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Large-Scale Analysis of Cell Cycle Regulators in Urothelial Bladder Cancer Identifies p16 and p27 as Potentially Useful Prognostic Markers

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Key Words
Bladder cancer · Prognosis · Cell cycle

Abstract
Aims: We investigated the value of multiple cell cycle markers for their prognostic impact on overall survival and recurrence-free survival in urothelial carcinoma (UC). ***Methods:*** A tissue microarray consisting of 99 UCs was evaluated for the expression of p53, p16, p21, p27, cyclin D1, cyclin E , Bcl-2, Ki-67 and PCNA. Statistical analysis was performed applying Kaplan-Meier and Cox regression models using receiver operator characteristic curves for determination of markers' cutoffs. ***Results:*** Expression above the cutoffs of Ki-67, p53 and p27, particularly in high-grade and early-stage UC, was associated with worse overall survival, while expression of p16 indicated a better outcome in low-grade and low-stage tumors. Recurrence-free survival was better in patients with high-grade UC expressing PCNA, p16 and cyclin E, and low-grade UC expressing Bcl-2 above the cutoffs, but worse in all tumors with high Ki-67. ***Conclusion:*** Cell cycle deregulation in UC is complex and the prognostic value of the various involved proteins should be differentially regarded with respect to this complexity and other tumor characteristics such as grade and stage. Our results point towards the role of p16- and p27-associated pathways in tumor progression and indicate that, by using standardized approaches for tissue antigen expression, evaluation and cutoff determination, single potentially useful prognostic markers could be identified.

Introduction

The normal cell cycle is characterized by the complex interactions of cyclins, cyclin-dependent kinases and their inhibitors [1]. Though the cell cycle regulation is not fully understood, the prognostic value of cell cycle-regulating proteins in malignant tumors has been widely studied and reported to be of significance in urothelial carcinoma (UC) of the bladder [1–7]. Among the most promising prognostic markers concerning survival, recurrences and progression are those involved in the G1/S-phase transition, such as cyclin D1 (CCND1) and E (CCNE), and cyclin-dependent kinase inhibitors, such as p27, p16 and p21, as well as p53 [1, 8]. Molecular studies revealed loss of chromosome 9 in up to 50% of UCs and p16 located at 9p21 was identified as a major target gene in early bladder cancer [9]. Expression of cell cycle markers has been linked to grade, stage and survival, but individual markers so far have not proved to be superior to

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Table 1. Antibodies, sources, dilutions, pretreatment and staining patterns

Antibody	Source	Clone	Dilution	Pretreatment	Staining pattern
Ki-67	Dako	MIB-1	1:100	wet autoclave; citrate buffer (pH 6)	nuclear
PCNA	Dako	PC10	1:300	microwave (10 min, 750W); distilled water	nuclear
p53	Dako	DO-7	1:50	wet autoclave; citrate buffer (pH 6)	nuclear
p21	Neomarkers	HZ52	1:400	microwave; citrate buffer (pH 6)	nuclear
p27	Dako	SX53G8	1:100	wet autoclave; citrate buffer (pH 6)	nuclear/cytoplasmic
p16	Neomarkers	16PO4 (JC2)	1:50	wet autoclave; citrate buffer (pH 6)	nuclear/cytoplasmic
CCND1	Vector/Al	SP4	1:100	wet autoclave; citrate buffer (pH 6)	nuclear
CCNE	Neomarkers	13A3	1:20	microwave; citrate buffer (pH 6)	nuclear
Bcl-2	Dako	124	1:50	wet autoclave; citrate buffer (pH 6)[1]	cytoplasmic

[1] Amplifier A/B (Ventana).

classical prognostic parameters [10–13]. Studies on combinations of different markers that might enhance the predictive power, including p53 together with p16, CCND1 and CCNE together with phosphorylated retinoblastoma protein as well as p21 and p27, suggest multiple alterations, especially in invasive UC that might contribute to recurrence, progression and, finally, worse survival [3–5, 14]. The expression of the antiapoptotic protein Bcl-2 and the proliferation index, assessed by Ki-67 and PCNA, have been reported to be potentially useful in predicting prognosis as well [4, 5, 7, 12, 14–17]. Nevertheless, results obtained in different studies remain controversial.

