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# Loss of P27$^{Kip1}$ Expression Correlates with Tumor Grade and with Reduced Disease-free Survival in Primary Superficial Bladder Cancers[1]

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## ABSTRACT

**p27$^{Kip1}$ is a member of the Cip1/Kip1 family of cyclin-dependent kinase inhibitors and is a potential tumor suppressor gene. We previously reported a deregulated expression of p27$^{Kip1}$ in a series of human cancer cell lines and in primary breast and colon cancers. Moreover, p27$^{Kip1}$ has been reported as an important prognostic factor in primary lung, breast, colon, and prostate cancers. In this study, we evaluated the prognostic value of p27$^{Kip1}$ in a series of 96 superficial (pTa-1) human bladder carcinomas. High (>50% positive cells), moderate (25–50%), and low (<25%) p27$^{Kip1}$ staining was observed in 39 (41%), 19 (20%), and 38 (39%) of the 96 primary superficial bladder cancers, respectively. No significant association was found between the expression level of p27$^{Kip1}$ and tumor stage. Decreased p27$^{Kip1}$ staining correlated with higher tumor grade ($P = 0.001$). Interestingly, a significant association was observed between increased expression of p27$^{Kip1}$ and positivity for p53 (>20% positive cells; $P < 0.001$). A significant correlation was also observed between low expression of p27$^{Kip1}$ and decreased disease-free survival ($P = 0.0003$ by log-rank test) and overall survival ($P = 0.01$ by log-rank test). Furthermore, on multivariate analysis, low p27$^{Kip1}$ protein expression was an independent predictor of reduced disease-free survival ($P = 0.018$; relative risk = 1.95) second only to tumor stage. These data indicate that p27$^{Kip1}$ protein is frequently expressed at low level in poorly differentiated tumors and suggest that this protein might represent a useful prognostic marker for disease recurrence and overall survival in superficial bladder carcinomas.**

## INTRODUCTION

Alterations in genes involved in the regulation of normal cell cycle progression are frequent events in human cancers. The CDI[3] proteins are potent negative regulators of the cell cycle and are potential tumor suppressor genes, the loss of which might play an important role in the development of human tumors (1, 2).

Indeed, loss of the CDIs p15$^{Ink4b}$, p16$^{Ink4a}$, and p18$^{Ink4c}$ by gene mutation, deletion, and/or methylation has been observed in a variety of human tumors (3, 4), and p16$^{Ink4a}$-deficient mice develop spontaneous tumors at an early age and are highly sensitive to carcinogens (5).

On the other hand, several independent studies have found that alterations in the integrity of the human p27$^{Kip1}$ and p21$^{Cip1}$ genes, which belong to the Kip/Cip family of CDIs, are rare events in a variety of human primary tumors and cancer cell lines (6–13). However, it has been reported that reduced expression of p27$^{Kip1}$ predicts poor survival in breast, colon, prostate, esophagus, and lung cancer patients (14–18). The decreased expression of p27$^{Kip1}$ is due to posttranscriptional events and has been associated with increased proteasome-mediated degradation of the protein (15, 16).

Alterations of the p53 tumor suppressor gene have been reported for a variety of tumors, including bladder carcinomas. The p53 protein is produced at a very low level in normal cells and can act as a transcriptional factor involved in many cellular functions, including the regulation of cell proliferation and apoptosis. In the absence of a functional p53 protein, cells fail to repair DNA damage or to undergo to apoptosis and are more susceptible to neoplastic transformation (19). Mutant p53 has, usually, an increased half-life and is more easily detected by immunohistochemistry than the wild-type protein. In addition to gene mutations, several viral and cellular proteins (*i.e.*, mdm2) can bind to and stabilize the p53 protein, thus, inhibiting its activity (20). p53 overexpression has been shown to correlate with tumor grade and stage in primary bladder carcinomas (21), and it has been suggested that p53 mutations might play an important role in bladder cancer progression (22).

In this study, we have evaluated by immunostaining the expression of p27$^{Kip1}$ in 96 primary superficial bladder carcinomas and have correlated the results obtained with tumor grade and stage. The expression of p27$^{Kip1}$ showed a significant relationship with tumor grade and with the expression of the p53 protein. p27$^{Kip1}$, but not p53, expression correlated with the prognosis of patients, both in terms of disease-free and overall survival, in our series. On a multivariate Cox regression analysis, loss of p27$^{Kip1}$ expression was an independent predictor of early recurrence. The implications of these findings are discussed.

