells was linked to downregulated expression of E-cadherin, which also induces tumor cells to exhibit obvious properties of cancer stem cells.[15] Normal or higher expression of E-cadherin has an inhibitory effect on EMT.

Ki-67 is a nuclear protein that is associated with ribosomal RNA transcription and is a marker of cellular proliferation.[16] Ki-67 is strongly expressed in the fraction of growing cancer cells, and the presence of Ki-67-positive tumor cells indicates a poor prognosis for survival and recurrence.[17] An expert consensus panel has found that certain markers, such as Ki-67 and p53, can predict the recurrence and progression of bladder cancer, but the inconsistency of the available data indicates that these markers are unreliable.[18]

The TP53 gene [17p13] encodes a nuclear transcription factor expressed in response to various stress signals such as DNA damage, heat shock, hypoxia, and oncogene overexpression. Upon activation, p53 maintains the integrity and stability of the genome, cell-cycle arrest and DNA repair or (if DNA could not be repaired) leads to apoptosis.[19] Extensive efforts have been made to study the effects of TP53 mutation on cancer prognosis and therapeutic responses and the role of TP53 mutations in cancer diagnosis.[20] For instance Hodgson and colleagues showed that abnormal p53 expression (null staining pattern or staining in >50% of cells) is useful for precise prognostics of high grade UBC.[21] From a more recent study by Sjödahl *et al*., is evident that changes in expression of p53 is associated with more aggressive molecular subtype of UBC and also with progression into advanced form of UBC.[22]

We conducted a retrospective study to confirm the prognostic value of E-cadherin, Ki-67, and p53 in terms of cancer-specific survival (CSS), progression-free survival (PFS), and recurrence-free survival (RFS) in a clinical subset of 224 UBC patients with five years of follow up. CSS represents the amount of time from either the date of diagnosis or the date of first treatment for a malignant disease, to the date of death caused by this disease. RFS specifically in cancer is the length of time after recieving the primary treatment for a malignant disease, that the patient survives without any signs or symptoms caused by this disease. PFS represents the amount of time after treatment for a malignant disease, that the patient lives without experiencing any worsening of his condition and without progression of his diagnosis.

Materials and Methods

Patients and tumor characteristics

The tumor tissues of 224 patients with UBC were analyzed, of which 116 were pTa, 43 were pT1, and 65 were pT2. Clinical management complied with the current EAU (European Association of Urology) guidelines.[4,23] Out of 65 pT2 patients, 25 patients were treated by radical cystectomy, 11 patients were unfit/not willing to undergo further treatment, 24 patents underwent platinum-based chemotherapy and external radiation therapy. This subgroup of patients was not involved in univariate and multivariate analysis. All tumors were diagnosed according to the TNM classification.[24] In NMIBC, after an initial transurethral resection of the bladder at a single center (2007-2018) and histologic assessment, a second resection was performed, and intravesical chemotherapy and adjuvant instillation of bacillus Calmette-Guérin were administered according to EAU guidelines. All samples were collected in accordance with the Helsinki Declaration and were approved by the local ethics committee.

Histomorphologic analysis and immunohistochemistry

A comprehensive retrospective histomorphologic examination of all the tumor samples according to the World Health Organization (WHO) classifications of 1973[25] was performed independently by two experienced uropathologists who had no knowledge of the clinical evolution of the patients. In case of contradictory results, a re-evaluation was done and a consensus was reached. Immunohistochemistry was evaluated semiquantitatively using a scoring system with 5% steps. The areas of strongest intensity were assessed. Clinical follow-up (CSS, PFS, and RFS) of all the patients was added to our data analysis. We prepared a tissue microarray (TMA) from formalin-fixed paraffin-embedded tissue blocks; one 1.5-mm core was utilized, and the representativeness of the TMA was proven by a histopathologic comparison of the TMA with original sections of the entire tumor. The 4 µm sections were prepared using a microtome and were mounted on poly L-lysine-coated microscopy slides. Immunohistochemistry was car-

ried out in a Leica ST 5050 Immunostainer (Leica, Wetzlar, Germany) utilizing the avidin-biotin peroxidase method with diaminobenzidine as the chromogen according to the instructions provided by the manufacturer.