To further assess the prognostic value of these multiple markers in UC and rationally address determination of prognostic cutoff levels applying receiver operator characteristics (ROC)-based methods, a large-scale immunohistochemical study on 99 specimens of patients with UC of the bladder in a tissue microarray (TMA) format was performed.

Material and Methods

Samples

Ninety-nine primary diagnoses of UC of the bladder, obtained by means of transurethral resection and diagnosed between January 1994 and December 1997 at the Institute of Pathology, Medical University of Innsbruck, were included in our study. All cases were reviewed by 2 pathologists (A.B. and G.M.) and reclassified according to the WHO 2004 classification. Our study group consisted of 27 pTa (22 low-grade and 5 high-grade UCs), 33 pT1 (2 low-grade and 32 high-grade UCs), 34 pT2–4 tumors (all high-grade UCs) and 5 carcinomata in situ (CIS). The clinical and pathological characteristics of the 99 patients have been reported elsewhere [18].

TMA Construction

Samples were brought into a TMA format as previously described [19]. Three tissue cores (0.6 mm) and, in small specimens, 1–3 cores were obtained based on tissue amount including superficial, invasive portion tumor and the adjacent stroma.

Immunohistochemistry

Immunohistochemistry was performed, except for CCNE and p21, which were manually incubated, on an automated immunostainer (Nexes; Ventana), applying the streptavidin-biotin peroxidase technique with diaminobenzidine as chromogen. The antibodies, sources, dilutions, pretreatment and staining patterns are summarized in table 1. Immunohistochemical staining was evaluated calculating the mean value of the relative proportion (percentage) of positively staining cells of the individual cores of each case based on at least 100 cells/case. For p16 and p27 cytoplasmic staining was also considered as previously reported [20, 21].

Statistics

The Statistical Package of Social Sciences (SPSS 12.0 for Windows) was used. The Spearman rank test was used to test correlations between individual markers. The analysis of variance (ANOVA) was used to compare means between groups. For de-

Table 2. Number of evaluable cases (n), number of positive cases (n_p), mean expression and cutoff values for OS and RFS determined by ROC of the studied markers

	n	n_p[1]	Mean expression[2]	Cutoff OS, %	Cutoff RFS, %
Ki-67	93	91 (98)	26.75 ± 20.81	9.5	8.75
PCNA	89	88 (99)	58.60 ± 31.82	58	74
p53	93	43 (46)	20.16 ± 31.47	14	4.5
p21	92	91 (99)	93.14 ± 19.46	98	98
p27	90	46 (46)	8.82 ± 16.42	5.5	4.5
p16	93	42 (45)	18.22 ± 30.29	1.5	23
CCND1	89	35 (39)	8.07 ± 17.20	0.15	7
CCNE	94	66 (70)	18.94 ± 23.07	8.5	0.2
Bcl-2	91	52 (57)	14.12 ± 19.70	13.15	1.55

[1] Figures in parentheses are percentages.
[2] Data are presented as mean percentages ± SD.

Table 3. Correlations between different markers

Ki-67						
PCNA	0.342 *0.001*	0.217 *0.044*				
p53	0.445 *0.000*		0.288 *0.006*			
p21	0.210 *0.047*					
p16		0.320 *0.002*		0.301 *0.004*	–0.241 *0.024*	
CCNE	0.292 *0.005*	0.262 *0.012*	0.433 *0.000*	0.311 *0.003*	–0.235 *0.028*	0.278 *0.007*
Bcl-2		0.231 *0.028*				0.284 *0.007*
	Ki-67	p53	p21	p27	CCND1	p16

Calculated by Spearman rank test. The upper figures are correlation coefficients, the lower italicized figures are p values.

termination of optimal cutoff values of continuous variables, ROC curves by plotting sensitivity versus 1 – specificity were used. The optimal cutoff point was calculated using Youden's index (Y) [22]. Overall survival (OS) and recurrence-free survival (RFS) were analyzed by the Kaplan-Meier method applying the cutoff values calculated by ROC/Y and compared by the log rank test, except for continuous variables, where a Cox regression model was applied. Multivariate analysis was performed to identify independent prognostic markers for OS and RFS using a Cox multistep regression model. $p < 0.05$ was considered significant.