## MATERIALS AND METHODS

**Patient Characteristics and Tissue Samples.** A cohort of 96 patients who underwent routine surgery for bladder cancer at the Division of Urology, County Hospital (Modena, Italy) between April 1990 and December 1995 were used for this study. They included 83 men (86%) and 13 women (14%), with a mean age at diagnosis of 68 years (range, 29–92) and a mean follow-up of 50 months (range, 24–102). All of the patients underwent transurethral resection with curative intent, and none of them had received any therapy before surgery. The follow-up of patients was conducted according to standard clinical practice, and all of the patients were treated by intravescical *Bacillus* Calmette-Guerin instillations, in case of recurrence. Tissue specimens were obtained from the files of the Service of Anatomical Pathology, University of Modena. For molecular analysis, fresh tissues were frozen and stored at −140°C immediately after surgery. The corresponding formalin-fixed paraffin-embedded specimens were used for immunohistochemical analyses. Histological grading and staging were assessed according to WHO (23) and tumor-node-metastasis (24) classification, respectively. Other clinic-pathological parameters, such as age and gender, were also recorded and are shown in Table 1. The samples were coded, and the names of the patients were not revealed.

**Immunohistochemistry.** All immunohistochemical analyses were performed on routinely processed, formalin-fixed, paraffin-embedded tissues using an avidin-biotin complex immunoperoxidase technique. Successive 5-$\mu$m tissue sections were cut from blocks selected for the presence of representative

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[3] The abbreviations used are: CDI, cyclin-dependent kinase inhibitor; CI, confidence interval; RR, relative risk.

Table 1 *p27Kip1 expression and clinicopathological parameters in 96 primary superficial bladder carcinomas*

| | Total | p27Kip1 expression | | | P[a] |
|---|---|---|---|---|---|
| | | High (%) | Moderate (%) | Low (%) | |
| Age (yr) | | | | | |
| ≥65 | 62 | 24 (39%) | 10 (16%) | 28 (45%) | |
| <65 | 34 | 15 (44%) | 9 (26%) | 10 (30%) | 0.26 |
| Sex | | | | | |
| Male | 83 | 33 (40%) | 16 (19%) | 34 (41%) | |
| Female | 13 | 6 (46%) | 3 (23%) | 4 (31%) | 0.78 |
| Tumor grade | | | | | |
| Well differentiated | 13 | 3 (23%) | 6 (46%) | 4 (31%) | |
| Moderately differentiated | 51 | 30 (59%) | 6 (12%) | 15 (29%) | 0.001[b] |
| Poorly differentiated | 32 | 6 (19%) | 7 (22%) | 19 (59%) | |
| Tumor stage | | | | | |
| A | 42 | 21 (50%) | 8 (19%) | 13 (31%) | |
| 1 | 54 | 18 (33%) | 11 (20%) | 25 (47%) | 0.22[a] |
| p53 expression[c] | | | | | |
| Positive (≥20%) | 52 | 30 (58%) | 9 (17%) | 13 (25%) | |
| Negative (<20%) | 44 | 9 (20%) | 10 (23%) | 25 (57%) | <0.001 |

[a] Statistical analyses were performed by the Pearson $\chi^2$ test. $P < 0.05$ was considered significant.

[b] The same values were also obtained when the Fisher's exact test was used for the analysis.

[c] The values refer to the results obtained using the monoclonal Pab1801 antibody. See text for more details.

tumor tissue and mounted on poly-L-lysine-coated slides. After incubation in an oven at 43°C for 12 h to prevent sections from lifting from the slide during the antigen-retrieval step, sections were dewaxed and rehydrated. They were then submerged in a citrate buffer (10 mM; pH 6.0) and microwaved for a total of 10 min at 750 W. Sections were then treated to block endogenous peroxidase and, after blocking with goat (for the polyclonal anti-p27Kip1 antibody) or horse (for the monoclonal anti-p53 antibodies) serum for 1 h at room temperature, the primary antibodies were applied overnight at 4°C in a high-humidity chamber. After washing, immunostaining was performed using the Vectastain ABC and diaminobenzidine kits (Vector Laboratories, Burlingame, CA), as described. Sections were then counterstained with 1% modified Harris hematoxylin, dehydrated, and mounted.

