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Protein Expression and Gene Copy Number Analysis of Topoisomerase 2α, HER2 and P53 in Minimally Invasive Urothelial Carcinoma of the Urinary Bladder - a Multitissue Array Study with Prognostic Implications

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Abstract. *Background: Due to their variable clinical course, there is a need for new prognostic parameters in minimally invasive (stage T1) bladder carcinomas. Materials and Methods: Using multitissue arrays, protein expression and gene copy numbers of topoisomerase (TOP2α), HER2 and p53 were investigated by immunohistochemistry and by fluorescence in situ hybridization (FISH) in 73 T1 tumors. The results were compared with tumor recurrence and progression. Results: The median TOP2α and p53 index was 21% (range, 3-59%) and 7% (range, 0-93%), respectively. HER2 overexpression (score 3+) was detected in 9 cases (12%). High TOP2α and p53 indices and HER2 overexpression were significantly associated with earlier tumor recurrence, but not with earlier tumor progression. While TOP2α and p53 gene amplification was detected in no case, 5 cases (8%) showed HER2 gene amplification, which was related to HER2 3+ score in 4 cases. Loss of TOP2α, HER2 and p53 gene was observed in 4 (8%), 8 (13%) and 6 cases (12%), respectively. By univariate analysis, TOP2α index (p=0.0267), HER2 score (p=0.028) and p53 index (p=0.0188) were significantly and loss of TOP2α gene (p=0.0575) tendentially correlated with tumor recurrence, while loss of HER2 gene (p=0.069) and loss of p53 gene (p=0.0587) were tendentially correlated with tumor progression. In a multivariate analysis, which also included tumor grade and T1 substage, TOP2α index (p=0.043) and p53 index (p=0.02) were identified as independent predictors of tumor recurrence and loss of p53 gene (p=0.012) and T1 substage (p=0.029) as independent predictors of tumor progression. Conclusion: Immunohistochemical TOP2α and p53 staining as well as FISH analysis of p53 gene copy numbers and T1 substaging are helpful means of providing additional information on the biological behavior of T1 transitional cell carcinomas.*

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Minimally invasive urothelial bladder carcinomas (stage T1) display a variable clinical course. Following transurethral resection (TUR), about three-quarters of patients develop tumor recurrence, whereas progression into a muscle-invasive stage (T>2) is observed in 20-30% of patients (1,2). Besides TUR, therapeutic options include intravesical instillation of *Bacillus Calmette-Guérin* (BCG) or cytotoxic agents, radiation therapy and radical cystectomy. Usually, patients with recurrent superficial tumor (Ta or T1) are treated by TUR followed by BCG or cytotoxic agent treatment, while radical cystectomy is recommended for those patients undergoing tumor progression into a muscle-invasive stage. Currently, tumor stage and grade are the most established prognostic factors of bladder carcinoma. However, these parameters cannot precisely predict the behavior of most bladder tumors (3).

Numerous studies have focused on the identification of new parameters that may allow more accurate prediction of the biological behavior of bladder carcinoma. Concerning pT1 tumors, the depth of submucosal invasion (</≥ 1.5 mm), which subdivides stages pT1a and pT1b, has been proposed as a prognostic factor (4). However, substaging of pT1 tumors is not commonly accepted and has not been included in the actual WHO staging system (5). Among immunohistochemical parameters, proliferation-associated markers were widely investigated in many studies. A predictive value has been reported for the mitotic index (6), S-phase fraction (7), thymidylate synthase activity (8), as well as for Ki67 antigen (6,9-12), proliferating cell nuclear antigen (PCNA) (13,14), Cyclin E (15), Cyclin D1 (12,16,17), Cyclin D3 (12), $p21^{WAF-1}$ (12,18) and $p27^{KIP1}$ immunoreactivity (12,15,19) in

superficial bladder carcinomas. Expression of topoisomerase 2α (TOP2α) represents another proliferation marker which has recently been reported to correlate with the survival of patients with superficial bladder carcinomas (20). The nuclear enzyme TOP2α plays a pivotal role in the condensation of chromatin and segregation of chromosomes during mitosis. Furthermore, it serves as a target for chemotherapeutic agents. Until now, no studies examining TOP2α expression in pT1 bladder carcinomas have been published.

A large number of other parameters, not strictly associated with proliferation, have been investigated in bladder cancer in the past. Among them, p53 (9,12,21,22) and HER2 immunoreactivity (23) have been reported to bear some prognostic impact in superficial bladder carcinomas. The HER2 gene, also referred to as the c-erbB-2 gene, is located on the long arm of chromosome 17 in close proximity to the TOP2α gene, while the p53 gene is located on the short arm of chromosome 17. In breast carcinoma, a large proportion of tumors has been found to co-amplify both genes (24), whereas a recent study on bladder carcinoma reported such co-amplification only in a very small minority of cases (25). No association between anomalies of HER2 or TOP2α gene copy numbers and the clinical course of superficial bladder carcinomas has yet been described. With regard to p53 gene copy numbers, correlation of p53 deletion with grade and stage of bladder carcinomas has been observed in one study (26), but no reports on a correlation between numerical anomalies of the p53 gene and prognosis of bladder tumors are available as yet.

The aim of the present study was to analyze the protein expression and anomalies of gene copy numbers of TOP2α, HER2 and p53 in a series of 73 minimally invasive bladder carcinomas and to evaluate their prognostic impact by comparing the results with recurrence- and progression-free survival of the patients. All stainings were performed using multitissue arrays, which are a highly representative and cost-effective measure of analyzing large series of tumors simultaneously (27).