Table 4. ROC and Kaplan-Meier analysis for markers showing prognostic significance concerning OS and RFS

	ROC			Kaplan-Meier analysis	
	AUROC	p value	cutoff, %	median OS	$p^{log\ rank}$
Ki-67	0.571	0.287	>9.5 <9.5	30 118	0.004*
p53	0.536	0.593	>14 <14	17 62	0.047*
p27	0.564	0.351	>5.5 <5.5	31 57	0.03*
p16	0.584	0.213	>1.5 <1.5	64 35	0.087
CCND1	0.663	0.021*	>0.15 <0.15	64 31	0.065
				median RFS	$p^{log\ rank}$
Ki-67	0.516	0.793	>8.5 <8.5	10 88	0.018*
PCNA	0.599	0.11	>74 <74	90 12	0.023*
p16	0.587	0.154	>23 <23	80 12	0.027*
CCNE	0.603	0.09	>0.2 <0.2	71 9	0.014*
Bcl-2	0.60	0.106	>1.55 <1.55	71 38	0.003*

AUROC = Area under ROC curve. * $p < 0.05$.

Results

TMA Quality

Three tissue cores/case could be arrayed in 90 cases, whereas 2 and 1 core could be obtained in 6 and 3 cases, respectively, resulting in a total number of 285 cores. After immunohistochemistry, between 225 (79%) and 262 (92%) cores and between 89 (90%) and 94 (95%) cases could be evaluated.

Expression and Correlations

The number of evaluable cases, the number of positive cases and the mean percentage ± SD of positively staining cells, as well as the cutoffs for OS and RFS are summarized in table 2. Nuclear and cytoplasmic expression of p16 was found in all positively staining specimens, while p27 expression was mainly restricted to the nuclei with only 6 specimens exhibiting additional cytoplasmic staining (fig. 1). The correlations between markers are shown in table 3. Compared with clinical and pathological parameters, such as age, grade, stage, recurrence and