The polyclonal anti-p27Kip1 antibody, raised against a peptide corresponding to amino acids 181–198 mapping in the COOH-terminus of the human p27Kip1 protein (Santa Cruz Biotechnology, Santa Cruz, CA), was used at a concentration of 1 μg/ml (in PBS with 10% goat serum) because this gave good nuclear staining with minimal background. As a negative control, a duplicate section of each tissue sample was immunostained in the absence of the primary antibody. A breast carcinoma with known positive immunostaining for p27Kip1 served as a positive control. A strong nuclear staining of lymphocytes provided a useful internal positive control for preservation of the p27Kip1 immunogenicity in most sections examined. Within tumor cells, the staining reaction for p27Kip1 was often localized both in the nucleus and in the cytoplasm, but the nuclear staining was predominant and, in most of the cases, exclusive. Some tumor cells also showed a clear nucleolar signal. The significance of this finding is unknown. The specificity of the reaction for p27Kip1 was demonstrated by inhibition of the immunohistochemical staining in positive samples by preincubating the antibody with a 100-fold excess of the immunizing peptide (Santa Cruz Biotechnology) for 1 h at room temperature (data not shown). Confirmatory results were obtained when a different monoclonal anti-p27Kip1 antibody (NCL-p27, clone 1B4; Novocastra, Newcastle, United Kingdom) was used to stain selected cases for which duplicate sections were available. The cytoplasmic staining was markedly reduced with this antibody (data not shown). The monoclonal PAb1801 (DAKO, Milan, Italy) and DO7 (Novocastra) anti-p53 antibodies were used diluted 1:750 in PBS. A p53-positive breast carcinoma with known positive nuclear reaction for p53 was used as a positive control. The immunostaining of duplicate sections in the absence of the primary antibody was used as a negative control.

The number of cells with positive nuclear reaction for both p27Kip1 and p53 was calculated semiautomatically by means of a computer-assisted cellular image analyzer on a total of 1000 nuclei/case. All scoring and interpretations of the results were made by two of the authors independently (A. S. and M. M.) without knowledge of clinical outcome or other clinic-pathological variables. The few cases with discrepant scoring were reevaluated jointly on a second occasion, and agreement was reached. Nuclei were considered positive when they showed a distinct brown color in the absence of background staining. Positive and negative control slides were included within each batch of slides. Tumors showing undetectable or negligible levels of p27 expression were scored as negative. Positivities were categorized as low (<25%), moderate (25–50%), and high (>50%; Refs. 14, 16–18). The cutoff value of p53 immunostaining used to define p53 overexpression for statistical analysis was 20% of positive tumor nuclei (25, 26).

**Statistical Analyses.** The association between p27Kip1 and p53 expression and other variables shown in Table 1 were calculated using contingency table methods and tested for significance using the Pearson's $\chi^2$ test. Interrater agreement of the results obtained with the two anti-p53 antibodies was evaluated by Cohen's k coefficient (27). Survival curves were calculated using the Kaplan-Meier method, and the log-rank test was used for the analysis. Patients who died of other causes during the follow-up period were treated as censored data in the survival analyses. Univariate and multivariate RRs were calculated using Cox proportional hazards regression. The RR for age represents the hazard increase/1-year increase in age. For sex, the RRs are given as male *versus* female. For grade and stage, the G1 grade and the pTa stage were used as baseline, respectively. All calculations were performed using the STATA 5.0 statistical software package (Stata Corporation, College Station, TX), and the results were considered statistically significant when the $P$ was <0.05.

## RESULTS

**Expression of p27Kip1 Correlates with Tumor Grade in Primary Superficial Bladder Cancers.** To investigate the significance of p27Kip1 in human bladder cancer, the expression of this protein was evaluated by immunostaining in a series of 96 primary human superficial bladder carcinomas. Within tumor cells, the staining was predominantly nuclear. However, a concomitant diffuse, less intense cytoplasmic staining was also observed in 37 (38.5%) of the 96 tumors (Fig. 1 and data not shown). Only cells with a clear nuclear staining were considered positive. The percentage of positive tumor cells within a microscopic field ranged from 0–90%, and intratumoral heterogeneity of p27Kip1 nuclear immunostaining was common (Fig. 1). When present, the normal mucosa was also assessed for p27Kip1 staining. We found that normal urothelium constantly demonstrated a weak nuclear reaction for p27Kip1 (data not shown).

High (>50% positive cells), moderate (25–50%), and low (<25%) p27Kip1 staining were observed in 39 (41%), 19 (20%), and 38 (39%) of the 96 primary superficial bladder cancers, respectively. High or moderate p27Kip1 expression was found in 9 of 13 (69%) well-differentiated tumors (G1), 36 of 51 (71%) moderately differentiated tumors (G2), and 13 of 32 (41%) poorly differentiated tumors (G3). Thus, p27Kip1 expression was strongly associated with tumor grade in superficial bladder cancer ($P = 0.001$). No correlation was found between p27Kip1 expression and tumor stage in superficial bladder cancers ($P = 0.2$; Table 1).

