Urol Res (2000) 28:285–292

ORIGINAL PAPER

T. Liukkonen · P. Lipponen · M. Raitanen
E. Kaasinen · M. Ala-Opas · P. Rajala · V.-M. Kosma
Finnbladder Group

Evaluation of $p21^{WAF1/CIP1}$ and cyclin D_1 expression in the progression of superficial bladder cancer

Received: 13 September 1999 / 22 March 2000

Abstract Immunoreactivity of $p21^{WAF1/CIP1}$ and cyclin D_1 proteins was assessed in a cohort of 207 patients with superficial (pTa-pT_1) bladder cancer followed up for a mean of 4.9 years. The results of the immunostainings were compared with T category, WHO grade, tumor cell proliferation rate (MIB-1 score), the expressions of p53 and *bcl-2* as well as survival. Sixty-eight percent and 75% of the tumors were $p21^{WAF1/CIP1}$ positive (≥5% of cells positive) and cyclin D_1 positive (≥10% of cells positive), respectively. The $p21^{WAF1/CIP1}$ expression was related to cyclin D_1 immunolabelling ($P < 0.001$) but not to the other variables studied. The expression of cyclin D_1 was inversely associated with T category ($P = 0.001$), WHO grade ($P = 0.006$), MIB-1 score ($P = 0.014$), p53 expression ($P = 0.001$), and *bcl-2* ($P = 0.011$) immunoreactivity. In univariate analysis, T category ($P = 0.0001$), WHO grade ($P < 0.0001$), MIB-1 score ($P < 0.0001$), *bcl-2* ($P = 0.0092$), p53 ($P = 0.0016$) and $p21^{WAF1/CIP1}$ ($P = 0.009$) expressions were significant prognostic factors with regard to tumor progression, whereas cyclin D_1 was without any prognostic significance ($P = 0.1$). Out of 123 p21 positive tumors 21 progressed, whereas only 2 out of 58 p21 negative tumors progressed. In multivariate analysis, the MIB-1 score was the only independent predictor of cancer-specific survival ($P = 0.03$), whereas tumor grade ($P = 0.002$) and cyclin D_1 expression ($P = 0.04$) were independent predictors of tumor recurrence. Only the WHO grade ($P = 0.04$) retained its prognostic value indicating the risk of progression. We suggest that in superficial bladder cancer $p21^{WAF1/CIP1}$ and cyclin D_1 immunohistochemistry provide no additional prognostic information compared with already established prognostic factors for predicting the risk of progressive disease.

Key words Superficial bladder cancer · $p21^{WAF1/CIP1}$ · Prognosis · Cyclin D_1

T. Liukkonen (✉)
Department of Surgery, Mikkeli Central Hospital, Porrassalmenkatu 35-37, Fin-50100 Mikkeli, Finland
Tel.: +358 (0)15 3511; Fax: +358 (0)15 3512200

P. Lipponen
Department of Pathology and Forensic Medicine, University of Kuopio, Finland

M. Raitanen
Department of Surgery, Tampere University Hospital, Finland

E. Kaasinen
Department of Surgery, Hyvinkää District Hospital, Finland

M. Ala-Opas
Department of Urology, Kuopio University Hospital, Finland

P. Rajala
Department of Surgery, Turku University Hospital, Finland

V.-M. Kosma
Department of Pathology and Forensic Medicine, Kuopio University Hospital, Finland