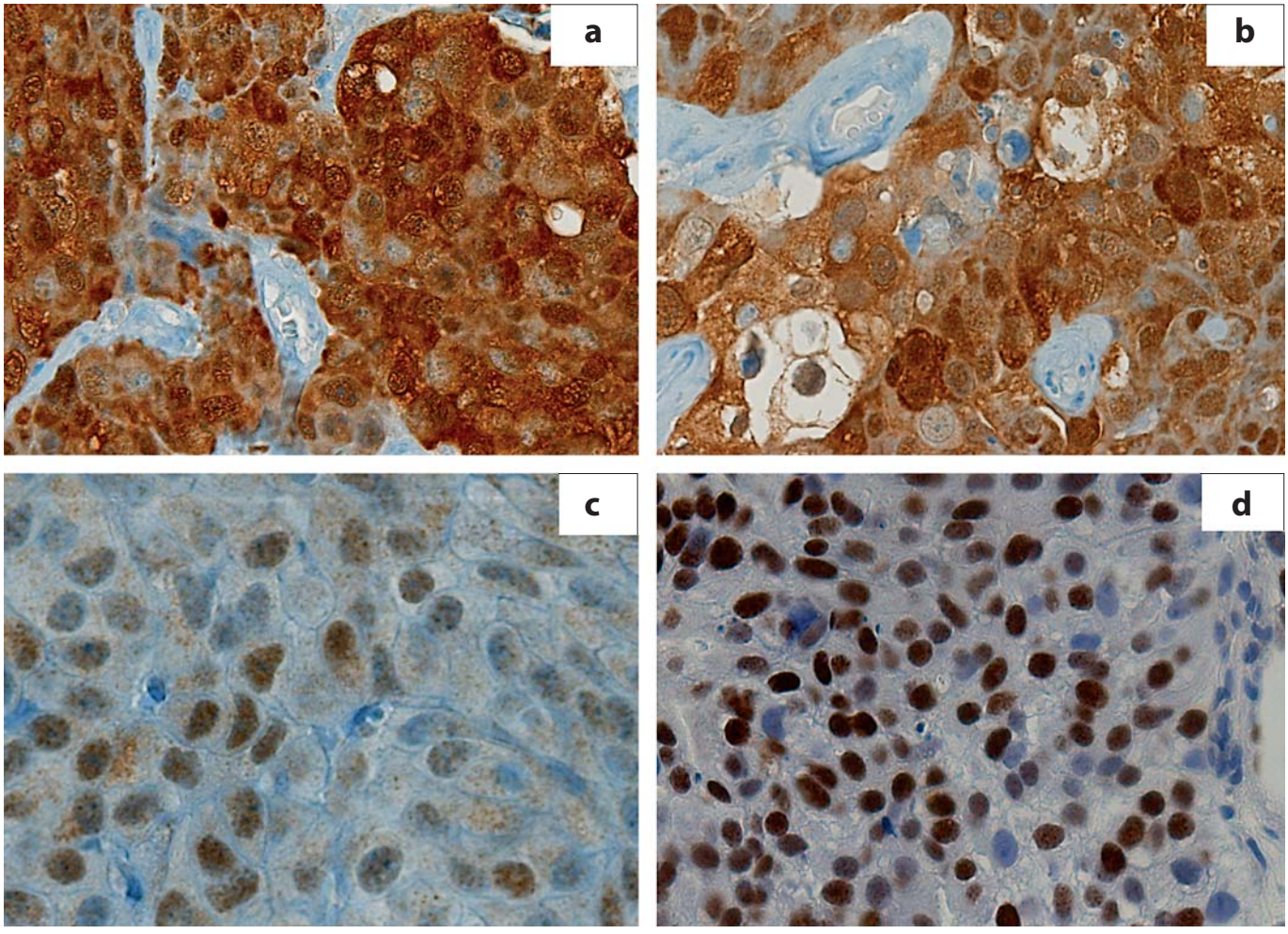

Fig. 1. High-grade bladder cancer with nuclear and cytoplasmic expression of p16 (**a**, **b**; ×200 and ×400); low-grade bladder cancer with nuclear p27 expression and slight cytoplasmic staining (**c**; ×400) as well as strong nuclear expression of CCNE in a high-grade tumor (**d**; ×200).

disease progression, only Ki-67, PCNA and p53 were associated with higher grade ($p^{ANOVA} < 0.001$, $p^{ANOVA} = 0.001$ and $p^{ANOVA} = 0.001$, respectively) and stage ($p^{ANOVA} < 0.001$, $p^{ANOVA} = 0.021$ and $p^{ANOVA} < 0.001$, respectively). Ki-67 and CCND1 expression were higher in tumors with subsequent disease progression ($p^{ANOVA} = 0.042$ and $p^{ANOVA} = 0.032$, respectively). High CCNE expression correlated with the presence of tumor-associated CIS ($p^{ANOVA} = 0.018$).

Receiver Operator Characteristics

ROC showed significant discriminatory power considering OS for the expression of CCND1, with an optimal cutoff value of 0.15% (table 4). For all other markers, ROC curves showed only borderline significance or lacked discriminatory power considering OS (table 4). ROC showed borderline discriminatory significance for RFS for PCNA, CCNE, Bcl-2, p16 and p27, but lacked significant discriminatory power for all other markers (table 4). Cutoff values for all markers considering OS and RFS determined by ROC/Y and used for Kaplan-Meier analysis are shown in table 2.

Overall Survival

OS was influenced by age, grade, stage and number of recurrences, but not by tumor-associated CIS and progressive disease [18]. A worse OS was found for Ki-67, p53 and p27 expression above the cutoff (table 4; fig. 2a).

