Predictive Value of Cell Cycle Markers p53, MDM2, p21, and Ki-67 in Superficial Bladder Tumor Recurrence[1]

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ABSTRACT

The aim of the study was to determine whether the expression of the cell cycle markers p53, MDM2, p21, and Ki-67 was predictive of superficial bladder cancer recurrence and to compare the relative predictive power for tumor recurrence of a cell cycle index based on the number of abnormally expressed cell cycle markers with a clinicopathological index based on primary clinical tumor characteristics. The expression of p53, MDM2, and p21 proteins and the value of the Ki-67 index were analyzed for 244 patients. One hundred ninety-four lesions were determined to be superficial papillary tumors (pT_a), whereas 50 tumors invaded the lamina propria (pT_1). Tumor grade was noted low (grade 1) in 83 cases and high (grades 2–3) in 161 cases. An avidin-biotin peroxidase method was performed using monoclonal antibodies against p53, MDM2, p21, and Ki-67 antigens after antigen retrieval treatment of formalin-fixed specimens. The cell cycle marker index was created using the number of abnormally expressed cell cycle markers according to the following cutoff points: p53 (>5%), MDM2 (>20%), p21 (<5%), and Ki-67 (>10%). The clinicopathological index was created using the following adverse tumor characteristics: grades G_2-G_3, stage pT_1, multifocality, and diameter of tumors >3 cm. Cox regression models were used to calculate the relative risks and their 95% confidence intervals associated with disease recurrence for the clinicopathological index and the cell cycle marker index. The χ^2 test was performed to describe the correlation between the Ki-67 index and p53, MDM2, and p21 protein expression. Kaplan-Meier survival curves were generated to demonstrate the disease-free survival according to these two prognostic indexes. The clinicopathological index was a strong, independent predictor of disease recurrence where tumors with three or four adverse tumor characteristics at initial resection had over four times the risk of recurrence than tumors with no risk factors (*P* for trend = 0.0001). A strong correlation was observed between the Ki-67 index >10% and both MDM2 and p21 proteins. MDM2 was overexpressed in 106 tumors (43%), and p53 was overexpressed in 47 (19%); Ki-67 was >10% in 171 cases (70%). Thirty-nine tumors (16%) were p21 negative. The risk of recurrence increased slightly with the number of abnormally expressed cell cycle markers, but when the clinicopathological index was taken into account in multivariate analysis, the cell cycle marker index was not predictive of disease recurrence (*P* for trend = 0.72). The cell cycle markers studied provided no added prognostic information on disease recurrence after initial resection of papillary superficial tumors when the clinicopathological parameters were taken into account.

INTRODUCTION

At the time of initial diagnosis, 80% of patients with transitional cell carcinomas have superficial papillary tumors confined to the mucosa (stage pT_a) or the lamina propria (stage pT_1). After their initial treatment by endoscopic resection, more than one-half of patients develop recurrences within 2 years, and the overall cumulative risk of muscle-invasive cancer is estimated at 7% after 5 years (1, 2, 3). Clinical prognostic variables contributing to recurrence, invasion, and survival have been widely investigated in cohorts of newly diagnosed papillary bladder tumors, with similar grade and stage distribution (4–7). Parmar *et al.* proposed dividing patients in three groups by combining two predictive factors of recurrence risk: the number of tumors at presentation and the result of the control cystoscopy 3 months after initial endoscopic resection (5, 6). In a previously described study, Allard *et al.* (7) defined a simple prognostic clinicopathological index taking into account four primary tumor characteristics: diameter, stage, grade, and multifocality. Although several tumor markers have been studied in bladder cancer, few have been evaluated in well-defined cohorts of patients to determine their independent prognostic value while taking into account known clinicopathological criteria. According to Cordon-Cardo (8), alteration of cell cycle regulation is a key event in determining the biological behavior of bladder cancer. Genetic alterations of the tumor suppressor gene *p53* have been reported as frequent events in bladder cancer, and overexpression of p53 protein is commonly associated with an aggressive clinical course (9, 10). More recently, a complex network of cell cycle regulatory proteins has been identified, including MDM2 and p21, which can modulate or be regulated by p53 activity (11). MDM2 and p53 proteins interact with each other, forming a self-regulation feedback loop (12). Overexpression of MDM2 was reported in a high proportion of superficial