The following individuals belong to the Finnbladder Group: M. Ala-Opas, K. Tuhkanen, Kuopio University Hospital; J. Viitanen, North Karelian Central Hospital, Joensuu; T. Forsell, Kymenlaakso Central Hospital, Kotka; P. Granbacka, Pietarsaari District Hospital; O. Hynninen, Lahti City Hospital; H. Juusela, District Hospital of Jorvi, Espoo; K. Jauhiainen, T. Liukkonen, Mikkeli Central Hospital; P. Kemppainen, Kainuu Central Hospital, Kajaani; H. Korhonen, Satakunta Central Hospital, Pori; T. Lahdes-Vasama, S. Rannikko, M. Ruutu, Helsinki University Hospital; K. Lehtoranta, M. Talja Päijät-Häme Central Hospital, Lahti; J. Häkkinen, P. Hällström, M. Kontturi, O. Lukkarinen, M. Raitanen, Oulu University Hospital; M. Nurmi, P. Rajala, Turku University Hospital; J. Ottelin, Kemi Central Hospital; P. Pellinen, Kokkola Central Hospital; J. Permi, V.-M. Puolakka, South Karelian Central Hospital, Lappeenranta; E. Rintala, Helsinki City Hospital; T. Tammela, H. Tainio, Tampere University Hospital; R. Usenius, Jyväskylä Central Hospital; E. Kaasinen, Hyvinkää District Hospital, Hyvinkää.

Introduction

The optimal management of superficial bladder cancer (SBC) requires the assessment of a tumor's biological potential. Tumor grade and stage determined histologically have been the primary prognostic variables. The role of subjective grading as a prognostic factor is, however, a matter of controversy due to its low

interobserver reproducibility [39]. Pathologists also have difficulties in interpretating the depth of invasion in superficial bladder cancers [2]. Therefore, a variety of molecular prognostic markers have been developed in order to predict more accurately those superficial tumors that recur and progress.

The control of cell proliferation plays an essential role in the progression of bladder cancer. Normal cellular proliferation occurs by an orderly progression through the cell cycle, which is regulated by cell cycle-associated protein complexes composed of cyclins and cyclin-dependent kinases (CDKs) [8]. Loss of this cell cycle control appears to be an early step in the development of carcinogenesis and, ultimately, cancer progression [49]. The wild-type p53 protein is capable of arresting the cell cycle in response to DNA damage by inducing $p21^{WAF1/CIP1}$ [13]. The $p21^{WAF1/CIP1}$ is a versatile, although not universal, inhibitor of CDKs; it appears to be effective against CDK_2, CDK_3, CDK_4 and CDK_6, less effective against CDK_1 and CDK_5, and ineffective against CDK_7 [38]. The $p21^{WAF1/CIP1}$ is observed to induce a G_1 phase arrest, suggesting that in vivo the inhibitor preferentially acts upon complexes with the G_1 cyclins, D and E [38]. However, $p21^{WAF1/CIP1}$ can also be upregulated in a p53-independent manner by a number of agonists, including transforming growth factor-beta (TGF-β), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and epidermal growth factor (EGF), as well as the intracellular signal transduction protein STAT1, for the purpose of inhibiting the progression of the cell cycle [38]. The $p21^{WAF1/CIP1}$ may therefore serve as an intermediary link between the core cell cycle machinery and a variety of upstream signal transduction pathways operating in cell proliferation control and differentiation.

A decrease in $p21^{WAF1/CIP1}$ expression is accompanied by an unfavorable prognosis in colorectal, lung, gastric and breast cancers [46, 23, 52]. In contrast, $p21^{WAF1/CIP1}$ overexpression emerged as an important indicator of shortened disease-free survival in gliomas [24], head and neck cancers [14] and prostatic cancers [1]. The role of $p21^{WAF1/CIP1}$ expression in bladder cancer is somewhat contradictory as some authors report a positive prognostic impact of $p21^{WAF1/CIP1}$ expression [48], while according to a study by Lipponen et al. [29] it provides no additional prognostic information compared with already established prognostic factors in bladder cancer. Makri et al. [33] suggest that $p21^{WAF1/CIP1}$ inhibits cell proliferation either in a p53-dependent or -independent manner but does not mediate p53-induced apoptosis in urothelial carcinoma cells. Finally, according to studies by Kawasaki et al. [22], $p21^{WAF1/CIP1}$ expression itself may have an important role in the induction of apoptosis by DNA-damaging agents.