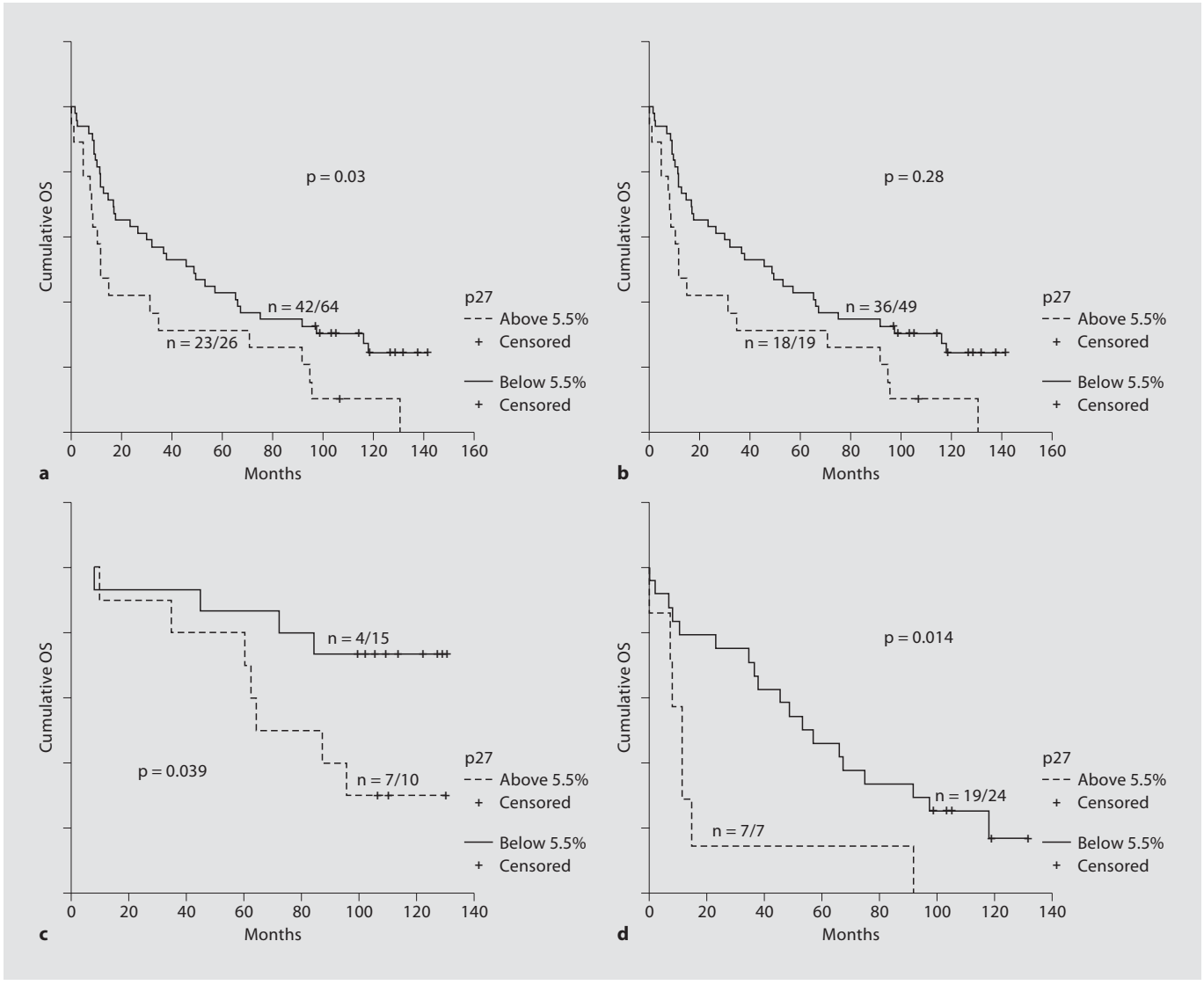

Fig. 2. a Expression of p27 above the cutoff is associated with a worse OS in UC of the bladder. Stratified by grade and stage, p27 expression above the cutoff indicates a worse OS in high-grade (**b**) as well as in pTa (**c**) and pT1 (**d**) tumors. p27 <5.5% versus p27 >5.5% compared by the log rank test. n = Number of events/number of cases.

Stratified by grade, p27 and Ki-67 expression above the cutoff, but not p53, were associated with a worse OS in high-grade tumors ($p^{log\ rank}$ = 0.028 and $p^{log\ rank}$ = 0.036, respectively). Stratified by stage, this finding held true for p27 expression in pTa and pT1 tumors ($p^{log\ rank}$ = 0.039 and $p^{log\ rank}$ = 0.014, respectively; fig. 2b–d). In addition, p16 expression in >1.5% of tumor cells showed an association with a better OS in low-grade (mean OS 103 months, median survival was not reached) and pTa tumors (mean OS 109 months, median survival was not reached) compared to p16 expression in <1.5% of tumor cells (mean OS 63 months, median OS 72 months and mean OS 73 months, median OS 84 months, respectively; p = 0.043 and p = 0.019, respectively; fig. 3a, b).

