Loss of p21^Waf1 Expression Is a Strong Predictor of Reduced Survival in Primary Superficial Bladder Cancers

Mario Migaldi, Alessandro Sgambato, Lorella Garagnani, Raffaele Ardito, Paolo Ferrari, Carmela De Gaetani, Achille Cittadini, and Gian Paolo Trentini


ABSTRACT

p21^Waf1 is a downstream effector of p53 and belongs to the Cip1/Kip1 family of cyclin-dependent kinase inhibitors. Thus, it is a potential tumor suppressor gene and likely plays an important role in tumor development. Moreover, reduced expression of p21^Waf1 has been reported to have prognostic value in several human malignancies. In this study, we evaluated the prognostic value of p21^Waf1 in bladder cancer compared with other clinicopathological features and with p27^Kip1 and p53 expression. A total of 96 superficial (pTa-1) human bladder carcinomas were immunohistochemically stained for p21^Waf1 protein expression. Positive p21^Waf1 staining (≥5% positive nuclei) was observed in 68 of the 96 (71%) tumors. p21^Waf1 expression was neither associated with tumor stage (P = 0.9) nor with tumor grade (P = 0.18) but was significantly associated with both p53 protein expression (≥20% positive nuclei; P = 0.007) and with p53 gene mutations (P = 0.017). A significant correlation was also observed between positivity for p21^Waf1 and high (>50% positive cells) p27^Kip1 expression (P = 0.04). With regard to prognosis, patients whose tumors showed absence of p21^Waf1 staining displayed a significantly shorter overall survival (P = 0.01 by log-rank test). However, p21^Waf1 expression did not correlate with disease-free survival (P = 0.15 by log-rank test). On a multivariate analysis that also included p53 and p27^Kip1 expression, negative p21^Waf1 staining was an independent predictor of reduced overall survival (P = 0.004; relative risk, 5.32), stronger than age and tumor stage. These data indicate that expression of p21^Waf1 protein strongly correlates with survival and might represent a useful prognostic marker in primary superficial bladder carcinomas.

INTRODUCTION

Mutations and deregulation of genes involved in the regulation of normal cell cycle progression are frequent events in human urothelial carcinomas. The most frequent alterations regard p16^{ink4a} and p15^{ink4b} (1), cyclin D1 (2), Rb (3), which is the major substrate of cyclin/CDK4 complexes, and p53 (4–6). More recently, numerous pieces of evidence have been accumulated regarding the role of p21^Waf1 in the pathogenesis of primary bladder cancers (7–10), and data have been reported regarding the prognostic role of p27^Kip1 (11) expression in the same tumors.

p21^Waf1 and p27^Kip1 belongs to the Kip/Cip family of CDIs. They are potent negative regulators of the cell cycle, and it has been suggested that they may function as tumor suppressor genes. Several independent studies, however, have found that alterations in the integrity of the human p27^Kip1 and p21^Waf1 genes are rare events in human tumors, including bladder cancer (12–16). Moreover, p21^Waf1-deficient mice undergo normal development and, unlike the p53-deficient mice, do not exhibit early tumorigenesis (17). The p27^Kip1-deficient mice also complete an apparently normal prenatal development, but they develop a multiple organ hyperplasia, retinal dysplasia, and pituitary tumors (18, 19).

p21^Waf1 is transcriptionally induced by p53 (20), the function of which is missing in a high percentage of human tumors. The p53 protein is produced at very low level in normal cells and is involved in many cellular functions including the regulation of cell proliferation and apoptosis. Mutant p53 has usually an increased half-life and is more easily detected by immunohistochemistry than the wild-type protein. Many reports have documented alterations in the p53 gene in bladder carcinomas (4–6). Moreover, 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 (5). In the absence of a functional p53 protein, the expression of p21 is often undetectable or present at a very low level, and immunocytochemical studies have demonstrated a global decrease of p21^Waf1 expression in human tumors compared with normal tissues (22). Moreover, loss of expression of p21^Waf1 has been
reported to have prognostic value in several human malignancies, including bladder cancers (9, 10, 23, 24). However, it has been shown that p21\textsuperscript{Waf1} expression can also be mediated through p53-independent pathways (25, 26). Therefore, p21\textsuperscript{Waf1} expression can be maintained even in the presence of p53 alterations. Indeed, absence of a correlation between decreased expression of p21\textsuperscript{Waf1} and p53 mutations has been observed in several human malignancies (27, 28). Moreover, increased expression of p21\textsuperscript{Waf1} has been reported in brain and ovarian tumors (29, 30).