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tumors and at lower frequency in high stage and grade tumors (13, 14). In contrast, the loss of p21 expression has been described as a poor prognostic factor and as an independent predictor of bladder cancer progression in muscle invasive cancer (15). However, the relationship between altered expression of p21 protein or MDM2 and superficial bladder tumor recurrence has not been reported. On the other hand, several groups have reported a correlation between the value of the Ki-67 index with regards to cell proliferation and biological aggressiveness of bladder tumors (16, 17).

The aim of the present study was to assess the relative risk of superficial bladder tumor recurrence according to abnormal p53, MDM2, p21, and Ki-67 expression. We also defined a cell cycle marker index according to the number of abnormally expressed cell cycle markers and compared the relative predictive power of this index with the clinicopathological index defined by Allard *et al.* (7).

MATERIALS AND METHODS

Tissues and Patients Characteristics. Study patients were selected from a cohort of 381 patients with superficial bladder tumors admitted for the first transurethral resection between September 1990 and April 1992 in 15 hospitals of the province of Quebec. Briefly, the patients' mean age was 65.1 years, and the male:female ratio was 3:1. Participants in the original study met the following eligibility criteria: the resected primary tumors were histologically confirmed as pT_a or pT_1 transitional cell carcinoma; and their tumor specimens were large enough for histopathological studies. Thus, these patients were representative of a general population of patients presenting with newly diagnosed bladder tumors, the exception being that patients with carcinoma *in situ* alone were systematically excluded (7). After the initial transurethral resection, cystoscopies were performed at 3-month intervals for 2 years, at 6-month intervals for another 2 years, and yearly thereafter. This schedule was repeated for any patient with tumor recurrence during the follow-up period. Small recurrent tumors were fulgurated immediately, and patients with larger tumors were admitted for new endoscopic resection under general anesthesia. In our series, tumor progression was defined as occurrence of muscle invasion, metastasis, or death by bladder cancer. Patients received no additional intravesical treatment after initial resection until the first recurrence.

In the present study, the immunostaining for p53, MDM2, p21, and Ki-67 was performed on unstained slides of paraffin-embedded material that were available for 269 patients. Of these, 18 patients were excluded because we had no information on one or more of the clinicopathological parameters, and 7 patients had no follow-up. The final study population is therefore 244 patients of the initial 381 patients reported by Allard *et al.* (7). The mean follow-up in the present cohort is 47 months, compared with 34 months in the previous report. According to the International Union Against Cancer (UICC) Tumor Node Metastases classification, 194 lesions were determined as superficial papillary tumors (pT_a), whereas 50 tumors invaded the lamina propria (pT_1). According to the last recommendations of WHO (18), we observed 83 tumors classified as low malignant potential papillary neoplasm (grade G_1) and 161 tumors as low and high grade papillary carcinoma (grades G_2-G_3). In cases with multiple tumors, the size of the largest tumor was always the one reported. At initial resection, most primary tumors were single (70%) and had a diameter of <3 cm (66%).