Immunohistochemistry for E-cadherin, Ki-67, and p53 was performed using standard staining procedures as described previously.[26] Each assay included positive and negative controls. E-cadherin, Ki-67, and p53 positive control slides (Cell marque, USA) were used as a positive controls. The negative control slides were probed with phosphate buffered saline instead of the primary antibody. The primary antibodies were as follows: E-cadherin: clone NCH38, DAKO, Glostrup, Denmark, dilution 1:100; Ki-67: mouse monoclonal, clone MIB-1, DAKO, Hamburg, Germany, dilution 1:50; and p53: mouse monoclonal, Bp53-12, Santa Cruz Biotechnology, Santa Cruz, CA, USA, dilution 1:1000.

Cut-off levels were chosen *a priori* in accordance with previous reports.[27-29] Immunoreactivity to Ki-67 was "low" if the nuclear staining of tumor cells was ≤15% and "high" if the staining of cells exceeded 15%. p53 was considered "negative" if the nuclear staining of tumor cells was less than 10% and "positive" if ≥10%. E-cadherin expression was considered "low" if the staining of tumor cells was less than 50% and "high" if staining was greater than 50%.

Statistical analysis

Statistical analysis was performed using SPSS v.10.0 (SPSS Inc., Chicago, IL, USA). Kaplan-Meier method was used to estimate the survival of patients in time. The log-rank test was used to compare the survival of patients compared to the reference group. The Cox proportional risk model was used to estimate the impact of variables on survival. Logistic regression was used to estimate the probability and impact of variables on the probability of death. The multiple logistic regression method used was Forward Selection Wald. p53, Ki-67, and E-cadherin alone and in combination were analyzed in terms of CSS, PFS and RFS. The P-values <0.05 were considered statistically significant. Primarily, only statistically significant results are shown.

Results

Table 1 presents the clinicopathologic variables of patients with UBC. Of 224 patients, 180 (80.4%) were men. The median patient age was 72.5 years (range: 42-87 years). A total of 51.8% of the patients presented with an initial diagnosis of stage pTa UBC, and 19.2% presented pT1 UBC. The rest of the patients were diagnosed with initial pT2 UBC (29%). The median follow-up period was 58 months (range: 36-89 months).

Figure 1 presents representative microphotographs from immunohistochmical analysis of E-cadherin, Ki-67, and p53 expression in urothelial bladder cancer, with positive and negative controls.

Association with CSS

The log-rank test did not show a significant association of CSS with p53 (Table 1), but statistical significance was demonstrated for Ki-67 and E-cadherin. There was no significant relationship with CSS (P=0.936) in the univariate Cox regression analysis. In contrast, Ki-67 (P<0.001) was significantly associated with CSS, but the strongest correlation was shown in the case of E-cadherin (P<0.001) with a hazard ratio (HR) of 0.38 (CI 0.24-0.61).

The Kaplan-Meier analysis showed that high p53 expression (P=0.936) was not significantly related to CSS (Figure 2). Low Ki-

Table 1. Clinicopathologic variables of patients with urothelial bladder cancer and univariate analyses regarding cancer-specific survival (reference groups are labeled).

		Patients		Kaplan-Meier (survival in months)		Log-rank test (P-value)	Cox regression	
		n	%	Mean (95% CI)	Median		P-value	Hazard ratio (95% CI)
Gender	Male	180	80.4%	65.6 (59.4-71.7)	78.0	0.316	0.320	0.80 (0.50-1.26)
	Female (ref)	44	19.6%	-	-			
Pattern	Papillary	168	75.0%	69.5 (63.2-75.8)	92.0	0.004	0.005	0.48 (0.27-0.83)
	Solid	32	14.3%	49.0 (35.4-62.7)	28.0			0.88 (0.45-1.71)
	Mixed (ref)	23	10.3%	43.1 (26.6-59.6)	30.0			-
Focality	No	117	47.8%	60.9 (52.6-69.2)	69.0	0.353	0.356	0.84 (0.58-1.22)
	Yes (ref)	177	52.2%	66.7 (59.2-74.3)	80.0			
Grading	G1	55	24.6%	77.0 (67.4-86.7)	-	0.008	0.003	0.43 (0.25-0.75)
	G2	122	54.5%	64.1 (56.3-71.9)	92.0			0.64 (0.41-0.99)
	G3 (ref)	47	21%	46.9 (35.5-58.3)	31.0			-
Recurrence	Yes	79	35.3%	55.3 (46.0-64.6)	46.0	0.069	0.072	1.42 (0.97-2.07)
	No (ref)	145	64.7%	68.0 (61.1-74.8)	80.0			-
Stage	Ta	116	51.8%	79.2 (72.4-86.0)	-	<0.001	<0.001	0.25 (0.14-0.46)
	T1	43	19.2%	46.0 (35.0-56.9)	42.0			0.68 (0.36-1.29)
	T2a	48	21.4%	50.4 (37.6-63.3)	23.0			0.64 (0.34-1.19)
	T2b (ref)	17	7.6%	35.3 (21.2-49.4)	28.0			-
Invasivity	Yes	109	48.7%	47.4 (39.4-55.4)	28.0	<0.001	<0.001	2.15 (1.97-4.34)
	No (ref)	115	51.3%	79.8 (73.0-86.5)	-			-
p53	< 10%	13	5.8%	66.2 (47.1-85.3)	78.0	0.936	0.936	0.97 (0.45-2.09)
	≥ 10% (ref)	211	94.2%	63.8 (58.0-69.7)	78.0			-
Ki-67	≤ 15%	73	32.6%	81.3 (73.8-88.8)	-	<0.001	<0.001	0.38 (0.24-0.61)
	> 15 %(ref)	151	67.4%	54.8 (47.8-61.7)	42.0			-
E-cadherin	≤ 50%	69	30.8%	45.8 (36.2-55.3)	26.0	<0.001	<0.001	2.17 (1.49-3.17)
	> 50% (ref)	155	69.2%	71.9 (65.4-78.4)	-			-