Reduced expression of p27\textsuperscript{Kip1} has been reported in a variety of human tumors and has been associated with increased proteasome-mediated degradation of the protein (31, 32). The levels of expression of p27\textsuperscript{Kip1} correlate with prognosis in breast, colon, prostate, esophagus, lung, and bladder cancer patients (11, 31–35).

In this study, we have evaluated by immunostaining the expression of p21\textsuperscript{Waf1} in 96 primary superficial bladder carcinomas and have correlated the results obtained with tumor grade and stage. The expression of p21\textsuperscript{Waf1} was also related with the expression levels of p27\textsuperscript{Kip1} and p53 in the same subset of tumors. No relationship was observed between positivity for p21\textsuperscript{Waf1} and both tumor stage and tumor grade. Positive p21\textsuperscript{Waf1} staining was significantly associated with both p53 and p27\textsuperscript{Kip1} expression. Moreover, p21\textsuperscript{Waf1} expression correlated with patients’ survival, and on a multivariate Cox regression analysis, loss of p21\textsuperscript{Waf1} expression was the only independent predictor of reduced overall survival, stronger than age and tumor stage. 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 years) and a mean follow-up of 50 months (range, 24–102 years). All of the patients underwent transurethral resection with curative intent, and none of them had received Gue ´rin instillations in case of recurrence. The collection of tissue specimens was described previously (11). The samples were coded, and the names of the patients were not revealed. Our series only included papillary tumors. Histological grading and staging were assessed according to the WHO (36) and Tumor-Node-Metastasis (37) classification, respectively. Thirteen (13.6%) cases were classified as well (G1), 51 (53.1%) as moderately differentiated (G2), and 32 (33.3%) as poorly differentiated (G3) tumors. Tumor stage was pT\textsubscript{1} in 42 (43.7%) and pT\textsubscript{1} in the remaining 54 (56.3%) cases (see Table 1).

**Immunohistochemistry.** All immunohistochemical analyses were performed on routinely processed, formalin-fixed, paraffin-embedded tissues using an avidin-biotin complex immunoperoxidase technique, as described previously (11). Briefly, successive 5-μm tissue sections were cut from blocks selected for the presence of representative tumor tissue. Sections were dewaxed, rehydrated, and then microwave pretreated (10 min at 750 W in 10 mM citrate buffer, pH 6.0), followed by incubation with 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase. After blocking with horse serum overnight at 4°C in a high-humidity chamber. Binding of the primary antibody was detected with a peroxidase-conjugated secondary antibody and a diaminobenzidine vector (Vector Laboratories, Burlingame, CA). Positive and negative control slides were included for each batch of slides. When present, normal urothelium showed a discrete staining of the umbrella cells and some of the cells of the intermediate zone, whereas the basal layer was constantly negative, thus providing a useful internal control for preservation of the p21\textsuperscript{Waf1} immunogenicity in most sections examined. The number of cells with positive nuclear reaction for p21\textsuperscript{Waf1} was calculated semi-automatically by means of a computer-assisted cellular image analyzer on a total of 1000 nuclear/total

<table>
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<th>Table 1</th>
<th>p21\textsuperscript{Waf1} expression and clinicopathological parameters in 96 primary superficial bladder carcinomas</th>
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<td>Age (yr)</td>
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<td>≥65</td>
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<td>p21\textsuperscript{Waf1} expression</td>
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<td>High (≥50%)</td>
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<td>Low (&lt;50%)</td>
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<td>Wild-type (&lt;20%)</td>
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* Statistical analyses were performed by the Pearson χ² test. P < 0.05 was considered significant. 

a The values refer to the results obtained using the monoclonal DO-7 antibody. See text for more details.
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. Tumor sections were counted as positive if nuclear staining was clearly observed in >5% of tumor cells. The value of 5% was used as the cutoff for p21Waf1 positivity to allow a better comparison with the data in the literature because the 5 and 10% cutoff have been mostly used in previous publications (9, 10, 23, 38–40). A 10% cutoff did not cause any significant change, and therefore, the results will be presented showing the 5% cutoff only. Detection of the p27Kip1 and the p53 proteins has been described previously (11).