Immunohistochemical Staining. Sections were deparaffinized, rehydrated, and washed in PBS, pH 7.2. Endogenous peroxidase activity was blocked in 1.0% hydrogen peroxide in PBS for 15 min. After a PBS wash, sections were immersed in prewarmed 0.01 M citric acid (pH 6.0), heated in a microwave oven for 15 min, and allowed to cool at room temperature to enhance antigen retrieval. Then, a three-step immunoperoxidase procedure (Ultra Steptavidine kit; Signet Ltd., Dedham, MA) was used after washing sections with flowing tap water and PBS. Normal horse blocking serum diluted 1:10 in 2% BSA in PBS was applied for 30 min to minimize background staining. A panel of mouse monoclonal antibodies from Oncogene Science, Inc. (Cambridge, MA) was used for: p53 (clone Pab1801; final dilution, 1:500), p21 (clone EA10; final dilution, 1:20); and MDM2 (clone IF2; final dilution, 1:50). The murine monoclonal antibody MiB1 (Dako Ltd., Mississauga, Ontario, Canada) against the Ki-67 antigen was used at 1:50 dilution. Primary antibodies were applied after suction removal of blocking serum, and sections were washed twice more and incubated at room temperature overnight. Sections were incubated with secondary antibodies, biotinylated horse antimouse IgG, 1:500 dilution (Vector Laboratories, Inc., Burlingame, CA) for 30 min at room temperature with avidin-biotin peroxidase complexes. Diaminobenzidine solution was used as the final chromogen, and sections were counterstained with Mayer's hematoxylin before mounting.

Immunohistochemical evaluation was done by two independent investigators (C. P. and B. T.), who scored the estimated percentage of tumor cells that showed nuclear staining. Only intense nuclear staining was considered as p53, MDM2, or p21 accumulation. On the basis of a previous study correlating p53 immunostaining and gene mutations in a subset of 51 high-grade tumors from this cohort, the cutoff point for p53 positivity was established at $>5\%$ cells with nuclear staining (19). As reported by other groups, tumors with $>20\%$ antigen-positive cells for MDM2 were regarded as positive (13, 14). The p21-negative group was defined as tumors with no detectable cells showing p21 expression or only very low percentage of p21 nuclear immunoreactivity in $<5\%$ of cells (15). Nuclei in morphologically malignant cells were considered positive for the Ki-67 antigen when they showed dark brown granular staining. A square grid (10×10 mm) in the ocular lens was used at a $\times 40$ to delineate the area in which both the stained and unstained cells were counted. This immunohistochemical analysis was performed in a blind fashion, without knowledge of the clinical data.

Statistical Analysis. Follow-up was calculated as the time between the date of diagnosis and the date of last contact or death. Cox regression models were used to calculate crude RRs[3] and their 95% CIs associated with disease recurrence for

[3] The abbreviations used are: RR, relative risk; CI, confidence interval; ATC, adverse tumor characteristic.

Table 1 RR of tumor recurrence according to clinical prognostic variables and different cell cycle markers

	n	RR	95% CI
Clinicopathological parameters			
Tumor stage pT_1	50	2.15	1.48–3.13
Tumor grade G_2–G_3	161	1.70	1.20–2.40
Multifocality	81	2.27	1.65–3.11
Tumor diameter >3 cm	92	1.56	1.13–2.14
Cell cycle markers			
p53+	47	1.33	0.90–1.96
MDM2+	106	1.05	0.77–1.45
p21−	39	1.10	0.73–1.65
Ki-67 > 10%	171	1.31	0.92–1.87

clinicopathological parameters and cell cycle marker abnormal expression. The χ^2 test was performed to describe the potential correlation between the Ki-67 index value, MDM2, p53, and p21 protein expression, respectively.

The clinicopathological index was constituted of four ATCs: grade G_2-G_3, stage pT_1, tumor diameter >3 cm, and multifocality. Patients with three or four ATCs were grouped together, leading to a 4-point index. The cell cycle marker index was created using the number of abnormally expressed cell cycle markers according to the following cutoff points: p53 (>5%), MDM2 (>20%), p21 (<5%), and Ki-67 (>10%). Patients with three or four positive markers were grouped together, which lead to a 4-point index.