**Prognostic Significance of p27Kip1 Expression in Primary Superficial Bladder Cancer.** Thirty-two of 38 superficial tumors (84%) with low expression of p27Kip1, 10 of 19 moderate expressors (53%), and 20 of 39 high expressors (51%) recurred in our series of tumors during the period of follow-up. This difference was highly significant ($P = 0.005$). Similarly, 10 of 38 low expressor tumors (26%), 2 of 19 moderate expressors (11%), and 2 of 39 tumors (5%) expressing high levels of p27Kip1 died of disease during the period of follow-up. This difference was also significant ($P < 0.03$). Hence, recurrence and death occurred more frequently when the patients with primary bladder cancers in our series showed a reduced expression of p27Kip1 compared with tumors showing high expression of p27Kip1.

The Kaplan-Meier curves of disease-free survival and overall survival within patients with low (<25% of cells) *versus* moderate or high expression of p27Kip1 showed a highly significant separation ($P = 0.0003$ and 0.01 by log-rank test, respectively). When all of the

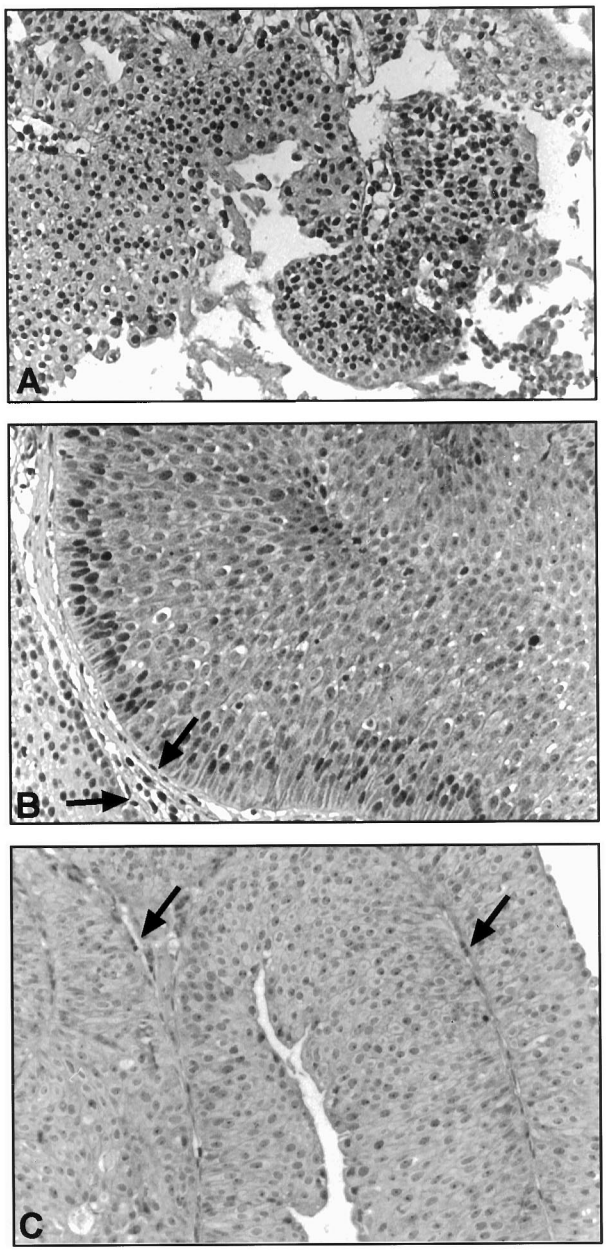



Fig. 1. Examples of $p27^{Kip1}$ immunostaining in three representative cases of bladder carcinomas. *A,* more than 90% of neoplastic cells show a strong, positive staining of the nuclei (high expressor) and a diffuse, faint cytoplasmic reaction. *B,* less than 50% of nuclei (moderate expressor) can be regarded as positive. *C,* only few nuclei can be regarded as positive (low expressor). A strong nuclear staining of lymphocytes (*arrows*) provided a useful internal positive control for preservation of the $p27^{Kip1}$ immunogenicity. (×100)

groups (*i.e.,* low, moderate, and high expressor tumors) were included in the analysis, there was a clear trend for a statistically significant association between reduced expression of p27 and worse prognosis both in terms of disease-free survival ($P = 0.001$) and overall survival ($P = 0.03$; Fig. 2).