**DNA Analysis and PCR-SSCP Analysis.** High molecular weight DNA was isolated from tumors by standard proteinase K digestion and phenol-chloroform extraction. All samples were screened for mutations in exons 5, 6, 7, 8, and 9 of the p53 gene by PCR-SSCP analysis. Primers used were: 5'-GCCCGACGCTGCTCACCA3' (sense) and 5'-TTCCTCTTCCTGCAGTAC3' (antisense) for exon 5; 5'-ACCAGGCGCTGTCAGAT-3' (sense) and 5'-AGTTGCAACAGACCTCAG-3' (antisense) for exon 6; 5'-CTGTGTGCTCTTCAAGTTG-3' (sense) and 5'-CAAGTCGGCTCTGACCTGGA-3' (antisense) for exon 7; 5'-TATCCTGAGTAGTGTAATC-3' (sense) and 5'-CCAGACATTAGTACCTGAAG-3' (antisense) for exons 8–9. The PCR reaction mix consisted of 1× PCR buffer, 50 μM deoxynucleotide triphosphates, 1 unit Taq polymerase (Boehringer Mannheim), 70 pmol of each primer, 1 μCi[α-32P]dCTP (specific activity, 3000 Ci/mmol; Amersham) and 1 μg of genomic DNA. After an initial denaturation at 94°C for 5', 40 cycles were performed under the following conditions: denaturation at 90°C for 45 s; annealing at 55°C for exons 6, 8, and 9; at 58°C for exon 5; and 63°C for exon 7 for 2 min; and extension at 72°C for 3 min, followed by a final extension for 10 min. Three μl of the PCR reaction were added to 3 μl of denaturing loading dye (95% formamide, 10 mM EDTA, and 0.05% of both bromphenol blue and xylene-cyanol), denatured at 95°C for 5 min, and flash cooled on ice. The mixtures were loaded on two different nondenaturing gels: the first containing 6% polyacrylamide and 10% glycerol was run at 8W for 16–18 h at room temperature; the second, without glycerol, was run at 40 W for 3–4 h at 4°C. Gels were dried and exposed to Kodak XAR-5 film at −70°C in a cassette with intensifying screens.

**Direct Sequencing of PCR Products.** PCR products, prepared as described previously, were isolated by electrophoresis in 1% low-melting-point agarose gel and purified (Wizard PCR Preps; Promega). Direct nucleotide sequencing of both strands was performed by the dideoxy chain termination method after asymmetric PCR of abnormal products (Thermo Sequenase Cycle Sequencing kit; Amersham). The primers used for direct sequencing were 5'-labeled by [γ-32P]ATP. Gels were fixed in 10% acetic acid and methanol, dried, and autoradiographed.

**Statistical Analysis.** The association between p21Waf1 expression and other clinicopathological variables were calculated using contingency table methods and tested for significance using the Pearson’s χ² test. 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 relative risks were calculated using Cox proportional hazards regression. The relative risk for age represents the hazard increase/1-year increase in age. For sex, the relative risks are given as male versus female. For grade and stage, the G1 grade and the pT1 stage were used as baseline, respectively. All calculations were performed using the STATA 5.0 statistical software package (Stata Corp., College Station, TX), and the results were considered statistically significant if P ≤ 0.05.

**RESULTS**

**Expression of p21Waf1 Did Not Correlate with Tumor Grade nor with Tumor Stage in Primary Superficial Bladder Cancers.** To investigate the significance of p21Waf1 in human bladder cancer, the expression of this protein was evaluated by immunostaining in a series of 96 primary human superficial bladder carcinomas. Only cells with a clear nuclear staining were considered positive. p21Waf1 nuclear immunostaining was frequently heterogeneous within one specimen, both in terms of percentage of positive cells and staining intensity (data not shown). The percentage of reactive nuclei ranged from 0 to 50%. When present, the normal urothelium showed a discrete staining of the umbrella cells and of ~10% of the cells of the intermediate zone, whereas the basal layer was constantly negative, thus providing a useful internal control for preservation of the p21Waf1 immunogenicity in most sections examined (Fig. 1).

Tumor specimens were counted as positive if nuclear staining was present in >5% of tumor cells. Expression of p21Waf1 was observed in 68 of the 96 (71%) primary superficial bladder cancers. No correlation was found between p21Waf1 expression and tumor stage. In fact, positive staining for p21Waf1 was observed in 30 of 42 (71%) pT1a and in 38 of 54 (70%) pT1b tumors (P = 0.9; Table 1). Similarly, p21Waf1 expression was found in 9 of 13 (69%) well-differentiated tumors (G1), 40 of 51 (78%) moderately differentiated tumors (G2), and 19 of 32 (59%) poorly differentiated tumors (G3). Thus, p21Waf1 expression was not associated with tumor grade in superficial bladder cancer (P = 0.18; Table 1).