The Cox model was also used to generate RRs for the clinicopathological index and the cell cycle marker index. Adjusted RRs were calculated by including both indexes in the statistical model to compare their independent prognostic value. Then, *P*s for trend were calculated by entering each index in the model as a linear variable with four categories. Kaplan-Meier survival curves were generated to demonstrate disease-free survival over the follow-up period according to the clinicopathological index and the cell cycle marker index.

RESULTS

Of the 244 available patients for this study, a total of 157 patients experienced tumor recurrence during a median follow-up time of 47 months. Individually, stage pT_1, grade G_2-G_3, multifocality, and tumor diameter >3 cm were all associated with a statistically significant greater risk of tumor recurrence (Table 1). MDM2 and p21 proteins were frequently overexpressed in these samples of primary papillary bladder tumors. Although normal urothelium shows no p21 immunoreactivity, 205 tumors (84%) showed >5% positive cells. In contrast, 39 tumors (16%) were p21 negative. MDM2 was expressed in >20% of cells in 106 patients (43%), whereas a Ki-67 index was >10% in 171 tumors (70%). A strong correlation (P = 0.001) was observed between increased Ki-67 index and increased expression of both MDM2 and p21 proteins. As shown in Fig. 1, 86% of MDM2-positive tumors had a Ki-67 index >10% compared with 58% of MDM2-negative tumors. Similarly, 75% of p21 positive tumors had Ki-67 index >10% compared with 48% of p21 negative tumors (P = 0.001 for both marker comparisons). p53 was overexpressed (>5% of tumor cells) in only 47 tumors (19%). Overexpression of the cell cycle

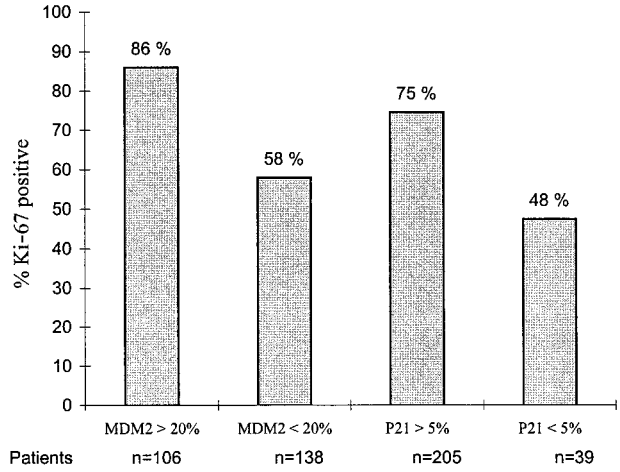

Fig. 1 Proportion of Ki-67 positive (>10%) according to MDM2 and p21. χ^2 P = 0.001 in both cases.

markers p53 (>5%), MDM2 (>20%), and Ki-67 (>10%) were all associated with a slightly increased risk of recurrence, but this difference never reached statistical significance (Table 1). In the present study, lack of p21 expression was not associated with higher recurrence.

The clinicopathological index defined by Allard *et al.* (7) was based on the accumulation of any combination of the four parameters that were found to be independent predictors of tumor recurrence in Table 1. The clinicopathological index was strongly predictive of disease recurrence, where tumors with three or four ATCs at initial resection had over four times the risk of recurrence of tumors with no clinicopathological risk factor (Table 2). Although none of the cell cycle markers studied here had independent predictive value, we hypothesized that based on the biological interaction of these markers, a cumulative cell cycle marker index reflecting the number of abnormally expressed cell cycle markers might have a better correlation with tumor recurrence. Overexpressed p53 and MDM2 (>5 and >20% positive cells, respectively) were considered as abnormally expressed; although p21 is not expressed in normal urothelium, negative p21 expression was considered as abnormal in view of the reported loss of p21 expression in high-grade, high-stage bladder tumors (15). A Ki-67 index >10% was also included in this cell cycle marker index. Indeed, the risk of recurrence increased as the value of cell cycle marker index increased, where tumors with three or four positive markers had 1.47 times the risk of those with no positive markers. This difference did not, however, reach statistical significance.