Cyclin D_1 is a protein derived from the cyclin D_1 gene (CCND 1) or bcl-1 gene on chromosome 11q13, which is involved in both normal regulation of the cell cycle and neoplasia [36, 10]. In the G_1 phase of the cell cycle, cyclin D_1 together with its cyclin-dependent kinase (CDK_4 and CDK_6) partner, is responsible for the transition to the S phase by phosphorylating the product of the retinoblastoma gene (pRb). Amplification of the $CCND_1$ gene or overexpression of the cyclin D_1 protein releases a cell from its normal controls and causes transformation to a malignant phenotype [10]. Indeed, the increased expression of cyclin D_1 has been shown in a number of human tumors and cell lines [10].

The purpose of the current study was to elucidate the expression of $p21^{WAF1/CIP1}$ and cyclin D_1 in superficial bladder cancer and to investigate whether they can provide predictive information of disease progression.

Materials and methods

From December 1991 to March 1994 in 23 Finnish hospitals (the Finnbladder III Group) altogether 273 patients with newly diagnosed superficial transitiocellular carcinoma of the urinary bladder were randomized into three different groups of treatment. In this Finnbladder III trial, one group was treated by transurethral resection (TUR) alone, the second group received 50 million IU interferon α-2b (Intronar®, Schering-Plough) for 2 h after TUR and the third group received 100 mg epirubicin (Farmorubicin®, Pharmacia-Upjohn) for 2 h after TUR. The three treatment groups were of equal size, evenly balanced and comparable with each other in terms of stage, grade and papillary status distribution. The primary diagnostics and staging were carried out according to the UICC 1978 classification [51]. The initial staging was based on urethrocystoscopy, cytological examination of voided urine and excretory pyelography.

The patients were followed every 3 months during the first 2 years and thereafter individually. At every visit, cystoscopy and urine cytology studies were performed to detect recurrences. The causes of death were analyzed separately in each case. The pertinent clinical data are summarized in Table 1.

Histological methods

The histological samples were either preoperative bioptic or peroperative TUR specimens. They were fixed in buffered formalin, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin for histological examination. Pathological staging and grading were performed in a blinded manner according to the

Table 1 Clinical data of the patients (*TUR* transurethral resection, *TUR + EPI* transurethral resection + 100 mg epirubicin immediately after TUR, *TUR + IFN* transurethral resection + 50 million IU interferon α-2b immediately after TUR)

Number of patients	207		
Mean age at diagnosis (years)	65.8 (range 30–89)		
Sex, females/males	55/152		
Mean follow-up (years)	4.9 (range3.7–6.0)		
Stage:			
pTa	169		
pT1	38		
Treatment			
Stage	pTa/pT1 1/2/3		
Grade	Papillary/nodular		
TUR only	54/11	37/21/7	54/7
TUR + EPI	59/13	37/27/8	65/4
TUR + IFN	56/14	29/31/10	63/3

WHO classification [37]. Slides of the tissue blocks from each participating hospital were evaluated by the referee pathologist to obtain a uniform diagnosis of pT category and grade. The total number of eligible patients was reduced to 207; in the majority of cases this was due to insufficient sample material, but in some cases also due to protocol violation and change in pT category by the referee pathologist. The papillary status of tumors could be evaluated in 196 cases (P.L.) and tumors were divided into papillary ($n = 182$) and nodular types ($n = 14$). Tumors were considered papillary if papillary stromal projections covered by tumor epithelium were present. In nodulary tumors, no such stromal structures could be detected.

Immunohistochemistry

The cohort was not entirely consecutive, since most of the tumors were small superficial TCCs and the amount of tissue for immunohistochemical analysis was therefore limited. For the same reason the data obtained from the evaluated parameters were not entirely uniform.