**Positive Correlation between $p27^{Kip1}$ and p53 Expression in Primary Bladder Cancer.** The expression of p53 protein was evaluated by immunostaining in the same series of bladder tumors using the Pab1801 and the DO7 monoclonal antibodies. As mentioned in "Materials and Methods," a tumor was considered positive for p53 only if definite nuclear staining was present. Tumor samples were divided in two categories, positive or negative, by estimating the percentage of stained nuclei. The presence of >20% positive cells was used as a cutoff to define positive tumors because, in previous studies, it gave the best prognostic value for p53 (25, 26). Normal urothelium demonstrated no immunoreactivity for p53 (data not shown).

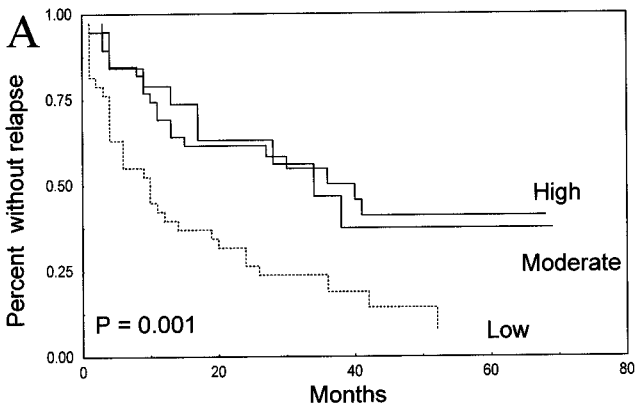
We observed a good, although not absolute, agreement between the results obtained with the two antibodies. The Cohen's K coefficient was 0.65, which corresponded to a substantial (82.41%) amount of agreement. In fact, 52 (54%) and 41 (43%) of 96 tumors were positive for p53 immunostaining when tested with the Pab1801 and the DO7 antibodies, respectively. Positivity for p53, as assessed with the Pab1801, was correlated with the differentiation status. In fact, overexpression of p53 was observed in 6 of 13 (46%) well-differentiated tumors, 39 of 51 (77%) moderately differentiated tumors, and 7 of 32 (22%) poorly differentiated tumors ($P < 0.0001$). No correlation was observed with tumor stage. In fact, overexpression of p53, as assessed with the Pab1801 antibody, was found in 25 of 42 $T_a$ (59%) and 27 of 54 $T_1$ (50%) tumors ($P = 0.3$). When assessed with the DO7 antibody, overexpression of p53 was observed in 4 of 13 G1 (31%), 29 of the 51 G2 (57%), and 8 of 32 G3 (25%) tumors ($P = 0.01$). No significant association was observed between p53 overexpression and tumor stage when the results obtained with the DO7 antibody were used for the analysis ($P = 0.4$).

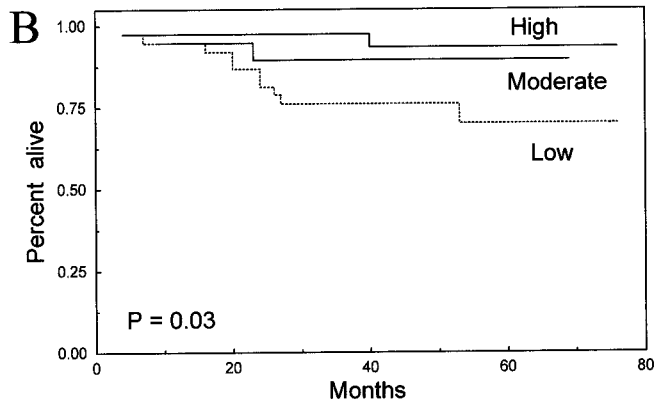
Interestingly, we observed a significant relationship between the expression levels of $p27^{Kip1}$ and positivity for p53 in our series of patients. In fact, 30 of 39 high (77%), 9 of 19 moderate (47%), and 13 of 38 low (34%) $p27^{Kip1}$-expressing tumors were positive for p53, as assessed by the Pab1801 antibody ($P = < 0.001$; Table 1). This association was also true, although weaker, when p53 expression was assessed using the DO7 antibody. With this antibody, positivity for p53 was found in 23 of 39 high (59%), 7 of 19 moderate (37%), and 11 of 38 low (29%) p27-expressing tumors ($P = 0.02$).