When both clinicopathological and cell cycle marker indexes were introduced into the Cox model, the strong predictive value of the clinicopathological index was unaffected by the cell cycle marker index (P for trend = 0.0001). However, when the clinicopathological index was taken into account, the cell cycle marker index was not predictive of disease recurrence (P for trend = 0.7). Kaplan-Meier curves in Fig. 2*A* show the difference in recurrence-free survival according to the clinicopathological index (Log-rank P < 0.0001). Fig. 2*B* shows Kaplan-Meier curves of recurrence-free survival according to the

Table 2 RR of tumor recurrence according to clinicopathological index and cell cycle marker index

	n	Crude RR	95% CI	Adjusted RR	95% CI
Clinicopathological index					
0	42	1.00		1.00	
1	77	1.33	0.77–2.30	1.34	0.78–2.31
2	77	2.77	1.64–4.67	2.79	1.64–4.74[a]
3/4	48	4.27	2.45–7.45	4.32	2.46–7.60
Cell cycle marker index					
0	39	1.00		1.00	
1	86	1.13	0.68–1.87	1.08	0.65–1.79
2	81	1.40	0.85–2.30	1.24	0.75–2.05[b]
3/4	38	1.47	0.81–2.66	0.99	0.54–1.81

[a] P for trend = 0.0001.
[b] P for trend = 0.72.

different values of the cell cycle marker index. Recurrence increased as the value of the cell cycle marker index increased, but the curves were not statistically different (Log-rank P = 0.40).

DISCUSSION

The clinical management of bladder tumors is problematic because of the difficulty in predicting the tumor profile in a given patient. Parmar *et al.* (5) have strongly advocated the use of a clinical management strategy that would depend on prognosis at initial diagnosis. One questionable feature of the prognostic index proposed by these authors is that it does not take into account several primary tumor characteristics such as diameter, stage, and grade, which most urologists consider important factors of bladder tumor recurrence (6). In contrast, the clinicopathological index defined by Allard *et al.* (7) is based on the accumulation of four independent ATCs: primary tumor multiplicity, diameter >3 cm, stage pT_1, and grades G_2-G_3. As the number of ATCs increased, an increased risk of tumor recurrence was consistently observed (7). In the present series, with an extended follow-up on a subset of 244 patients from the same cohort, the clinicopathological index had a similar predictive power to that reported previously. The main interest of this simple prognostic index in clinical practice remains the potential selection of patients for adjuvant intravesical therapy after endoscopic resection and for the determination of the optimal periodicity of systematic control cystoscopy.

Recently, molecular phenotyping has provided another dimension to the characterization of the biological potential of tumors that may help better predict their clinical outcome. Different studies have indicated that alteration in cell cycle regulation is a key event in determining the biological behavior of bladder cancer (8, 20). Although several tumor markers have been studied in bladder cancer, few have been evaluated in well-defined cohorts of patients to determine their independent prognostic value above known clinicopathological criteria. Abnormalities of the *p53* tumor suppressor gene have been reported to occur in early stages of bladder carcinogenesis and to be associated with an aggressive biological behavior (9, 10). Results from many published studies suggest that p53 status analysis may help physicians to predict which superficial tumors