Multivariate analysis for OS considering age, grade, stage, number of recurrences, Ki-67, p53, p27 and p16 revealed that only increased age (relative risk = 1.062, 95% CI 1.108–1.018, p = 0.005), low number of recurrences (relative risk = 0.682, 95% CI 0.499–0.933, p = 0.017) and p53 expression in >14% (relative risk = 1.026, 95% CI

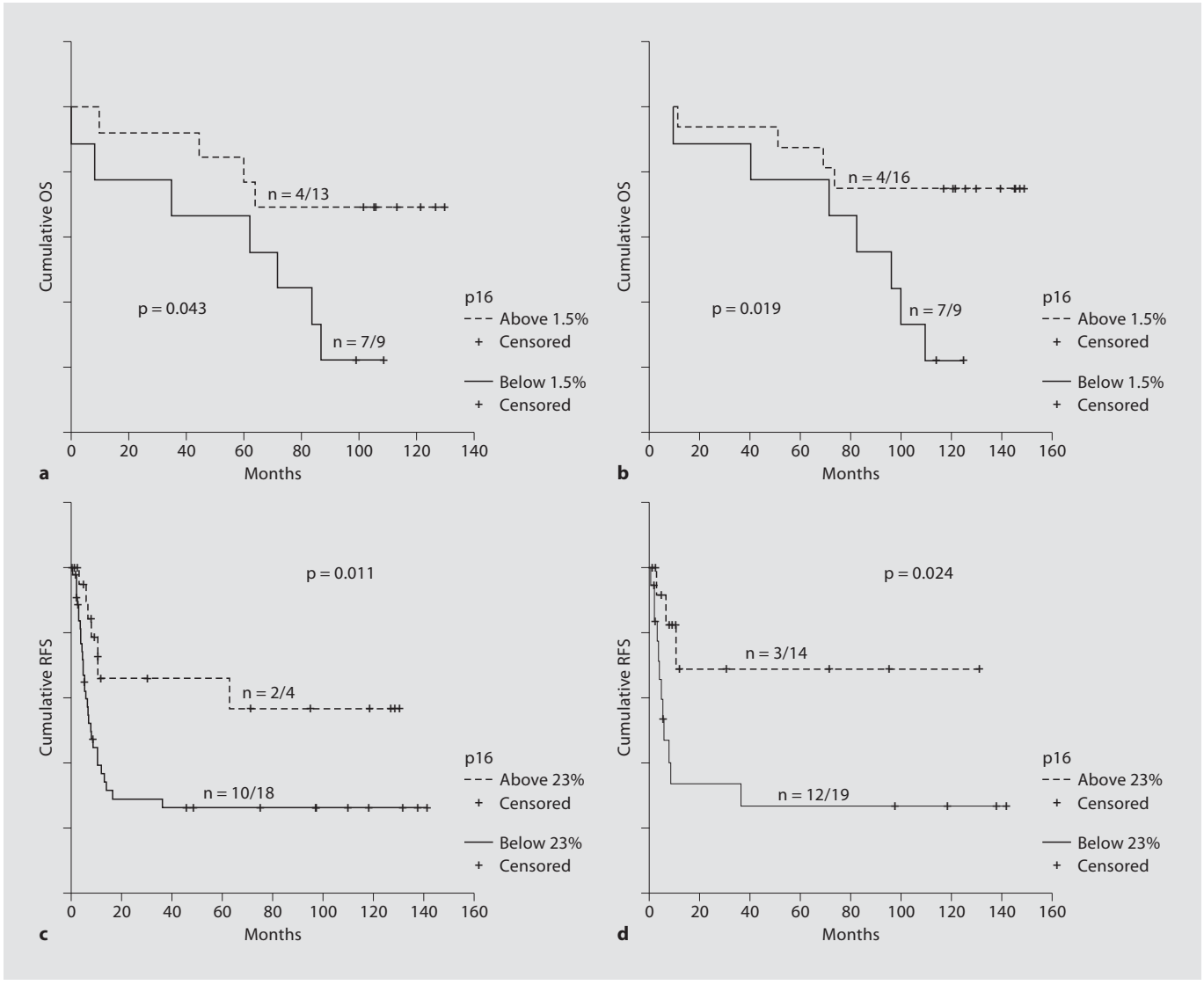

Fig. 3. Expression of p16 above the cutoff is significantly associated with a better OS in low-grade (**a**) and pTa (**b**) tumors and predicts a better RFS in high-grade (**c**) and pT2–4 tumors (**d**). p16 <1.5% versus p16 >1.5% and p16 <23% versus p16 >23% compared by the log rank test. n = Number of events/number of cases.

1.008–1.044, p = 0.004) were independent predictors of a worse OS.

Recurrence-Free Survival

Ki-67 expression in >8.75% of tumor cells was associated with a shorter RFS when compared to tumors with Ki-67 expression in <8.75%. Patients with PCNA expression of more than 74% had a longer RFS than those with PCNA expression below 74%. Similarly, Bcl-2, CCNE and p16 expression above the cutoff was associated with a better RFS (table 4). Considering all other known clinical and morphological parameters, only progress at relapse was associated with a worse RFS [18].

Stratified by grade and stage, PCNA expression above the cutoff was associated with a longer RFS only in high-grade tumors ($p^{\log\ rank} = 0.023$). CCNE expression above the cutoff was only associated with a better RFS in high-grade ($p^{\log\ rank} = 0.02$), pTa ($p^{\log\ rank} = 0.043$) and pT1 tumors ($p^{\log\ rank} = 0.008$). Expression of p16 above the cutoff predicted a longer RFS in high-grade ($p^{\log\ rank}$ =