$p21^{WAF1/CIP1}$ and cyclin D_1 immunohistochemistry

In order to demonstrate the presence of $p21^{(WAF1/CIP1)}$ protein, 5-μm serial sections from the primary bladder cancers were heated in a microwave oven for 5×5-min in 0.01 M citrate buffer (pH 6.0). Thereafter, the slides were processed according to standard practice. The sections were incubated with the monoclonal anti-p21 protein (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK) antibody diluted at 1:20 in phosphate-buffered saline (PBS). Secondary antibody (Vectastain ABC Elite kit, Vector, Calif., USA) was used at a dilution of 1:200 in PBS, and then the slides were incubated for 20 min in preformed avidin-biotinylated peroxidase complex (Vectastain ABC Elite Kit, Vector, Calif., USA). The color was developed with diaminobenzidine tetrahydrochloride (DAB) substrate (Sigma, Poole, UK), slightly counterstained with Mayer's hematoxyline, dehydrated, cleared, and mounted with DePex (BDA Ltd., Poole, UK). Cyclin D_1 was demonstrated using the same staining procedure as in $p21^{(WAF1/CIP1)}$ immunohistochemistry. The antibody was purchased from Novocastra Laboratories (Newcastle upon Tyne, UK) and was used at a dilution of 1:100. Positive and negative controls were used in all the patches of the stainings. The $p21^{WAF1/CIP1}$ and cyclin D_1 status could be evaluated in 181/207 (87%) and 187/207 (90%) of cases, respectively.

Scoring of $p21^{WAF1/CIP1}$ and cyclin D_1 expression

The nuclear expression of $p21^{WAF1/CIP1}$ and cyclin D_1 proteins was analyzed in the entire section in at least ten microscopic fields. The mean fraction of these fields was calculated and used in further statistical analyses. Tumor nuclei were considered positive for $p21^{WAF1/CIP1}$ when a clear staining signal was present. The expression of cyclin D_1 was considered positive only when distinct nuclear positivity was present. Faint expression of cyclin D_1 was present in some of the tumor cell nuclei but it was not included in the scoring process as recommended previously [15]. Based on previous studies [26, 50] the cut-off limit for cyclin D_1 was set at 10%. Likewise, we chose 5% as a cut-off limit for $p21^{CIP1}$ according to previous reports [7, 25].

p53 Protein

The p53 protein was detected by a routine method as described before [27]. The p53 expression (CM1 antibody, Novocastra Laboratories Ltd., Newcaste-upon-Tyne, UK; dilution 1:1200) was scored from the area that contained the highest fraction of positive nuclei. The fraction of positive cells was recorded and at least 2000 cells were evaluated. A bladder cancer biopsy specimen showing intense positivity for p53 was used as a positive control. A negative control was processed without primary antibody. The cut-off value for p53 positivity (20%) was based on the study by Sarkis et al. [44]. The p53 status could be evaluated in 205/207 (99%) cases.

Ki-67 nuclear antigen (MIB-1)

From routinely processed representative paraffin blocks 5-μm sections were cut and placed on poly-L-lysine coated slides. Overnight drying of the sections at 37 °C was followed by dewaxing and hydration. The sections were dewaxed in xylene and rehydrated in a graded series of ethanol to water. Citrate buffer (pH 6.0) was used for antigen retrieval in a microwave processor. The sections were treated twice for 7 min at 850 W power in a household microwave oven, after which the sections were allowed to cool in the buffer for 30 min. For the immunostaining of Ki-67 antigen, the monoclonal antibody (IgG1), Immunotech S.A. Marseille, France) was used at a 1:40 concentration. The sections were incubated at +4 °C overnight, and the primary antibody was demonstrated with a streptavidin-biotin technique (Zymed Laboratories Inc., Calif., USA). Diaminobenzidine was used as the final chromogen. The counterstaining was performed using 0.4% ethyl green in acetate buffer for 15 min.