67 (P<0.001) and normal E-cadherin (P<0.001) expression is significantly related to improved CSS (Figures 3 and 4). Table 2 presents a multiple logistic regression (Forward Selection Wald) analysis of the UBC clinicopathological variables, where Ki-67 and E-cadherin were significantly associated with CSS.

Association with PFS

Kaplan-Meier analysis and log-rank test showed significantly worse PFS for patients with higher levels of Ki-67 and lower levels of E-cadherin compared to a reference group with inverted values as shown in Table 3 and Figure 5.

Table 2. Multiple logistic regression analysis of p53, E-cadherin and Ki-67 expression of NMIBC patients in relation to CSS.

Method	Variable	P-value	Odds ratio (OR)	95% CI
Multiple logistic regression (Forward Selection Wald)	Ki-67	0.007	0.406	0.210-0.0785
	E-cadherin	0.010	2.391	1.227-4.657
	Age	0.001	1.049	1.020-1.080
	p53	0.155	2.743	0.782-9.623
	Gender	0.702	1.124	0.509-2.480
	Grading	0.089	0.749	0.257-1.455
	Pattern	0.262	0.918	0.501-1.679
	Focality	0.767	0.576	0.216-1.533

NMIBC, non-muscle invasive bladder cancer; CSS, cancer-specific survival.