Materials and Methods

Seventy-three patients with stage T1 papillary urothelial bladder carcinomas, diagnosed in our University Hospital between 1988 and 1998, were included in the study. The median age was 67 years (range, 37-85 years), and the male-to-female ratio was 4.6 : 1. In all cases, tumors were removed by TUR, followed by intravesical BCG therapy, and the results of regular follow-up monitoring (at least twice a year) for a period of at least two years were available. Histologically proven tumor recurrence or progression were regarded as events for the survival analyses, while patients whose follow-up ended without manifestation of tumor recurrence or progression were treated as censored data in the survival analyses. Tumor recurrence was observed in 52 patients (71%) after an average interval of 30 months and tumor progression into a muscle-invasive stage (pT>2) in 27 patients (37%) after an average interval of 45 months.

Hematoxylin-eosin-stained sections of all tumors were reviewed by two pathologists (S.K., I.R.), who confirmed diagnosis, tumor grade according to the WHO criteria (28) as well as stage T1 in all cases. Additionally, T1 substaging was performed in all cases according to Cheng *et al.* (4) by measuring the maximum depth of tumor infiltration. In cooperation with Euroimmun Laboratory Diagnostics (Lübeck, Germany), representative, 2x2 mm-sized areas were cut from 5-µm-thick paraffin sections of all tumors and mounted on multitissue arrays (Biochips®), as previously described (29). Immunohistochemical staining was performed according to a standard three-step immunoperoxidase technique with diaminobenzidine (DAB) as chromogen. The HercepTest® (Dako, Glostrup, Denmark), which contains a prediluted polyclonal rabbit antibody (A0485), was used for HER2 staining, while the monoclonal mouse antibodies Ki-S1 (diluted 1:200; Dako) and pab 1801 (dilution 1:50; Novocastra, Newcastle, UK) were used for TOP2α and p53 staining. By counting the proportion of tumor cells with stained nuclei within 200 representative tumor cells, TOP2α and p53 indices were calculated. The immunohistochemical HER2 score was evaluated semiquantitatively (0, 1+, 2+ or 3+) as recommended by the HercepTest®.

Numerical anomalies of the TOP2α, HER2 and p53 gene were evaluated by fluorescence *in situ* hybridization (FISH). The PathVysion® kit (Abbott-Vysis, Wiesbaden, Germany), the TOP2α-pharmDX® kit (Dako) and the Chromosome 17p13/Alphasatellite 17 dual-color cocktail probe (Q-Biogene, Heidelberg, Germany) were used for analysis of HER2, TOP2α and p53 gene copy numbers, respectively. All of these kits provided visualization of the genes as red signals and additional reference staining of the centromer region of chromosome 17 as green signals. Nuclei were counterstained with diaminidophosphoindole (DAPI). According to the strict criteria proposed by the manufacturer of the PathVysion® kit, red and green signals were counted within 60 tumor cell nuclei in each tumor using an Axioplan epi-illumination fluorescence microscope (Zeiss, Göttingen, Germany) and appropriate multi-bandpass filter sets. The ratio of red: green signals was calculated for each case. Gene amplification was defined by a fluorescence ratio of >2.0. Genes were considered to be gained (over-presented) with a ratio of >1.1 and lost (under-presented) with a ratio of <0.9. All numerical evaluations were performed independently by two pathologists (S.K., I.R.), and only the mean value of each case was included in the study.

All statistical analyses were performed using the SPSS® statistical software package (SPSS, Munich, Germany). The level of statistical significance (p) was ≤0.05. A tendential, but not statistically significant, relationship was assumed when a p value of >0.05 but ≤0.10 was reached. Correlation between numerical parameters was calculated by linear regression and between categorized parameters by Pearson's χ^2 test. Association between categorized and numerical parameters was assessed using the Mann-Whitney U-test (for parameters with two categories) or Kruskal-Wallis test (for parameters with at least three categories). Kaplan-Meier survival curves were plotted from recurrence- and progression-free survival data. Apart from the HER2 score, which was stratified as score 3+ (overexpression) *vs.* score 0-2+ (lack of overexpression), all other parameters were stratified into two groups on the basis of their median values (≤ *vs.* > median). The log-rank test and the univariate Cox Proportional Regression Hazard model were used to analyze differences between groups. Also, other parameters with potential prognostic relevance (tumor

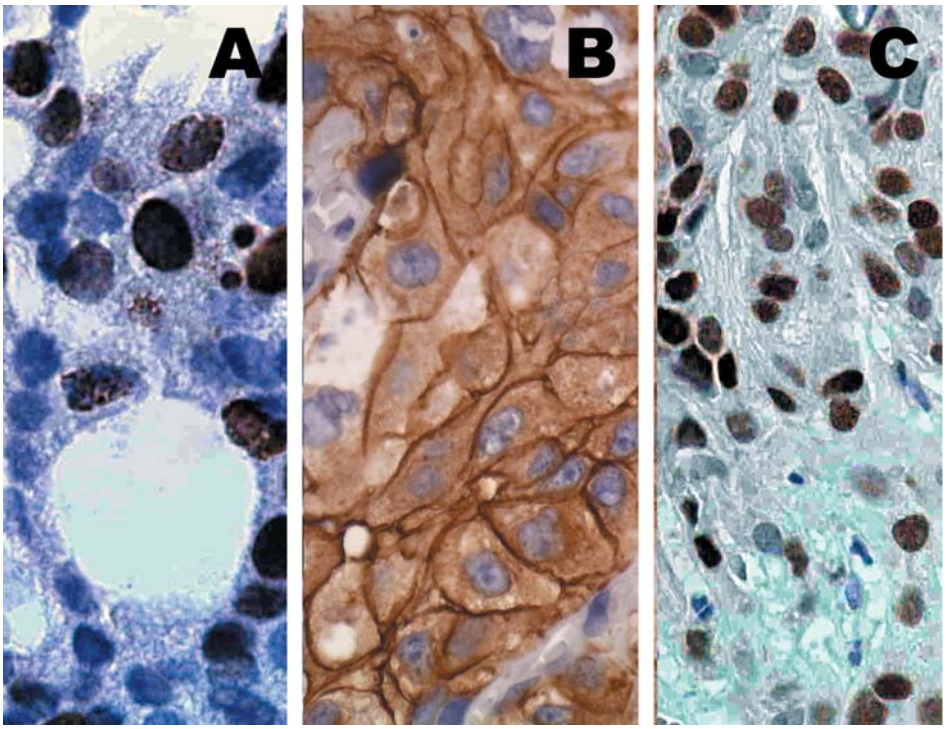

Figure 1. *Immunohistochemical stainings of minimally invasive urothelial bladder carcinomas. TOP2α (A) and p53 (C) are detectable within numerous tumor cell nuclei, indicating high TOP2α and p53 indices. HER2 (B) displays strong membrane-bound expression in this score 3+ tumor (magnification 400x in A and B, 200x in C).*