**Prognostic Significance of p21Waf1 Expression in Primary Superficial Bladder Cancer.** Forty-one of the 68 superficial tumors (60%) expressing p21Waf1 (≥5% of cells) and 21 of the 28 negative tumors (75%) recurred in our series of tumors during the period of follow-up. Thus, recurrence was less frequent in p21Waf1-expressing tumors, but the difference was not significant (P = 0.2). Similarly, 6 of the 68 (8.8%) p21Waf1-expressing tumors and 8 of the 28 negative tumors (28.6%) died of disease during the period of follow-up. This difference was significant (P = 0.02).

As expected, the Kaplan-Meier curve of overall survival within patients with positive versus negative tumors showed a highly significant separation (P = 0.01 by log-rank test). On the other hand, the Kaplan-Meier curve of disease-free survival showed no significant difference between positive versus negative tumors (P = 0.15 by log-rank test; Fig. 2).

**Positive Correlation between p21Waf1 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 DO-7 monoclonal
antibodies. As reported previously (11), the presence of at least 20% positive cells was used as a cutoff to define positive tumors, and there was a good agreement between the results obtained with the two antibodies. When the results obtained with the DO-7 antibody were used for the analyses, we found a significant correlation between the expression of p21\textsuperscript{Waf1} and positivity for p53 in our series of patients. In fact, 35 of the 68 p21\textsuperscript{Waf1}-expressing tumors (51%) and only 6 of the 28 (21.4%) p21\textsuperscript{Waf1}-negative tumors were positive for p53, as assessed by the DO-7 antibody ($P = 0.007$; Table 1). This association was also true, although weaker, when p53 expression was assessed using the Pab1801 antibody ($P < 0.05$). Thirteen of the 96 tumors in our series showed mutation of the p53 gene, as assessed by SSCP and sequencing of exons 5–9. Fourteen mutations were identified, because in one case, two mutations were simultaneously detected in the same tumor, and they include: 10 missense mutations (3, 1, 4, and 2 in exons V, VI, VII, and VIII, respectively); 3 frame-shift mutations, one for each of the exons V, VI, and VIII; and one nonsense mutation in exon V. Interestingly, a significant relationship was observed between the expression of p21\textsuperscript{Waf1} and the status of the p53 gene. In fact, p53 gene mutations were found in only 5 of the 68 p21\textsuperscript{Waf1}-expressing tumors (7%) and in 8 of the 28 (29%) p21\textsuperscript{Waf1}-negative tumors. This difference was significant ($P = 0.017$; Table 1).

\textbf{Relationship between p21\textsuperscript{Waf1} and p27\textsuperscript{Kip1} Expression in Primary Bladder Cancer.} We then examined the relationship between p21\textsuperscript{Waf1} staining and p27\textsuperscript{Kip1} expression in our series (Table 1). As reported previously, 39 (41%), 19 (20%), and 38 (39%) of the 96 primary superficial bladder cancers showed a high (>50% positive cells), moderate (25–50%), and low (<25%) p27\textsuperscript{Kip1} staining, respectively and high p27\textsuperscript{Kip1} expression was strongly associated with tumor grade ($P = 0.001$) but not with tumor stage ($P = 0.2$) in the same series of tumors (11). High p27\textsuperscript{Kip1} expression was found in 32 of the 68 (47%) p21\textsuperscript{Waf1}-positive and in 7 of the 28 (21%) p21\textsuperscript{Waf1}-negative tumors. This difference was slightly significant ($P = 0.04$; Table 1).

\textbf{p21\textsuperscript{Waf1} Expression Is an Independent Prognostic Factor of Overall Survival in Superficial Bladder Cancers Stronger Than p53 and p27\textsuperscript{Kip1}.} As reported previously, in an univariate analysis tumor stage was significantly associated with disease-free survival ($P = 0.001$ by log-rank test) and overall survival ($P = 0.01$), whereas tumor grade was only associated with disease-free-survival ($P = 0.01$) but not with overall survival in our series of patients. In an univariate analysis, reduced expression of p27\textsuperscript{Kip1} was also significantly associated with poorer prognosis both in terms of disease-free survival ($P = 0.001$) and overall survival ($P = 0.03$). No statistically significant association of the p53 staining was observed with both disease-free survival and overall survival (11).