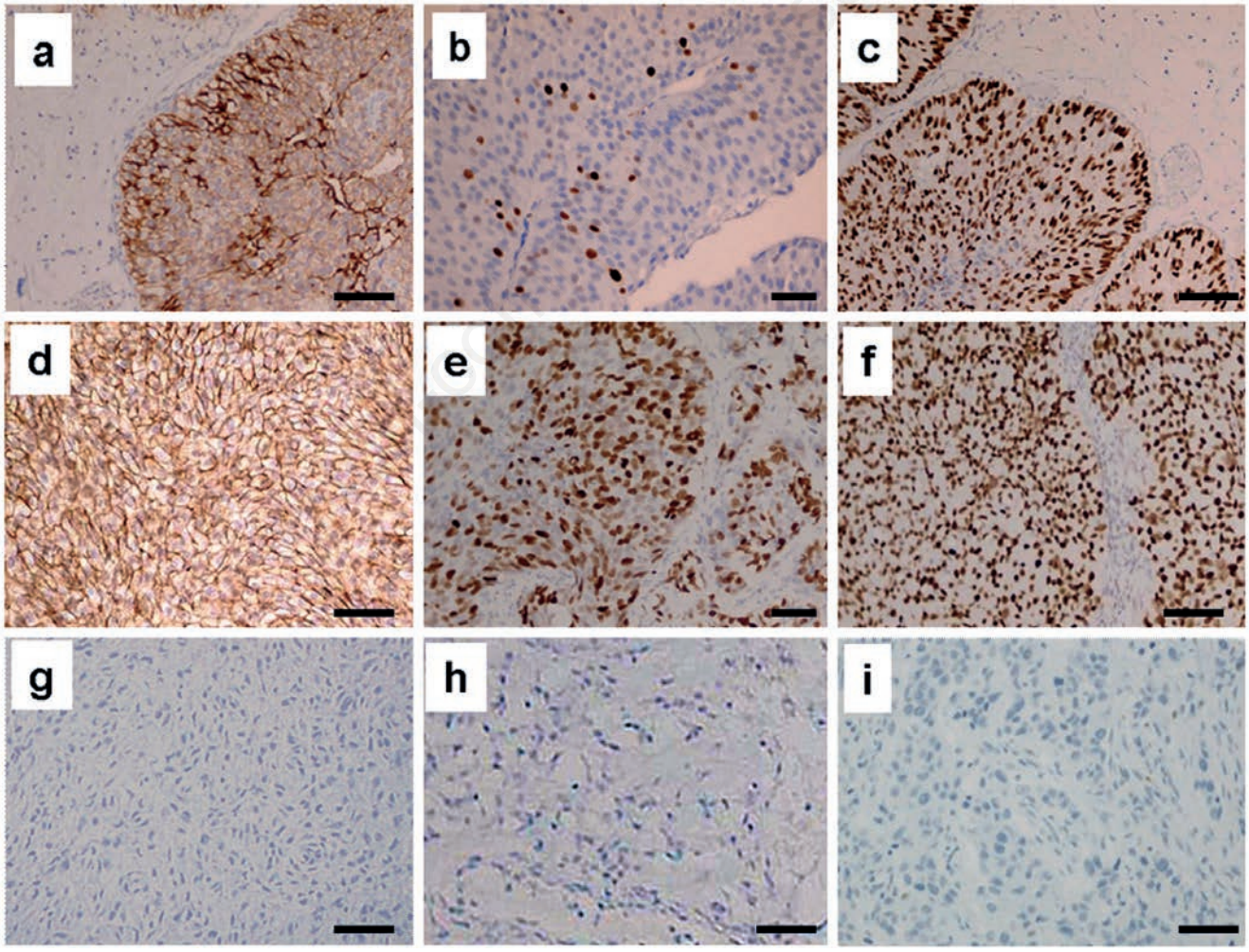

Figure 1. Representative microphotographs of E-cadherin (a), Ki-67 (b), and p53 (c) expression in urothelial bladder cancer (UBC). Positive and negative controls, respectively, for each biomarker: E-cadherin (d,g); Ki-67 (e,h); p53 (f,i). Scale bars: 100 μm.

Association with RFS

In the univariate logistic and Cox regression analyses, p53, Ki-67 and E-cadherin did not show statistical significance. Table 4 indicates that lower E-cadherin and higher Ki-67 expression levels significantly decreased the time to recurrence in patients with UBC compared to the reference group with inverted values.

Discussion

UBC, stages pTa and pT1, is a disease with heterogeneous and variable clinical behavior (recurrence and progression), and risk groups have been created to improve clinical management.[2-4] However, optimal treatment decisions and clinical management remain a challenge, especially for pT1 UBC patients.[5,30] As uncertainties remain in the prediction of clinical outcome in pTa UBC, it is very important to search for alternative ways in diagnostics, e.g. molecular diagnostics to improve the stratification of these patients.[31]

Many different markers (including proliferation markers) have already been studied in association to UBC. Most of these studies have shown that reduced expression of E-cadherin has an ill effect on prognosis in patients with UBC.[32,33] A minority of studies on markers reported no association of poor prognosis and aberrant E-cadherin expression.[34] Several studies and meta-analyses have confirmed that reduced E-cadherin expression is significantly correlated with a poor prognosis in breast cancer, gastric cancer, and head and neck squamous cell carcinoma[7,35] and that reduced E-cadherin expression significantly predicted unfavorable overall survival, PFS, and RFS.[8]

In univariate statistical analyses, our data showed that a low expression level of E-cadherin is associated with worse CSS in the pT1 UBC patients. In the multivariate analysis, a low expression level of E-cadherin was an independent predictor of worse CSS. This is in concordance with most other studies.