We then examined whether positivity for p53, as assessed with the Pab1801, was associated with disease-free survival and overall survival after surgery of the 96 cases of superficial bladder tumors in our series. The Kaplan-Meier survival curve showed a tendency for patients with positive tumors to have a poorer prognosis than patients with negatively stained tumors. However, no statistically significant association of the p53 staining was observed with both disease-free survival ($P = 0.07$ by log-rank test) and overall survival ($P = 0.05$ by log-rank test). When assessed with the DO7 antibody, overexpression of p53 was not associated with disease-free survival ($P = 0.6$ by log-rank test), nor with overall survival ($P = 0.6$ by log-rank test).

**$p27^{Kip1}$ Expression Is an Independent Prognostic Factor of Disease-free Survival in Superficial Bladder Cancers.** As expected, in an univariate analysis, tumor stage was significantly associated with disease-free survival ($P = 0.0001$ by log-rank test) and overall survival ($P = 0.01$) in our series of patients. On the other hand, tumor grade was significantly associated with disease-free survival ($P = 0.01$) but not overall survival ($P = 0.07$).

When a Cox proportional hazards model was constructed that included age of patients at the diagnosis, sex, tumor grade and stage, and p53 and $p27^{Kip1}$ expression, $p27^{Kip1}$ was a strong independent predictor of disease-free survival ($P = 0.018$; RR = 1.95), second only to tumor stage (Table 2). For overall survival, age at diagnosis was the only independent prognostic marker ($P = 0.038$) in our series of patients when all of the variables were included in the analysis (Table 3). As shown in Table 3, the power of this variable was also not very strong (RR = 1.084; 95% CI = 1.005- 1.170) and needs to be confirmed by a study that includes larger patient numbers and a longer follow-up period. It is to be noted, however, that the RR given represents the hazard ratio/1-year increase in age. We also observed a tendency for patients with low $p27^{Kip1}$-expressing tumors to have a shorter survival than did subjects with moderate or high $p27^{Kip1}$ expression (RR = 2.38), but this association was not significant




| | p27 | Total | Events observed | expected |
|---|---|---|---|---|
| High | >50% | 39 | 20 | 28.96 |
| Moderate | 25-50% | 19 | 10 | 13.93 |
| Low | 0-25% | 38 | 32 | 19.11 |
| Total | | 96 | 62 | 62 |

| | p27 | Total | Events observed | expected |
|---|---|---|---|---|
| High | >50% | 39 | 2 | 5.85 |
| Moderate | 25-50% | 19 | 2 | 2.75 |
| Low | 0-25% | 38 | 10 | 5.85 |
| Total | | 96 | 14 | 14 |

Fig. 2. Kaplan-Meier curve for disease-free survival (*A*) and overall survival (*B*) in 96 patients who underwent surgery for primary superficial bladder carcinomas, stratified according to $p27^{Kip1}$ expression. Decreased $p27^{Kip1}$ expression was significantly associated with early recurrence ($P = 0.001$ by log-rank test) and dead of disease ($P = 0.03$ by log-rank test). Significance was stronger when negative or low expressor tumors (<25% of positive tumor cells) were plotted against both moderate (25–50%) and high (>50%) expressor tumors, both in terms of disease-free survival ($P = 0.0003$ by log-rank test) and overall survival ($P = 0.01$ by log-rank test).

Table 2 *Contribution of various potential prognostic factors to disease-free survival by Cox regression analysis in 96 superficial bladder cancers*

| Variable | RR | 95% CI | *P* |
|---|---|---|---|
| Age | 1.005 | 0.976–1.035 | 0.741 |
| Sex | 1.04 | 0.455–2.378 | 0.926 |
| Grade | 1.112 | 0.399–3.098 | 0.839 |
| Stage | 2.489 | 1.293–4.791 | 0.006 |
| p27[a] | 1.951 | 1.122–3.391 | 0.018 |
| p53[b] | 0.917 | 0.516–1.629 | 0.767 |

[a] The RR is given as low (<25% positive cells) *versus* moderate or high expression.
[b] The RR is given as positive (>20% positive cells) *versus* negative tumors.

($P = 0.17$; 95%CI = 0.69–8.18). Age was included in the model because it is essential in this type of analysis. It is noteworthy that when age was not included in the model, only tumor stage and $p27^{Kip1}$ expression were statistically significant predictors of overall survival ($P = 0.006$ and 0.009, respectively).