0.011) and pT2–4 tumors ($p^{log\ rank}$ = 0.024; fig. 3c, d). Bcl-2 expression above the cutoff was a predictor for a better RFS in low-grade ($p^{log\ rank}$ = 0.0005) and pTa tumors ($p^{log\ rank}$ = 0.0001). Ki-67 did not show any association with RFS when either stratified for grade or stage.

Multivariate analysis for RFS considering progressive disease, Ki-67, PCNA, CCNE, p16 and Bcl-2 showed that only lacking progressive disease (relative risk = 0.234, 95% CI 0.109–0.503, $p < 0.001$), Bcl-2 (relative risk = 0.978, 95% CI 0.958–0.998, p = 0.032) and CCNE expression (relative risk = 0.983, 95% CI 0.966–0.999, p = 0.04) were independent prognostic factors considering better RFS.

Discussion

In UC a variety of studies have investigated cell cycle markers for their predictive value concerning disease-free, overall and disease-specific survival, but results often remained contradictory [2, 10]. Possible reasons include the heterogeneity of evaluated cases in different studies. Another important point is the use of different cutoffs for marker positivity, with only few studies determining the optimal cutoff values by ROC, rather than by stepwise analysis or by median or mean percentages of positively staining cells [6, 7, 12]. Similarly to previous studies, increased expression of Ki-67 and p53 proved to be associated with a worse OS in our collective [4, 12, 15–17]. Indeed, p53 mutations have been suggested as a key event in the development of aggressive UC phenotype [23].