The quantitation of immunohistochemistry was done as described before [30]. The evaluation was done by one observer (T. L.) using a computer-assisted image analysis system (CAS-200 Software, Beckton Dickinson, USA). The cutoff point of 15% was chosen. The method has been tested in previous studies [42, 43]. The quantitation of MIB-1 score could be reliably done (the result of immunostaining was acceptable, there was no confounding background staining and the specimen was representative, containing sufficient cancer tissue) in 196/207 (94%) of cases.

bcl-2 protein

For immunohistochemical demonstration of *bcl-2* protein, 5-μm sections from the primary bladder carcinomas were processed as detailed in connection with MIB-1 immunohistochemistry. After microwave heating the tissue sections were incubated with the monoclonal anti-*bcl-2* protein (Dako, Denmark) antibody diluted at 1:400 in PBS. Several dilutions of the antibody were tested to avoid background staining and to find optimal staining before the entire series was processed. The sections were washed twice for 5 min with PBS, incubated for 20 min with biotinylated secondary antibody (Vector, Calif., USA) diluted at 1:200 in PBS. Sections were washed twice for 5 min with PBS, developed with diaminobenzidine tetrahydrochloride substrate (Sigma, Poole, UK), slightly counterstained with Mayer's hematoxyline, dehydrated, cleared and mounted with DePex (BDA Ltd., Poole, UK). Histologically confirmed B-cell lymphoma biopsy specimens were used as positive controls. Tumor infiltrating lymphocytes served as internal controls and a part of them was positive in all sections. Sections prepared without primary antibody were used as negative controls.The status of *bcl-2* could be evaluated in 202/207 (98%) of cases.

Scoring of *bcl-2* protein expression

Twenty microscopic fields with a magnification ×250 were evaluated. The immunoreactivity of *bcl-2* protein in basal cells (1–3 cell layers) was scored negative or positive. The immunoreactivity of *bcl-2* protein in non-basal tumor cells was scored as positive or negative (0) and the immunoreactivity of *bcl-2* in positive cases was further classified as weak (1) or strong (2) The fraction of *bcl-2* positive cells was also estimated.

In normal bladder mucosa, *bcl-2* is weekly expressed in 2–3 basal cell layers and not at all in other cell layers. In papillary

tumors *bcl-2* is expressed in basal cells and in some tumors, also in non-basal cells. Based on a previous study [28] we analyzed the expression of *bcl-2* in nonbasal cells and the group limit was set at 0.

Analysis of prognostic factors

As disease outcomes, the time elapsed from the initial treatment to the first recurrence (recurrence-free interval), the first evidence of progressive disease (progression free interval) and the time of death from bladder cancer were considered. Progressive disease was defined as a recurrence with a higher tumor stage (from pTa to pT1 or from pT1 to muscle invasive disease) or the development of regional or distant metastases.

Statistical methods

The basic statistics were done by using SPSS 7.5.1 for Windows. The associations between $p21^{WAF1/CIP1}$, cyclin D1, p53, MIB-1, *bcl-2*, tumor stage, grade and number of recurrent or progressive tumors as well as cancer-specific deaths were assessed by Fisher's exact test. Univariate survival analysis was based on a life table (log rank analysis) method with the statistics by Gehan. Multivariate survival analysis (the analyzed parameters were tumor stage, grade, MIB-1 score, $p21^{WAF1/CIP1}$, cyclin D_1, *bcl-2* and p53 expression) [19] was performed in a stepwise manner.

Results

The clinical data, the distribution of patients in the pathological stages, WHO grades, papillary status as well as treatment arms are shown in Table 1. Twenty-eight out of 207 cases progressed (13.5%) during a mean follow-up period of 4.9 years (range 3.7–6.0). One hundred and thirty-two out of 207 (64%) patients developed a recurrence and 10/207 (5%) died of bladder cancer.

The status of $p21^{WAF1/CIP1}$ could be evaluated in 181/207 cases. The expression of $p21^{WAF1/CIP1}$ protein was always nuclear (Fig. 1). The fraction of positive nuclei ranged from 0 to 95% and intratumor heterogeneity of $p21^{WAF1/CIP1}$ expression was common. One hundred and twenty-three out of 181 cases (68%) were positive (≥5% of cells positive) and 58 (32%) negative (<5% of cells positive) for $p21^{WAF1/CIP1}$.