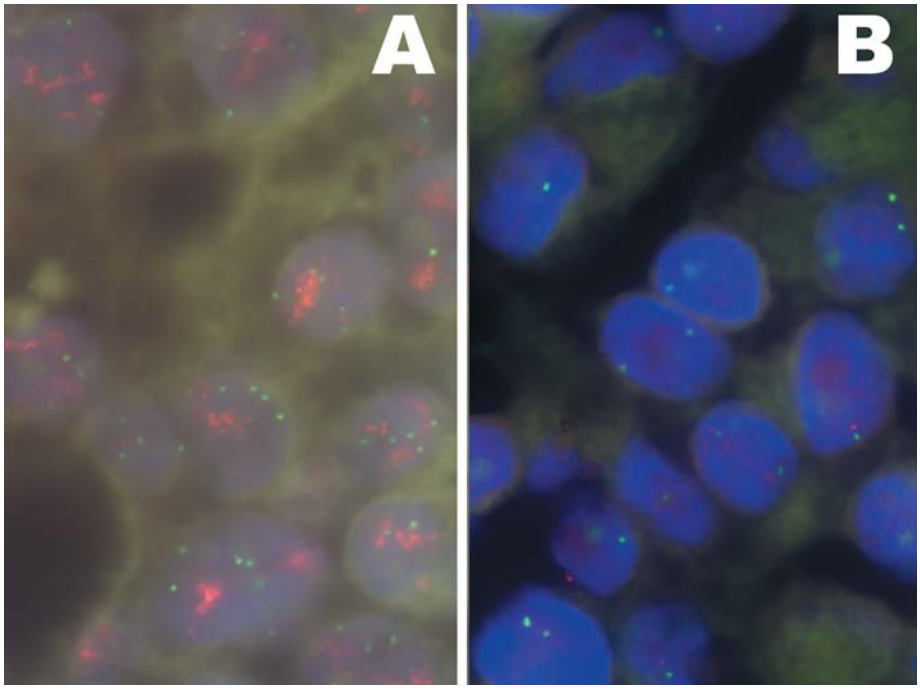

Figure 2. *Demonstration of HER 2 gene amplification (A) and p53 gene loss (B) in T1 bladder carcinomas by FISH (magnification 1000x, respectively). The gene copies are indicated by red signals and centromer regions of chromosome 17 by green signals. Tumor cell nuclei are counterstained with DAPI. In (A), tumor cells demonstrate polysomy 17 (4-5 signals per cell), but the multiplicity of HER2 gene copies supplies evidence of HER2 gene amplification. In (B), the tumor cells are diploid for chromosome 17, and only a few cells contain a single p53 gene copy (red signal), which indicates p53 gene loss.*

Table I. *Correlation of potentially prognostic parameters with recurrence-free and progression-free survival of patients with minimally invasive transitional cell carcinomas of the bladder (univariate Cox Regression analysis).*

Parameter	Correlation with recurrence-free survival			Correlation with progression-free survival		
	p	RR	95% CI	*p*	RR	95% CI
Tumor grade						
G2 (n=33) *vs.* G3 (n=40)	**0.006**	2.23	1.67-3.91	0.307	1.49	0.69-3.21
T1 substage						
T1a (n=53) *vs.* T1b (n=20)	**0.048**	1.79	1.01-3.18	**0.038**	2.28	1.05-4.97
Sex						
M (n=60) *vs.* F (n=13)	0.377	1.35	0.69-2.64	0.318	1.55	0.65-3.69
Age						
≤ (n=34) *vs.* > 67 yrs (n=39)	0.130	1.54	0.88-2.68	0.337	1.46	0.67-3.16
TOP2α index						
≤ (n=26) *vs.* > median (n=25)	**0.031**	2.14	1.07-4.27	0.679	1.24	0.45-3.45
p53 index						
≤ (n=31) *vs.* > median (n=30)	**0.023**	2.09	1.11-3.96	0.471	1.37	0.58-3.26
HER2 score						
3+ (n=9) *vs.* 0-2+ (n=57)	**0.036**	2.46	1.06-5.71	0.165	2.19	0.72-6.62
TOP2α gene gain						
yes (n=12) *vs.* no (n=39)	0.407	1.41	0.63-3.17	0.429	1.61	0.50-5.19
TOP2α gene loss						
yes (n=3) *vs.* no (n=48)	0.074	3.03	0.90-10.18	0.410	2.38	0.30-18.70
p53 gene gain						
yes (n=4) *vs.* no (n=45)	0.228	2.09	0.63-6.93	0.926	1.07	0.24-4.83
p53 gene loss						
yes (n=6) *vs.* no (n=43)	0.370	1.62	0.56-4.66	0.075	3.25	0.89-11.91
HER2 gene amplification						
yes (n=5) *vs.* no (n=55)	0.754	0.83	0.25-2.72	0.864	0.84	0.11-6.32
HER2 gene gain						
yes (n=14) *vs.* no (n=46)	0.733	0.88	0.43-1.81	0.583	1.30	0.51-3.34
HER2 gene loss						
yes (n=8) vs. no (n=52)	0.169	1.78	0.78-4.03	0.081	2.45	0.90-6.71

Abbreviations: RR, relative risk; CI, confidence interval; M, male; F, female; yrs, years

grade, age, sex, T1 substage) were tested by univariate analysis. In order to prove an independent prognostic value of a parameter, a multivariate Cox analysis was performed, in which a *p* value of 0.10 was adopted as the limit for entering and removing covariates. Of the prognostic parameters that contributed significantly to the model, the effect was calculated in terms of relative risks (RR) and the associated 95% confidence intervals (CI).