When a Cox proportional hazards model was constructed that included age of patients at the diagnosis, sex, tumor grade and stage, p53, and p27\textsuperscript{Kip1} and p21\textsuperscript{Waf1} expression, low expression of p27\textsuperscript{Kip1} was the only independent predictor of disease-free survival ($P = 0.015$; relative risk, 1.97) second only to tumor stage (Table 2). We observed only a tendency for patients with absence of p21\textsuperscript{Waf1} staining to have a shorter survival (relative risk, 1.40), but this association was not significant ($P = 0.23$; 95% confidence interval, 0.81–2.42). For overall survival, however, negative p21\textsuperscript{Waf1} staining was a powerful independent predictor of reduced survival ($P = 0.004$; relative risk, 5.32), even stronger than age at diagnosis ($P = 0.011$; relative risk,
rank test). Decreased p21Waf1 expression was significantly associated with overall survival (P = 0.01 by log-rank test) but not with early recurrence (P = 0.15 by log-rank test).

DISCUSSION

The CDI p21Waf1 plays an important role in the regulation of cell cycle transitions in normal cells. It can inhibit several cyclin/CDK complexes and can block cell cycle progression. Moreover, expression of p21Waf1 can be induced by p53, which is an important tumor suppressor. Thus, p21Waf1 has been proposed as a tumor suppressor gene and as a potential mediator of the p53-associated tumorigenesis. However, p21Waf1-deficient mice undergo normal development and, unlike the p53-deficient mice, do not exhibit early tumorigenesis (17). Moreover, alterations in the integrity of the human p21Waf1 genes are rare events in human tumors (14). On the other hand, it has been shown that p21Waf1 expression can also be mediated through p53-independent pathways (25, 26). Thus, p21Waf1 expression can be maintained, even in the presence of p53 alterations, and p21Waf1 might represent an independent marker of tumor behavior. Indeed, reduced expression of the CDI p27Kip1, the role of which in regulating cell cycle progression is highly analogous to that of p21Waf1, predicts a poor prognosis in several human malignancies, including breast, colon, prostate, esophagus, lung, and bladder cancer patients (11, 31–35).

In this study, the expression of p21Waf1 was evaluated by immunostaining in 96 superficial bladder carcinomas, and its possible prognostic significance was analyzed in comparison with other classical prognostic factors (i.e., stage and grade) and with the expression levels of p53 and p27Kip1. Superficial bladder cancers are localized in the mucosal layer (pTa) or invade the lamina propria without extension into the muscularis propria (pT1). Thus, they have mostly a good prognosis and rarely metastasize. However, the majority of these tumors recur after surgery and require additional treatments, and we still lack useful markers able to accurately predict their clinical behavior.

To our knowledge, this is the first study in which the expression levels and the prognostic significance of p21Waf1, p27Kip1, and p53 proteins are evaluated in parallel in a large series of superficial bladder carcinomas. We reported previously that expression of p27Kip1 is associated with tumor grade and stage and with disease-free survival and overall survival in superficial bladder cancers (11). These results were in agreement with previous data on the correlation between loss of p27Kip1 and tumor progression in a number of tumor systems (31–35). On the other hand, 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 survival (11). These results were also in agreement with previous studies that found that overexpression of p53 is not a prognostic marker in superficial bladder carcinomas (4, 41, 42) and are consistent with the hypothesis that nuclear accumulation of the p53 protein becomes more important and apparent in the late stages of bladder carcinogenesis (4).

We found that p21Waf1 expression (≥5% positive nuclei) was present in 68 of the 96 (71%) tumors and was neither associated with tumor stage (P = 0.9) nor with tumor grade (P = 0.18). A significant correlation was observed, however, with both p53 protein expression (≥20% positive nuclei; P = 0.007) and with p53 gene mutations (P = 0.017). Positivity for p21Waf1 was also correlated with high expression (>50% positive cells) of the p27Kip1 protein (P = 0.04). In a univariate analysis, tumor expression of p21Waf1 was associated with an increased overall survival (P = 0.01 by log-rank test). On a multivariate analysis that also included p53 and p27Kip1 expression, negative p21Waf1 staining was an independent predictor of reduced overall survival (P = 0.004; relative risk, 5.32) stronger than age and tumor stage. Thus, p21Waf1 was the strongest independent and highly significant predictor of survival in patients with superficial bladder carcinomas.