In pTa and pT1 bladder tumors, significant correlations between the Ki-67 index and tumor recurrence, progression, and CSS has been described.[36] Our study indicated that patients with altered (higher) expression levels of Ki-67 were associated with a worse histopathology of pT1 UBC and a worse outcome.[37] In addition, Ki-67 positivity is prognostic for predicting tumor recurrence and progression. A combination of EORTC (European Organization for Research and Treatment of Cancer) risk scores and the expression of Ki-67 can improve the risk stratification for both recurrence and progression in NMIBC. Moreover, recent meta-analysis showed that a high Ki-67 expression level was associated with poor RFS, poor PFS, and a short CSS for pTa UBC patients.[38]

Ki-67 has also been studied in correlation with other markers. In the largest series of pT1 UBC patients analyzed to date, Ki-67 and CK20 have been shown to be potential prognostic markers improving the risk stratification of pT1 UBC. These markers are among the reliable indicators of biologic aggressiveness and may contribute to decision making regarding the therapeutic approach for pT1 UBC.[39] Our data show that Ki-67 is an independent marker

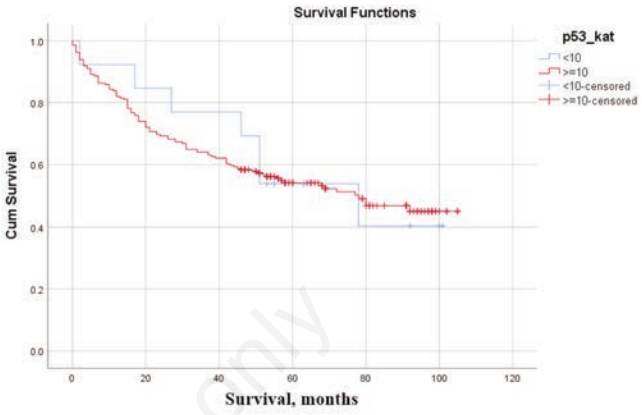

Figure 2. Kaplan-Meier analysis of p53 NMIBC patients in relation to CSS. (Log-rank P-value = 0.936).

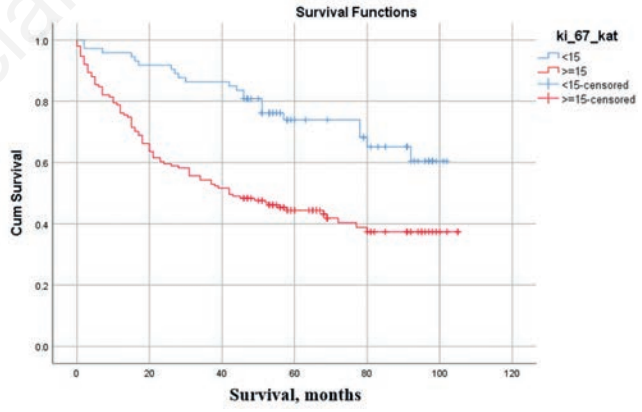

Figure 3. Kaplan-Meier analysis of Ki-67 NMIBC patients in relation to CSS. (Log-rank P-value <0.001).

Table 3. Univariate statistical analysis regarding progression-free survival of NMIBC patients analysed by Kaplan-Meier analysis and log-rank test.

Variable	Kaplan-Meier (time until progression measured in months)			Log-rank test (P-value)
	Mean (95% CI)	Mean (ref)	Median	
p53 <10%	66.2 (44.0-111.9)	64.0	78	0.834
Ki-67 >15%	54.8 (22.8-61.1)	64.0	42	<0.001
E-cadherin <50%	45.8 (11.7-40.2)	64.0	26	<0.001

NMIBC, non-muscle invasive bladder cancer.

of CSS and PFS. Genetic instability with mutations in and the inactivation of the p53 tumor suppressor gene characterizes a prognostically unfavorable group of UBC patients.[40] Mutations result in p53 nuclear accumulation and overexpression detectable by immunohistochemistry. In UBCs of different stages, p53 has been described as an independent predictor of cancer-specific mortality.[41] A meta-analysis of p53 as a prognostic marker could not reveal sufficient evidence to recommend the routine use of p53 as a marker for patient outcomes in BC,[42] and a study that included 309 patients with pT1 UBC reached the same conclusion.[39] In the present series, we found no association of p53 expression with CSS, PFS or RFS. In addition, Ki-67, p53, and E-cadherin in combination have been extensively studied. A study that analyzed NMIBC found that the combined use of p53 and Ki-67 had a predictive value in NMIBC recurrence. The expression of p53 and Ki-67 could be used to predict the risk of NMIBC recurrence postoperatively.[43] p53 can provide 33% more information than that obtained with classical prognostic factors alone, and positivity for p53 has a prognostic value in predicting the progression of pT1 UBC tumors.[44] However, the correlation of p53 expression with stage and grade has been questioned, either alone or in combination with Ki-67.[45] In the present study, a high Ki-67 proliferation index combined with abnormal E-cadherin expression was correlated with worse survival rates compared with tumors with a low proliferation index and normal E-cadherin expression.