Results

Immunohistochemical and FISH stainings of multitissue arrays were generally successful. In all staining runs, however, on average one-fifth of all tumors were not interpretable, mainly because of detachment of some chips from the array and because of lack of representative tumor cells in single cases. Nevertheless, data from about 80% of tumors could be obtained. The TOP2α and p53 indices varied from 5 to 59% (mean, 23%; median, 21%) and from 0 to 93% (mean, 27%; median, 7%), respectively. Examples of tumors with high TOP2α and p53 indices are illustrated in Figures 1A and 1C. A significant correlation was found between the TOP2α and p53 indices ($p=0.001$). Immuno-histochemical HER2 overexpression (score 3+) was registered in 9 cases (12%; an example is illustrated in Figure 1B), while 15 (20%) were assigned score 2+, 29 (40%) score 1+ and 13 (18%) score 0. No significant relationship existed between the HER2 score and TOP2α index ($p=0.906$) or between the HER2 score and p53 index ($p=0.543$).

In all tumors, the average number of TOP2α, HER2 and p53 gene copies per tumor cell was 2.4 (median, 2.2; range, 2.0-4.0), 3.3 (median, 2.3; range, 1.7-18.0) and 2.3 (median, 2.1; range, 1.7-3.5), respectively. A significant correlation existed between copy numbers of TOP2α and the p53 gene

Table II. *Multivariate Cox regression analysis on the correlation of parameters with recurrence-and progression-free survival of patients with minimally invasive transtitional cell carcinomas of the bladder*.*

Parameter	Correlation with recurrence-free survival			Correlation with progression-free survival		
	p	RR	95% CI	*p*	RR	95% CI
Tumor grade						
G2 (n=33) *vs.* G3 (n=40)	0.316	1.60	0.64-3.98	-	-	-
T1 substage						
T1a (n=53) *vs.* T1b (n=20)	0.703	1.24	0.41-3.70	**0.029**	3.68	1.14-11.8
TOP2α index						
< (n=26) *vs.* > median (n=25)	**0.043**	2.54	1.03-6.27	-	-	-
p53 index						
< (n=31) *vs.* > median (n=30)	**0.020**	3.28	1.21-8.89	-	-	-
HER2 score						
3+ (n=9) *vs.* 0-2+ (n=57)	0.450	1.62	0.46-5.71	-	-	-
TOP2α gene loss						
yes (n=3) *vs.* no (n=48)	0.517	1.57	0.40-6.12	-	-	-
p53 gene loss						
yes (n=6) *vs.* no (n=43)	-	-	-	**0.012**	6.63	1.52-28.9
HER2 gene loss						
yes (n=8) *vs.* no (n=52)	-	-	-	0.269	2.28	0.53-9.83

Abbreviations: RR, relative risk; CI, confidence interval.
*: multivariate analysis of recurrence-free and progression-free survival includes only those parameters yielding a *p* value of ≤0.10 by univariate analysis

(p=0.018), but not between TOP2α and the HER2 gene (p=0.392) or between p53 and the HER2 gene (p=0.239). The average ratio between gene copy number and chromosome 17 number was 1.06 for TOP2α (median, 1.00; range, 0.74-1.71), 1.24 for HER2 (median, 1.00; range, 0.76-6.15) and 0.99 for p53 (median, 0.99; range, 0.76-1.95). Only the HER2 gene was found amplified in five cases (8%), whereas the TOP2α and p53 gene were not found amplified in any case. Gains of TOP2α, HER2 and the p53 gene were observed in 24%, 23% and 8% of cases, and losses occurred in 6%, 13% and 12% of cases, respectively. Examples of tumors with HER2 gene amplification and p53 gene loss are illustrated in Figures 2A and 2B.

When examining the relationship between protein expression and gene copy number, no significant relationship was found for the TOP2α (p=0.630) or the p53 gene (p=0.148). With regard to HER2, tumors with protein overexpression (score 3+) had significantly higher fluorescence ratios (mean, 3.87; median, 4.74; range, 0.75-6.42) than tumors with lesser scores (means, 1.02-1.06; medians, 0.98-1.03; range, 0.76-2.02; p<0.05). Of the five tumors with HER2 gene amplification, four were associated with score 3+ and one with score 2+.

By univariate Cox regression analysis (Table I) and by log-rank test, all immunohistochemical parameters (TOP2α index, p53 index, HER2 score) as well as tumor grade and T1 substage were found to correlate significantly and one FISH parameter (TOP2α gene loss) to correlate tendentially with recurrence-free survival. With regard to progression-free survival, only T1 substage, but none of the other parameters, reached the level of statistical significance (Table I). However, two FISH parameters (HER2 and p53 gene loss) showed a tendential correlation. When performing a multivariate analysis which included all parameters that yielded a p value of <0.10 by univariate analysis, p53 index (p=0.020) as well as the TOP2α index (p=0.043) were identified as parameters with independent prognostic value for predicting tumor recurrence and p53 gene loss (p=0.012), as well as T1 substage (p=0.029) as parameters with independent prognostic value for predicting tumor progression (Table II). The Kaplan-Meier curves of these four parameters are illustrated in Figures 3A-D.