A particular new aspect of our study concerns the prognostic value of p16 and p27 protein expression. p16 was assessed as nuclear and cytoplasmic staining, as previously reported, though some authors regard cytoplasmic staining for p16 as nonspecific [20]. Nuclear expression of p16 above the cutoff values was associated with a better OS in low-grade and low-stage tumors and predicted a better RFS in high-grade and high-stage tumors in our patient cohort. Hitchings et al. [20] and Korkolopoulou et al. [24], who studied the influence of p16 on disease survival and progression, emphasized the protective effect of p16 in UC, especially in tumors without p53 accumulation and normal expression of p16, which is in accordance with our finding of improved OS in low-grade and pTa tumors, believed to lack p53 mutations [23]. Indeed, p16 promotor hypermethylations resulting in inactivation are more frequently observed in invasive than noninvasive tumors, indicating that inactivation of p16 is associated with a more aggressive phenotype, but the reported hypermethylation frequencies in UC of the bladder vary considerably [9, 25]. Additionally, lost p16 expression could also be due to deletions at 9p21, which are commonly observed in UC, but such deletions are as frequent or even more frequent in pTa and low-grade tumors compared to high-grade tumors [9, 26, 27]. Thus, retained p16 expression in UC, as observed in our cohort, may identify a distinct subgroup of both low- and high-grade as well as low- and high-stage UC patients without *p16* gene alterations, which are probably accompanied by a better prognosis. Strong cytoplasmic p16 staining was often seen in high-grade tumors (all with accompanying nuclear staining), pointing towards a probable deregulation leading to cytoplasmic retention of p16, analogously to p27 [21]. Nevertheless, in our study RFS was better in these patients as well and one may speculate that cytoplasmic p16 accumulation could simply reflect a compensatory mechanism to overcome other defects in cell cycle regulation. We observed a negative correlation between p16 and CCND1 expression. Loss of p16 and high CCND1 has been shown to be associated with hyperphosphorylated retinoblastoma protein, resulting in increased cell proliferation [8]. Intact p16 could probably counterbalance the oncogenic potential of CCND1 in UC [2, 4, 8]. Interestingly in that consideration, we observed a tendency towards better OS for patients with CCND1 expression and several authors have linked CCND1 expression to low grade, low stage and better OS, too [2, 5]. It seems likely that the proposed oncogenic role of CCND1 can be modified, particularly in UC, by alterations in other cell cycle-controlling molecules, such as loss of p16 or p53 mutations. Such complex interactions may probably be responsible for the observed association of CCND1 expression with subsequent UC progression despite its potential positive prognostic importance considering OS [4, 6].

Loss of p27 in bladder cancer has been reported to indicate a worse prognosis in UC [28]. In contrast, we showed that p27 expression in >5.5% of tumor cells is associated with worse OS in high-grade and pTa/pT1 tumors. Expression of p27 has been reported to be associated with an adverse disease outcome in diffuse large B cell lymphomas and posttranslational modifications were found to be responsible for the loss of the growth-inhibitory function of p27 despite its cellular accumulation [21, 29]. Increased p27 expression in superficial high-grade tumors may probably represent accumulation of a nonfunctional protein, which is supported by the pres-

ence of cytoplasmic p27 staining in some cases in our study. The impact of p27 on prognosis only in high-grade pTa/pT1 UCs further underlines differences between superficial low- and high-grade tumors. In addition, we observed a correlation of p27 and CCNE expression. Loss of p27 expression has been reported to be associated with loss of CCNE expression and a more aggressive tumor phenotype [3, 28, 30]. Indeed, high CCNE expression has been linked to a longer disease-specific survival and a lower risk of recurrence in UC [3, 30]. In our study, a better RFS in tumors with CCNE expression above the cutoff could be found, but no prognostic significance of CCNE concerning OS. On the other hand, gains of the *CCNE* gene have been detected in high-grade tumors, suggesting CCNE as a potential oncogene [30]. This finding is supported by several studies in other cancer types [31–35]. Therefore, we speculate that the impact of CCNE on prognosis probably depends on its expression causes (gene amplification, degradation failure, supraordinal cascades) and the functional state of p27 or other cell cycle regulatory molecules, including p53 and p21.

The prognostic value of other cell cycle-related proteins remains contradictory. Bcl-2, though associated with a better RFS, correlated with p53 accumulation, strongly suggesting complex interactions, which are reflected in the conflicting results on its prognostic impact obtained in different studies [14, 36, 37]. PCNA, though associated with Ki-67 expression, predicted a better RFS in high-grade tumors, which may be due to the interaction with other molecules, such as p21, modulating its functional activity [38].

To summarize, our results based on standardized analysis approaches suggest complex partially redundant, partially concurrent interactions between the proteins involved in cell cycle control, proliferation and apoptosis in UC and point towards the role of p16- and p27-associated pathways. However, profound changes in complementary or concurrent complex cell cycle control pathways probably result in different impacts of single molecules on disease outcome. Therefore, though studies on the expression of cell cycle markers may result in new insights of their function, the use of these molecules as prognostic markers still needs to be particularly prospectively evaluated.

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