As mentioned above, controversy for the use of these biomarkers still exists. In our opinion, future research should focus on prospective multicenter studies to reduce bias and the combination of markers to develop a panel of markers that can accurately predict the prognosis and clinical behavior of NMIBC. These markers are greatly needed.

The limitations of the present analysis include that this study was done retrospectively. Additionally, it is well known that tumor distribution may vary in the tissue cores used for the construction of the TMA. This leads to a variable number of spots being tumor-free in the deeper sections, resulting in a decreased number of analyzable spots. In our study, approximately 15% of the analyzable tumor tissue was lost due to this technical drawback. The present data warrant prospective series with case-by-case staining in light of these limitations.

In conclusion, the present analysis of immunohistochemical markers for pT1 UBC demonstrates that Ki-67 and E-cadherin are potentially predictive of CSS in pT1 UBC. Our data showed that Ki-67 is an independent marker of tumor progression in pTa and pT1 UBC. The analysis of Ki-67 and E-cadherin as combined factors can potentially improve the risk stratification of pT1 UBC regarding CSS. Our data suggest a potential role of Ki-67 and E-cadherin alone and in combination for predicting a worse CSS rate in pT1 UBC that may prompt more radical treatment in these patients. Ki-67 is also related to a worse PFS in pTa and pT1 patients, making Ki-67 a very valuable marker in UBC prognosis. We advocate further research to establish a panel of markers that could be used for better clinical management and prediction for patients with pT1 UBC.

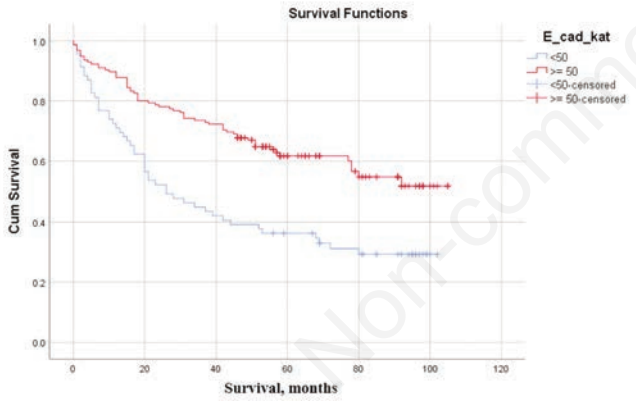

Figure 4. Kaplan-Meier analysis of E-cadherin NMIBC patients in relation to CSS. (Log-rank P-value <0.001).

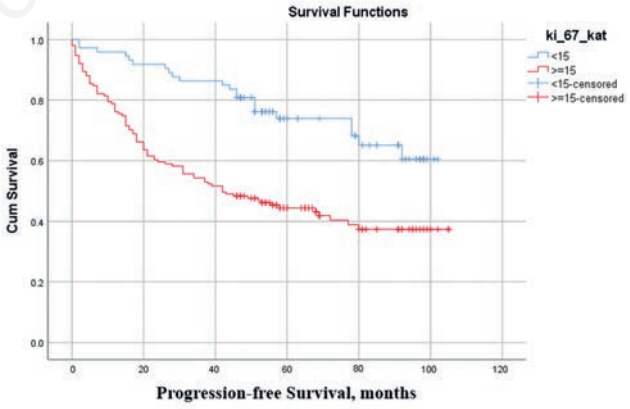

Figure 5. Kaplan-Meier analysis of Ki-67 NMIBC patients in relation to PFS. (Log-rank P-value <0.001).

Table 4. Univariate statistical analysis regarding recurrence-free survival of NMIBC patients analysed by the Kaplan-Meier method and log-rank test.

Variable	Kaplan-Meier (time to recurrence measured in months)			Log-rank test (P-value)
	Mean (95% CI)	Mean (ref)	Median	
p53 < 10%	51.3 (29.2-73.5)	57.6	27	0.666
Ki-67 > 15%	49.1 (42.1-56.0)	57.6	31	<0.001
E-cadherin < 50%	41.4 (32.0-50.6)	57.6	21	<0.001

NMIBC, non-muscle invasive bladder cancer.

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