Discussion

Because the clinical outcome of minimally invasive papillary urothelial bladder carcinomas is heterogeneous, criteria which would allow assessment of the biological behaviour more accurately are highly desirable. If patients with a high risk of tumor progression could be specifically identified,

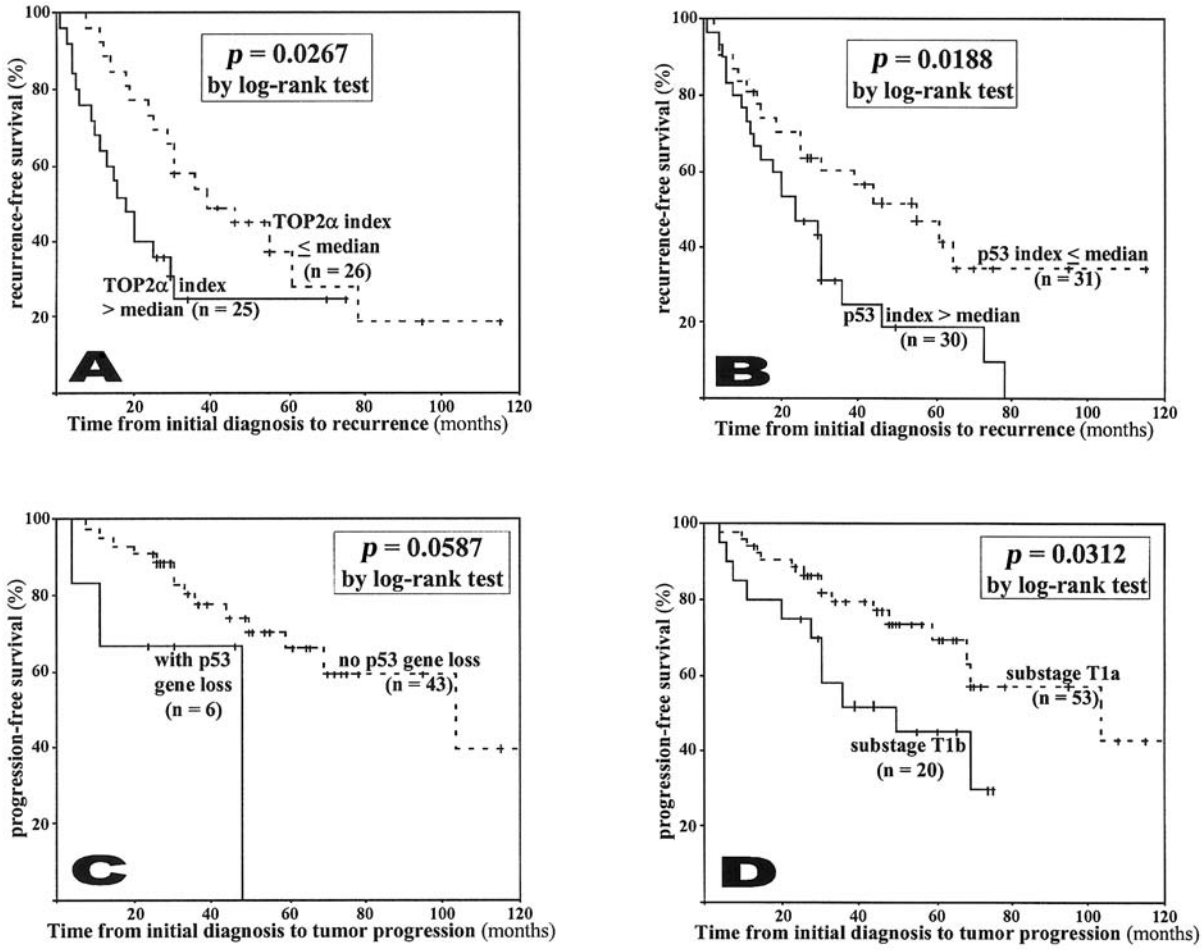

Figure 3. *Kaplan-Meier survival curves on the influence of immunohistochemical TOP2α index (A), p53 index (B), p53 gene loss (C) and T1 substage (D) on recurrence-free (A, B) and progression-free survival (C, D) of patients with minimally invasive bladder carcinomas. Censored data are marked by small vertical bars.*

they would profit from an aggressive therapy to prevent tumor spread. On the other hand, patients with a low risk of tumor recurrence or progression could be spared aggressive treatment and could be followed-up at longer intervals. Moreover, in cases where conventional prognostic parameters point to divergent prognostic outcomes, new prognostic parameters may provide additional arguments for therapeutic decisions.

In the present study, we evaluated the protein expression and gene copy number of three parameters – a proliferation marker, a growth factor receptor and a tumor suppressor – for their usefulness as predictors of recurrence and progression of pT1 bladder carcinomas. One of these genes (p53) is located on the short arm and the other two genes (TOP2α and HER2) in close proximity on the long arm of chromosome 17. Among the three parameters, p53 has been most extensively studied. However, most authors have focused on p53 protein expression, and no reports on the prognostic value of numerical aberrations of the p53 gene on the prognosis of bladder cancer are available in the literature. In this respect, our finding that p53 gene loss is of independent value in predicting progression of minimally invasive bladder tumors is unique. However, our finding is slightly restricted by the fact that the group of tumors with p53 gene loss is very small (12% in our study). Interestingly, deletion of the p53 gene, in association with increased p53 protein expression, has been found to be correlated with tumor genomic heterogeneity (30), and one study described a significant relationship between p53 deletion and high tumor grade (26). G3 tumors also showed a higher incidence of p53 deletion (17%) than G2 tumors (8%) in the present study, but the difference was not statistically significant ($p=0.355$ by Pearson's χ^2 test).