## DISCUSSION

$p27^{Kip1}$ belongs to the Cip1/Kip1 family of CDIs and it likely plays an important role in regulating cell proliferation during vertebrate development and tumorigenesis. In fact, p27-deficient mice exhibit multiorgan hyperplasia and develop pituitary tumors. Moreover, although alterations in the integrity of the human *p27Kip1* gene are rare events in human primary tumors and cancer cell lines (6–13), it has been reported that reduced expression of $p27^{Kip1}$ predicts poor survival in breast, colon, prostate, esophagus, and lung cancer patients (14–18). In this study, expression of $p27^{Kip1}$ was evaluated by immunostaining in 96 superficial bladder cancers. Superficial bladder cancers are localized in the mucosal layer (pTa) or invade the lamina propria without extension into the muscularis propria (pT1) and have mostly a good prognosis, being that metastasis is a rare event. However, the majority of them recur after surgery and require additional treatments.

To our knowledge, this is the first study investigating the expression of the $p27^{Kip1}$ protein in a large series of superficial bladder carcinomas. We found that a reduced expression of $p27^{Kip1}$ is associated with tumor grade ($P = 0.001$) and with disease-free survival ($P = 0.0003$ by log-rank test) and overall survival ($P = 0.01$ by log-rank test) in superficial bladder cancers. In a multivariate analysis, low expression of $p27^{Kip1}$ (<25% positive cells) was a powerful predictor of recurrence ($P = 0.018$; RR = 1.95), second only to tumor stage ($P = 0.006$; RR = 2.48), which is currently the strongest independent prognostic parameter in superficial bladder cancers. These results are in agreement with previous data on the correlation between $p27^{Kip1}$ expression and tumor grade and on the prognostic role of $p27^{Kip1}$, and they further support the hypothesis that loss of $p27^{Kip1}$ confers a selective growth advantage to tumor cells and might play an important role in the development of a variety of human tumors (14–18).

In preliminary studies, we observed that the frequency of loss of $p27^{Kip1}$ in invasive tumors is almost identical to that observed in superficial tumors (data not shown). Regardless of the underlying molecular mechanism, these findings strongly suggest that alterations in the expression of $p27^{Kip1}$ may be an early event in the multistep process of bladder carcinogenesis. Previous studies have demonstrated that the reduced expression of $p27^{Kip1}$ observed in tumor cells may result from an accelerated degradation of the protein mediated by the ubiquitin-proteasome pathway (16, 28). Further *in vitro* and *in vivo* studies are required to elucidate whether alterations in the ubiquitin pathway are also responsible for the reduced expression of $p27^{Kip1}$ observed in bladder cancer cells.

Table 3 *Contribution of various potential prognostic factors to overall survival by Cox regression analysis in 96 superficial bladder cancers*

| Variable | RR | 95% CI | *P* |
|---|---|---|---|
| Age | 1.084 | 1.005–1.170 | 0.038 |
| Sex | 1.626 | 0.2–13.229 | 0.649 |
| Grade | 0.497 | 0.032–7.793 | 0.618 |
| Stage | 5.333 | 0.763–37.282 | 0.092 |
| p27[a] | 2.381 | 0.693–8.178 | 0.168 |
| p53[b] | 1.784 | 0.541–5.878 | 0.951 |

[a] The RR is given as low (<25% positive cells) *versus* moderate or high expression.
[b] The RR is given as positive (≥20% positive cells) *versus* negative tumors.

An unexpected finding of the present study was the significant relationship between the expression levels of $p27^{Kip1}$ and that of the p53 protein in our series of superficial bladder cancers. This relationship was true when the level of the p53 protein was assessed using both the Pab1801 and the DO7 monoclonal antibodies ($P < 0.001$ and 0.02, respectively). Analysis of the *p53* gene by PCR-single strand conformation polymorphism analysis and sequencing was able to detect mutations only in 13 tumors. Most of these tumors (9 of 13) were G3 tumors, and there was no significant relationship between the level of expression of $p27^{Kip1}$ and the status of the *p53* gene (data not shown). Thus, this relationship only occurs at a posttranscriptional level for both proteins.