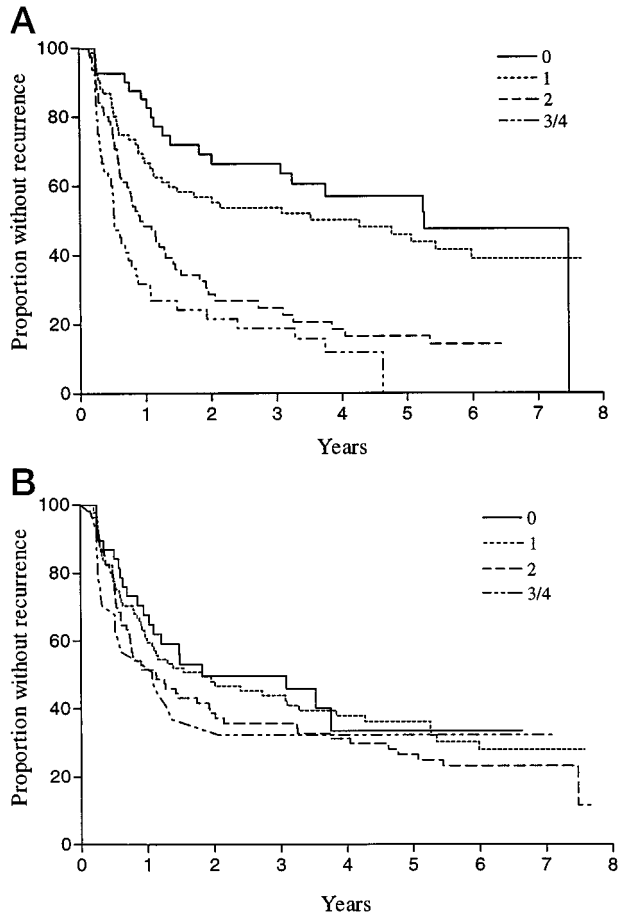

Fig. 2 *A*, recurrence-free survival according to the clinicopathological index. Log-rank $P < 0.0001$. *B*, recurrence-free survival according to the cell cycle marker index. Log-rank $P = 0.4$.

will progress to muscle invasion, allowing possible early aggressive intervention in this group of patients (9, 10, 21). The present study of initial papillary superficial tumors was not designed to address this question because only 12 patients (*i.e.*, 5% of cases) progressed to muscle invasive cancer with recurrence, even after a median time of 4 years of follow-up. Also, p53 overexpression was not an independent predictor of higher recurrence, but the frequency was low as expected in this population of mostly low-grade and low-stage tumors.

The MDM2 and p21 proteins have been identified as important modulators along the p53 pathway, leading to control cell proliferation (8, 11). Biological models have shown that when p53 is bound to MDM2, it is targeted for destruction by the ubiquitin-dependent proteasome pathway (22, 23). Consequently, MDM2 is thought to protect the normal cells from p53 overactivity and may be regarded as the big-brother of p53 in this role (24). A constant finding in bladder cancer has been the significant association between MDM2 overexpression and low-stage and -grade tumors, contrasting with a decreased frequency of MDM2 overexpression in muscle invasive cancers (13, 14). In the present study, MDM2 was overexpressed in >40% of tumors, whereas p53 was overexpressed in <20% of tumors.

This result is also consistent with the previous report by Lianes *et al.* (13). The absence or the very low progression rate observed in low-grade primary superficial tumors, despite a high recurrence rate, presents some analogies with meningiomas, where p53 and MDM2 have been studied recently (25). Although a high frequency of p53 overexpression was observed in recurrent meningiomas, *p53* gene mutations in exons 4–8 were not detected. MDM2, on the other hand, was overexpressed in most p53-positive tumors, which in turn had higher Ki-67 proliferative activity, suggesting that wild-type p53 inactivation by MDM2 may be involved in controlling the proliferative activity of meningiomas. In a subset of 51 patients from the present study with high-grade, low-stage tumors, we found only 14 tumors (28%) with *p53* mutations in exons 5–8 using direct sequencing (19), a frequency that is lower than that (~50%) reported in other series involving high-grade, high-stage tumors (10, 13). As in other studies, 80% of our tumors with mutated *p53* gene were positive by immunohistochemistry, but only 50% of positive tumors (even those with >20% positive cells) had *p53* mutations compared with ~70% in studies of higher stage cancers (10, 13). These results suggest that in a significant proportion of superficial tumors, p53 overexpression is the result of altered wild-type p53 function through mechanisms such as MDM2 interaction.