With regard to p53 protein expression, our finding that the p53 index is an independent predictor of tumor recurrence confirms the results of other studies reporting a correlation between p53 expression and the clinical outcome of superficial bladder carcinoma (9,12,21,22). However, several studies have not found such a relationship (10,18,31-35). The different results reported in the literature may be caused by differences in tumor collectives (superficial / only non-invasive / only minimally invasive carcinomas), immunohistochemical techniques (antibodies, antigen retrieval) and evaluation of p53 indices.

The prognostic impact of HER2 in bladder carcinoma has been examined in several studies, and some of them reported a relationship between HER2 overexpression and worse clinical outcome (23,35-39). These reports are confirmed by the results of the present study, but we found a significant correlation between immunohistochemical HER2 score and tumor recurrence only by univariate and not by multivariate analysis. Corresponding to other reports (25,39-41), we observed HER2 gene amplification only in a minority of tumors (8%), and most of them displayed immunohistochemical score 3+. However, two of six score 3+ tumors with known HER2 gene status showed no amplification, which is in accordance with the reported finding that molecular mechanisms other than gene amplification account for HER2 protein overexpression in a considerable proportion of bladder carcinomas (25,39,41). A significant correlation between HER2 gene amplification and prognosis was neither observed in this nor in other studies. Nevertheless, we found a tendential relationship between loss of HER2 gene and progression-free survival ($p=0.081$ by univariate analysis), which we regard as a noteworthy finding that should be further validated in a future study.

TOP2α represents a proliferation-associated protein that is overexpressed in many tumors with high proliferative activity. It catalyzes the topological isomerisation of DNA by passing one strand of DNA through a reversible break in a second DNA strand (42). Thus, it has different functional properties compared with the Ki-67 antigen, which represents a more commonly used proliferation marker. Nakopoulou *et al.* (20) reported no quantitative relationship between TOP2α and Ki-67 antigen protein expression in bladder cancer. In their study, which included a mixed cohort of non-invasive, minimally invasive and muscle invasive tumors, a correlation between an elevated TOP2α index and inferior survival was found by univariate, but not by multivariate analysis. The results of the present study, in which only minimally invasive tumors were included, exceeded those of Nakopoulou *et al.* by identifying the TOP2α index as an independent predictor of early tumor recurrence ($p=0.043$ by multivariate analysis). Additionally, a tendential correlation between loss of the TOP2α gene and tumor recurrence was registered ($p=0.074$ by univariate analysis). TOP2α gene amplification could not be detected in any of our tumors. In a similar multitissue array study, Simon *et al.* (25) reported TOP2α gene amplification in a very small percentage of T1 bladder carcinomas (1.8%). However, a different definition of gene amplification and different DNA probes were used in the cited study.

In accordance with the findings of Simon and coworkers, the TOP2α gene copy number showed no significant relationship with TOP2α protein expression, prognosis or HER2 gene copy number in our study. These findings are contrary to those reported in breast cancer, where TOP2α gene amplification occurs in a considerable proportion of tumors and is associated with decreased survival (43). Moreover, co-amplification of TOP2α and HER2 gene is a wide-spread phenomenon in breast cancer (24). Thus, our findings underline the view that, in comparison with breast cancer, different molecular events may determine cancerogenesis and tumor progression in bladder carcinomas. Additionally, our results suggest that immunohistochemical quantification of TOP2α protein expression may be a useful means of assessing tumor recurrence of T1 bladder carcinomas.

Also T1 substaging was proposed to provide additional prognostic information in minimally invasive bladder carcinomas (4,22,44). Originally, substages T1a and T1b were defined according to the maximum tumor infiltration above or beneath the muscularis mucosae (45). However, according to this definition, it was found that a large proportion of up to 48% could not be staged (46). Thus, Cheng *et al.* (4) introduced a substaging that is based on the depth of infiltration as measured by micrometer, and they suggested a maximal depth of 1.5 mm as a useful threshold to stratify patients with low and high risk of tumor progression. The present study, which applied the criteria proposed by Cheng and coworkers, confirms their findings by demonstrating a significant, independent prognostic impact of T1 substage for assessing tumor progression ($p=0.029$ by multivariate analysis). Therefore, we regard declaration of T1 substage as a useful adjunct in histopathological reports.

In conclusion, the results obtained in the present multitissue array study document that T1 substaging, assessment of immunohistochemical TOP2α and p53 indices as well as evaluation of p53 gene loss by FISH represent useful means of providing additional prognostic information in minimally invasive bladder carcinomas.

References

1 Malmström P, Busch C and Norlen BJ: Recurrence, progression and survival in bladder cancer: a retrospective analysis of 232 patients with ≥5-year follow-up. Scand J Urol Nephrol *21*: 185-195, 1987.

2 Holmäng S, Hedelin H, Anderström C and Johansson SL: The relationship among multiple recurrences, progression and prognosis of patients with stages Ta and T1 transitional cell carcinoma of the bladder followed for at least 20 years. J Urol *153*: 1823-1827, 1995.

3 Stein JP, Grossfeld GD, Ginsberg DA, Esrig D, Freeman JA, Figueroa AJ, Skinner DG and Cote RJ: Prognostic markers in bladder cancer: a contemporary review of the literature. J Urol *160*: 645-659, 1998.

4 Cheng L, Neumann RM, Weaver AL, Spotts BE and Bostwick DG: Predicting cancer progression in patients with stage T1 bladder carcinoma. J Clin Oncol *17*: 3182-3187, 1999.

5 Sobin LH and Wittekind C: TNM Classification of Malignant Tumours, 6 ed. Indianapolis, Wiley 2002, pp. 187-189.

6 Oosterhuis JW, Schapers RF, Janssen-Heijnen ML, Smeets AW and Pauwels RP: MIB-1 as a proliferative marker in transitional cell carcinoma of the bladder: clinical significance and comparison with other prognostic factors. Cancer *88*: 2598-2605, 2000.