Accumulation of the p53 protein is associated with stabilization and functional inactivation of the protein, which can be due not only to gene mutations but also to binding of the p53 protein to several cellular and/or viral proteins (*i.e.*, mdm2; 20). Our results suggest that when p53 protein is overexpressed, and thus presumably inactive, cells most frequently retain $p27^{Kip1}$ expression which, on the other hand, is most frequently lost in the presence of a functional p53 protein. Both p53 and $p27^{Kip1}$ regulate cell cycle progression through a similar mechanism (29–32). In fact, a functional p53 can block cell cycle progression by inducing increased expression of $p21^{Cip1}$, which belongs to the same family of CDKI than $p27^{Kip1}$ and targets the same cyclin/CDK complexes (30). Thus, it can be assumed that cells would not get any growth advantage from alterations (*i.e.*, inactivation of p53 and loss of $p27^{Kip1}$) that target the same pathway of cell growth regulation (29–32). This would explain why loss of $p27^{Kip1}$ and accumulation of p53 are mutually exclusive in our series of tumors. A similar correlation has been observed between other important regulators of cell cycle progression, such as overexpression of cyclin D1 and loss of the pRb protein (33, 34) and loss of pRb and loss of $p16^{Ink4a}$ (32, 35, 36), which also occur in distinct subsets of human tumors.

On the other hand, our data suggest that the relationship between p53 and $p27^{Kip1}$ expression only occurs at a posttranscriptional level. In fact, there was no relationship between $p27^{Kip1}$ expression and the status of the *p53* gene (data not shown). Moreover, most of the p53 overexpressing tumors retained a normal *p53* gene, as is the case for the $p27^{Kip1}$ gene, which is rarely mutated in tumor cells (6–13). An alteration in a common mechanism regulating the abundance of both proteins at a posttranscriptional level might be responsible for this relationship. (16, 28, 37, 38). It is also possible that a more general deregulation of the mechanisms controlling protein stability occurs in tumor cells and might involve several proteins other than p53 and $p27^{Kip1}$. Studies are ongoing to test this hypothesis in *in vitro* studies.

We could not find any significant association between p53 expression and the clinical outcome of the patients in our series, both in terms of disease-free survival and overall survival. Conflicting data have been reported in the literature regarding the prognostic significance of p53 accumulation in superficial bladder carcinomas (22, 25, 26). Our results are in agreement with previous studies that also found that overexpression of p53 is not a prognostic marker in superficial bladder carcinomas (25, 39, 40). We could not find any association between p53 expression and prognosis in our series of patients, even when different cutoffs were used to discriminate between negative and positive tumors (data not shown). These findings are consistent with the hypothesis that nuclear accumulation of the p53 protein becomes more important and apparent in the late stages of bladder carcinogenesis (25).

We previously reported a significant relationship between the level of expression of $p27^{Kip1}$ and that of cyclin D1 and cyclin E in a series of human cancer cell lines including breast, colon, prostatic, and esophageal cancer (41). A significant association between the expression levels of $p27^{Kip1}$ and that of cyclin D1 has been also observed in primary breast (42) and colon (42, 43) carcinomas and in esophageal cancer cell lines (44, 45). This is the first study evaluating the levels of expression of the $p27^{Kip1}$ protein in primary bladder cancer. It has been reported that the expression level of the cyclin D1 protein correlates with disease-free survival in superficial bladder cancers (46). It will be of interest to evaluate whether any relation exists between the expression levels of $p27^{Kip1}$ and that of cyclin D1, cyclin E and/or other cell cycle-related proteins, such as $p21^{Cip1}$, in these tumors. Studies are ongoing to evaluate the expression levels of these molecules in our series of superficial bladder cancers and to extend these studies on a larger series of patients with a longer follow-up.

In conclusion, we demonstrated a clear correlation of $p27^{Kip1}$ expression with tumor grade and p53 expression in superficial bladder carcinomas. Decreased $p27^{Kip1}$ expression was also shown to be associated with an increased risk of recurrence and death for disease in patients with superficial bladder carcinomas who undergo potential curative surgery. Bladder cancer is the eighth most common cancer worldwide in men, and over 50,000 new cases are diagnosed each year in the United States. Fortunately, about 80% of the patients present with superficial tumors and respond to transurethral resection. However, even patients with tumor confined to the mucosa (pTa) frequently recur (more than 50% during the 1st year) and can progress into invasive disease or metastases (10–15% of the cases; Ref. 47). Thus, our findings will certainly be of interest, considering the scarcity of useful prognostic factors able to accurately predict the clinical outcome of patients with superficial bladder cancers. Low $p27^{Kip1}$ expression might help to identify patients at higher risk of recurrence and to select candidates for an adjuvant treatment. Additional studies on a larger series of cases are required, however, to confirm these results and to elucidate the possible functional significance of the observed relationship between $p27^{Kip1}$ and p53.

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# Loss of $P27^{Kip1}$ Expression Correlates with Tumor Grade and with Reduced Disease-free Survival in Primary Superficial Bladder Cancers

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