The nuclear antigen Ki-67 is related to cell proliferation and is expressed in the S, G_2, and M phases of the cell cycle but absent in the G_0 phase (26). In previous studies, no significant correlation was found between the Ki-67 index and tumor stage; however, patients with tumors with a high Ki-67 index value had a worse prognosis than those with tumors with a low Ki-67 index value (27, 28). In the present study characterized by an increased production of p53 wild-type, we observed a significant correlation between a high Ki-67 index value and both MDM2 and p21 protein overexpression. The cyclin-dependent kinase inhibitor p21 appears to play a critical role in the p53 pathway. Alterations in p53 function can result in loss of p21 expression and may be one of the mechanisms by which altered p53 influences tumor progression as suggested recently by Stein *et al.* (15). In a multivariate analysis of 242 patients who underwent radical cystectomy for invasive bladder cancer, they reported that the loss of p21 expression was an independent predictor of treatment failure and survival. However, it has been demonstrated that p21 expression can also be mediated through p53-independent pathways (29). Fredersdorf *et al.* (30) in a recent study have also suggested that the expression of p21/WAF1/CIP1 varies among different human tissues, with a selective expression of this protein, restricted to some cell populations in certain tissues. Mateo *et al.* (31) confirmed that *in vivo* p21 expression is restricted to certain cells and tissues, preferentially the squamous and glandular epithelium. As reported by Stein *et al.* (15), we observed no p21 immunoreactivity in the normal urothelium specimens used as negative control.

To our knowledge, no study has been reported on the combined analysis of p53, MDM2, p21, and Ki-67 in bladder cancer. However, such analysis was reported in few other tumor types. Stefanaki *et al.* (32) investigated by immunohistochemistry the expression of p53, MDM2, p21, and Ki-67 in 38 patients with lymphomas. They reported that significant expression of p53, MDM2, p21, and Ki-67 proteins occurs more frequently in aggressive lymphoma histotypes. The pattern p53+, MDM2−, and p21− may represent tumors with *p53* gene mutations unable to activate expression of MDM2 and p21 proteins. Mateo *et al.* (31) have analyzed the expression of p21/WAF1/CIP1 in relation to Ki-67 and p53 in adult and fetal tissues using immunohistochemical techniques. They observed that tissues in which p21/WAF1/CIP1 was found showed a mutually exclusive topographical sequential expression between p21 and Ki-67, indicating that p21-positive cells are in the G_0 phase. These conclusions are in contradiction with our data, where p21 overexpression in bladder tumors was associated with increased Ki-67 index. The exact significance of p21 overexpression in different tissues and the tumors derived from them remains to be clarified.

In the present study, the different cell cycle markers were all associated with a slightly increased risk of recurrence, but this difference never reached statistical significance. Even when the analysis was restricted to the group of patients with pT_1G_2-G_3 tumors, the cell cycle marker index remained unpredictive of disease recurrence. Our data demonstrated that the risk of recurrence increased slightly as the value of cell cycle marker index increased, whereas patients with three or four abnormally expressed markers had 1.47 times the risk of recurrence than those with no abnormal marker expression. However, when the clinicopathological index based on grade, stage, size, and multiplicity of tumors was taken into account, the cell cycle marker index was not predictive of disease recurrence. It is surprising that cell cycle markers or the proliferative index of tumors was not associated with higher recurrence rate in these superficial tumors. Nevertheless, in a previous publication reporting on the same tumor population, the expression of the tumor antigen M344 was found to be an independent predictor of higher tumor rate, even when taking into account the clinicopathological parameters used in the present study (33). Interestingly, this M344 mucin antigen of superficial bladder tumors, which is not expressed in urothelium of normal individuals, was detected in a high proportion of biopsies from the normal-appearing urothelium in patients with tumors (34, 35). Taken together, these results and those of the present study suggest that recurrence of papillary superficial bladder tumors is more likely associated with field changes rather than to the proliferative potential of the resected tumors.

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