7 deVere-White RW, Deitch AD, Daneshmand S, Blumenstein B, Lowe BA, Sagalowsky AI, Smith Jr JA, Schellhammer PF, Stanisic TH, Grossman HB, Messing E, Crissman JD and Crawford ED: The prognostic significance of S-phase analysis in stage Ta/T1 bladder cancer. A Southwest Oncology Group Study. Eur Urol *37*: 595-600, 2000.

8 Mizutani Y, Wada H, Ogawa O, Yoshida O, Fukushima M, Nonomura N and Miki T: Prognostic significance of thymidylate synthase activity in bladder carcinoma. Cancer *92*: 510-518, 2001.

9 Popov Z, Hoznek A, Colombel M, Bastuji-Garin S, Lefrere-Belda MA, Bellot J, Abboh CC, Mazerolles C and Chopin DK: The prognostic value of p53 nuclear overexpression and MIB-1 as a proliferative marker in transitional cell carcinoma of the bladder. Cancer *80*: 1472-1481, 1997.

10 Gontero P, Casetta G, Zitella A, Ballario R, Paccioni D, Magnani C, Muir GH and Tizzani A: Evaluation of p53 protein overexpression, Ki67 proliferative activity and mitotic index as markers of tumour recurrence in superficial transitional cell carcinoma of the bladder. Eur Urol *38*: 287-296, 2000.

11 Wu TT, Chen JH, Lee YH and Huang JK: The role of bcl-2, p53, and Ki-67 index in predicting tumor recurrence for low grade superficial transitional cell bladder carcinoma. J Urol *163*: 758-760, 2000.

12 Lopez-Beltran A, Luque RJ, Alvarez-Kindelan J, Quintero A, Merlo F, Carrasco JC, Requena MJ and Montironi R: Prognostic factors in stage T1 grade 3 bladder cancer survival: the role of G1-S modulators (p53, p21waf1, p27kip1, cyclin D1, and cyclin D3) and proliferation index (ki67-MIB1). Eur Urol *45*: 606-612, 2004.

13 Kuczyk MA, Serth J, Bokemeyer C, Hervatin C, Oelke M, Oelert F, Hofner K and Jonas U: Value of the proliferation status (PCNA) and p53 immunohistochemistry as a prognostic factor for the clinical course of superficial cancer of the urinary bladder. Urologe A *34*: 146-152, 1995.

14 Shiina H, Igawa M, Nagami H, Yagi H, Urakami S, Yoneda T, Shirakawa H, Ishibe T and Kawanishi M: Immunohistochemical analysis of proliferating cell nuclear antigen, p53 protein and nm23 protein, and DNA content in transitional cell carcinoma of the bladder. Cancer *78*: 1762-1774, 1996.

15 Kamai T, Takagi K, Asami H, Ito Y, Oshima H and Yoshida KI: Decreasing of p27 (Kip1) and cyclin E protein levels is associated with progression from superficial into invasive bladder cancer. Br J Cancer *84*: 1242-1251, 2001.

16 Tut VM, Braithwaite KL, Angus B, Neal DE, Lunec J and Mellon JK: Cyclin D1 expression in transitional cell carcinoma of the bladder: correlation with waf1, pRb and Ki67. Br J Cancer *84*: 270-275, 2001.

17 Sgambato A, Migaldi M, Faraglia B, De Aloysio G, Ferrari P, Ardito R, De Gaetani C, Capelli G, Cittadini A and Trentini GP: Cyclin D1 expression in papillary superficial bladder cancer: its association with other cell cycle-associated proteins, cell proliferation and clinical outcome. Int J Cancer *97*: 671-678, 2002.

18 Leissner J, Hohenfellner R, Thüroff JW, Köppen C and Wolf HK: Prognostic significance of histopathological grading and immunoreactivity for p53 and p21/WAF1 in grade 2 pTa transitional cell carcinoma of the urinary bladder. Eur Urol *39*: 438-445, 2001.

19 Sgambato A, Migali M, Faraglia B, Garagnani L, Romano G, De Gaetani C, Ferrari P, Capelli G, Trentini GP and Cittadini A: Loss of P27Kip1 expression correlates with tumor grade and with reduced disease-free survival in primary superficial bladder cancers. Cancer Res *59*: 3245-3250, 1999.

20 Nakopoulou L, Zervas A, Lazaris AC, Constantinides C, Stravodimos C, Davaris P and Dimopoulos C: Predictive value of topoisomerase 2α immunostaining in urothelial bladder carcinoma. J Clin Pathol *43*: 309-313, 2001.

21 Raitanen MP, Tammela TL, Kallioniemi M and Isola J: p53 accumulation, deoxyribonucleic acid ploidy and progression of bladder cancer. J Urol *157*: 1250-1253, 1997.

22 Bernardini S, Billerey C, Martin M, Adessi GL, Wallerand H and Bittard H: The predictive value of muscularis mucosae invasion and p53 over expression on progression of stage T1 bladder carcinoma. J Urol *165*: 42-46, 2001.

23 Serio G: C-erbB-2 gene product-like expression in urothelial carcinomas of the human bladder. Its value as a prognostic indicator in superficial tumors. Pathologica *83*: 413-420, 1991.

24 Järvinen TA, Tanner M, Rantanen V, Bärlund M, Borg A, Grenman S and Isola J: Amplification and deletion of topoisomerase II-alpha associates with erbB-2 amplification and affects sensitivity to topoisomerase II inhibitor doxorubicin in breast cancer. Am J Pathol *156*: 839-847, 2000.

25 Simon R, Atefy R, Wagner U, Forster T, Fijan A, Bruderer J, Wilber K, Mihatsch MJ, Gasser T and Sauter G: HER-2 and TOP2A amplification in urinary bladder cancer. Verh Dtsch Ges Path *86*: 176-183, 2002.