Our results on the expression levels of p21Waf1 in superficial bladder cancer are consistent with previous data demonstrating that p21Waf1 is frequently expressed in tumors, despite
its putative role as a tumor suppressor (27, 28, 30, 38). The significance of this finding is still unknown. However, it is of interest that increased expression of p27Kip1, which is strictly related to p21Waf1, is also observed in human tumors (43, 44). Studies are ongoing to evaluate whether the increased expression of p21Waf1 might be related to changes in the expression and/or function of other cell cycle-related proteins, such as cyclin D1, which has been reported as overexpressed in bladder cancers (2).

Other studies have evaluated previously the expression levels of p21Waf1 protein and its relationship with clinical outcome in bladder cancers, but the results are controversial (7–10). In fact, some studies failed to find any significant association between expression of p21Waf1 and clinical outcome of patients with bladder cancers (7, 8). On the other hand, our results are in agreement with other reports that also found that expression of p21Waf1 provides important prognostic information in bladder cancer patients (9, 10). The discrepancies among these studies can depend on several factors that include differences in the selection of the patients, in their treatment, in the type of antibody, and in the cutoff used for the analyses (7–10).

Another interesting observation of our study is the lack of correlation between p21Waf1 expression and tumor grade or stage. This finding is consistent with most of the previous studies that also did not find any relationship between expression of p21Waf1 and tumor grade (8–10) or stage (7, 8, 10, 38) and further support the hypothesis that p21Waf1 might represent an independent prognostic factor in bladder cancers.

Our study is the first that analyzes p21Waf1 expression in relation with both p53 protein expression and p53 gene mutations in the same series of bladder cancers. As expected, the staining for p21Waf1 was inversely correlated with p53 gene mutations in our study (P = 0.017). This observation is in agreement with the results of a previous study that also analyzed the relationship between p21Waf1 expression and p53 gene status (9) and agrees with our knowledge that expression of p21Waf1 is transcriptionally activated by wild-type p53 protein (20). However, we also found that the expression of p21Waf1 positively correlated with p53 overexpression in our series (P = 0.007). This finding is in agreement with previous studies that also found a positive relationship between p21Waf1 and p53 protein expression in bladder cancer (7, 8). The p53 protein is produced at a very low level in normal cells, and its intracellular levels are usually below the threshold for detection by immunohistochemistry. Mutant p53 has usually an increased half-life and is more easily detected by immunohistochemistry than the wild-type protein. Several viral and cellular proteins (i.e., MDM2) can also bind to the p53 protein, stabilize it, and inhibit its activity (45). Indeed, a significant association between accumulation of p53 and overexpression of MDM2, in the absence of gene mutations, has been reported in bladder cancer (46, 47). Thus, detectable nuclear accumulation of p53 is considered a marker for a functionally inactive p53, even in the absence of gene mutations (48). On this basis, our results suggest that p21Waf1 expression can be maintained in bladder cancer cells in the absence of a functional p53 protein. Indeed, it is now well known that there are also p53-independent pathways that mediate p21Waf1 expression (25, 26). However, because we did find an inverse relationship with p53 gene mutations, we cannot exclude that, in tumors showing p53-positive staining, the p53 protein might still retain, at least in some cases, the ability to transcriptionally activate the p21Waf1 gene. Further studies are needed to verify this hypothesis.

Our study for the first time analyzes the expression levels and the prognostic significance of both p21Waf1 and p27Kip1 in the same series of primary superficial bladder cancers. Although supporting a role for p21Waf1 in predicting overall survival in these patients, our results further confirm our previous finding that p27Kip1 expression is a strong independent predictor of disease-free survival in patients with primary superficial bladder cancer (11).

In conclusion, our study demonstrates that expression of the CDI p21Waf1 provides important prognostic information in patients with primary superficial bladder carcinomas. Moreover, together with the results of our previous study on the prognostic significance of p27Kip1 in the same tumors, data suggest that the knowledge of the expression levels of these two CDIs may help to select patients for adjuvant treatment, because patients that are p21Waf1-, and/or p27Kip1-negative are at high risk of recurrence and death and would benefit from an aggressive adjuvant treatment.
treatment after surgery. These findings are of interest considering the scarcity of useful prognostic factors able to accurately predict the clinical outcome of these patients. The conflicting data on the prognostic role of p21Waf1 and the scarcity of data regarding p27Kip1 underscores the need for further investigations and for a standardization of the methods of analyses of these two proteins to allow a better comparison of the results obtained in different studies. Moreover, our results suggest that the simultaneous assessment of different molecules in the same series of tumors can help to identify useful molecular prognostic markers that can favorably influence treatment options and result in overall improved clinical outcome for patients with superficial bladder cancers.

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