26 Mahdy E, Pan Y, Wang N, Malmström PU, Ekman P and Bergerheim U: Chromosome 8 numerical aberration and C-MYC copy number gain in bladder cancer are linked to stage and grade. Anticancer Res *21*: 3167-3173, 2001.

27 Nocito A, Bubendorf L, Maria-Tinner E, Suess K, Wagner U, Forster T, Kononen J, Fijan A, Bruderer J, Schmid U, Ackermann D, Maurer R, Alund G, Knönagel H, Rist M, Anabitarte M, Hering F, Hardmeier T, Schoenenberger AJ, Flury R, Jäger P, Luc Fehr J, Schraml P, Moch H, Mihatsch MJ, Gasser T and Sauter G: Microarrays of bladder cancer tissue are highly representative of proliferation index and histologic grade. J Pathol *194*: 349-357, 2001.

28 Mostofi F, Sobin LH and Torloni H: Histological typing of urinary bladder tumours. *In*: International Histological Classification of Tumours, No. 10. Geneva, WHO 1973, pp 17-17.
29 Stöcker W, Teegen B, Meyer W, Müller-Kunert E, Proost S, Schlumberger W and Sonnenberg K: Differenzierte Auto-antikörper-Diagnostik mit Biochip-Mosaiken. *In*: Autoantikörper (Conrad K, ed). Lengerich, Pabst 1998, pp 78-99.
30 Sauter G, Moch H, Gasser TC, Mihatsch MJ and Waldman FM: Heterogeneity of chromosome 17 and erbB-2 gene copy number in primary and metastatic bladder cancer. Cytometry *21*: 40-46, 1995.
31 Gardiner RA, Walsh MD, Allen V, Rahman S, Samaratunga MLTH, Seymour GJ and Lavin MF: Immunohistological expression of p53 in primary pT1 transitional cell bladder cancer in relation to tumour progression. Br J Urol *73*: 526-532, 1994.
32 Nakopoulou L, Constantinides C, Papandropoulos J, Theodoropoulos G, Tzonou A, Giannopoulos A, Zervas A and Dimopoulos C: Evaluation of over-expression of p53 tumor suppressor protein in superficial and invasive transitional cell bladder cancer: comparison with DNA ploidy. Urology *46*: 334-340, 1995.
33 Vatne V, Maartmannmoe H and Hoestmark J: The prognostic value of p53 in superficially infiltrating transitional cell carcinoma. Scand J Urol Nephrol *29*: 491-495, 1995.
34 Underwood MA, Reeves J, Smith G, Gardiner DS, Scott R, Bartlett J and Cooke TG: Overexpression of p53 protein and its significance for recurrent progressive bladder tumours. Br J Urol *77*: 659-666, 1996.
35 Korkolopoulou P, Christodoulou P, Kapralos P, Exarchakos M, Bisbiroula A, Hadjiyannakis M, Georgountzos C and Thomas-Tsagli E: The role of p53, MDM2 and c-erb B-2 oncoproteins, epidermal growth factor receptor and proliferation markers in the prognosis of urinary bladder cancer. Pathol Res Pract *193*: 767-775, 1997.
36 Lipponen P: Expression of c-erbB-2 oncoprotein in transitional cell bladder cancer. Eur J Cancer *29*: 749-753, 1993.
37 Mellon JK, Lunec J, Wright C, Horne CH, Kelly P and Neal DE: C-erbB-2 in bladder cancer: molecular biology, correlation with epidermal growth factor receptors and prognostic value. J Urol *155*: 321-326, 1996.
38 Ravery V, Grignon D, Angulo J, Pontes E, Montie J, Crissman J and Chopin D: Evaluation of epidermal growth factor receptor, transforming growth factor alpha, epidermal growth factor and c-erbB2 in the progression of invasive bladder cancer. Urol Res *25*: 9-17, 1997.
39 Krüger S, Weitsch G, Büttner H, Matthiensen A, Böhmer T, Marquardt T, Sayk F, Feller AC and Böhle A: Overexpression of c-erbB-2 oncoprotein in muscle-invasive bladder carcinoma: relationship with gene amplification, clinicopathological parameters and prognostic outcome. Int J Oncol *21*: 981-987, 2002.
40 Schraml P, Jononen J, Bubendorf L, Moch H, Bissig H, Nocito A, Mihatsch MJ, Kallioniemi OP and Sauter G: Tissue microarrays for gene amplification surveys in many different tumor types. Clin Cancer Res *5*: 1966-1975, 1999.
41 Latif Z, Watters AD, Dunn I, Grigor KM, Underwood MA and Bartlett JMS : HER2/neu overexpression in the development of muscle-invasive transitional cell carcinoma of the bladder. Br J Cancer *89*: 1305-1309, 2003.
42 Ohashi Y, Sasano H, Yamaki H, Shizawa S, Kikuchi A, Shimeha R, Akaichi T, Satomi S and Nagura H: Topoisomerase IIα expression in squamous cell carcinoma. Anticancer Res *19*: 1873-1880, 1999.
43 Depowski PL, Rosenthal SI, Brien TP, Stylos S, Johnson RL and Ross JS: Topoisomerase IIα expression in breast cancer: correlation with outcome variables. Mod Pathol *13*: 542-547, 2000.
44 Smits G, Schaafsma E, Kiemeney L, Caris C, Debruyne F and Witjes JA: Microstaging of pT1 transitional cell carcinoma of the bladder: identification of subgroups with distinct risks of progression. Urology *52*: 1009-1013, 1998.
45 Platz CE, Cohen MB, Jones MP, Olsen DB and Lynch CF: Is microstaging of early invasive cancer of the urinary bladder possible or useful? Mod Pathol *11*: 1035-1039, 1996.
46 Weizer A, Green A, Shaw C, Wheeler T and Lerner S: Risk assessment of T1 transitional cell carcinoma of the bladder using p53 immunohistochemistry and pathologic substaging. J Urol *161(Suppl)*: 315A, 1999.

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