The positivity of $p21^{WAF1/CIP1}$ was neither related to tumor stage ($P = 0.5$) nor grade ($P = 0.4$). Furthermore, no relationship was found between immunostaining of $p21^{WAF1/CIP1}$ and tumor proliferation rate (MIB-1 score) ($P = 0.7$) or p53 immunoreactivity ($P = 0.6$). The expression of *bcl-2* was also independent of $p21^{WAF1/CIP1}$immunostaining ($P = 0.3$) (Table 2) and there was no correlation found between p21 and *bcl-2* expression in p53 negative tumors ($P = 0.9$) (Table 3).

Cyclin D_1 status could be evaluated in 187/207 cases. The expression was nuclear (Fig. 2). Of the 187 cases, 140(75%) were cyclin D_1 positive (≥10% of cells positive). The expression of cyclin D_1 was inversely associated with tumor stage ($P = 0.001$), grade ($P = 0.006$), MIB-1 score ($P = 0.014$), p53 immunoreactivity ($P = 0.001$) and *bcl-2* expression ($P = 0.011$). There was, however, a positive relationship between cyclin D_1 and $p21^{WAF1/CIP1}$ immunostaining ($P < 0.001$) (Table 4).

In univariate analysis, T category ($P = 0.0001$), WHO grade ($P < 0.0001$), MIB-1 score ($P < 0.0001$), *bcl-2* ($P = 0.0092$), p53 ($P = 0.0016$) and $p21^{WAF1/CIP1}$ ($P = 0.0089$) (Fig. 3) were significant prognostic factors with regard to tumor progression, whereas cyclin D_1 was without any prognostic significance ($P = 0.1$) (Fig. 4). Out of 123 $p21^{WAF1/CIP1}$ positive tumors, 21 progressed whereas only two out of 58 $p21^{WAF1/CIP1}$ negative tumors developed a progression. Seventy-two cases expressed p53 and p21 concomitantly. One out of 17 $p21^{WAF1/CIP1}$negative/p53 positive and 11 out of 30 $p21^{WAF1/CIP1}$positive/p53 positive tumors, respectively, progressed ($P = 0.058$).

In multivariate analysis the MIB-1 score was the only independent predictor of cancer-specific survival ($P = 0.03$). Tumor grade ($P = 0.002$) and cyclin D_1 expression ($P = 0.04$) were independent predictors of tumor recurrence. Only the WHO grade ($P = 0.04$) retained its prognostic value with regard to predicting the risk of progression.

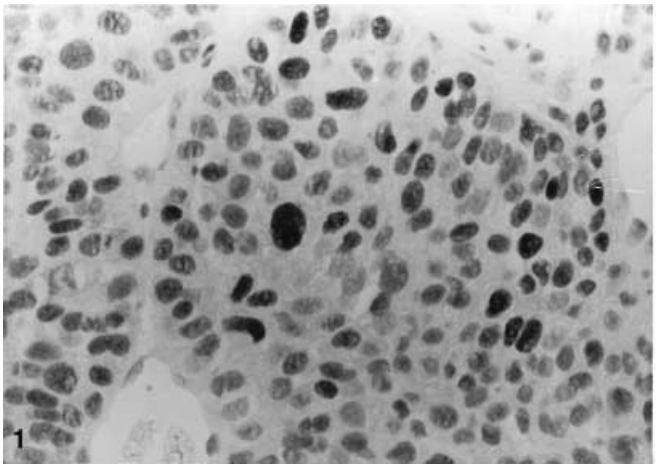

Fig. 1 Nuclear expression of $p21^{WAF1/CIP1}$ in a grade 2 transitional cell bladder cancer. Magnification ×400

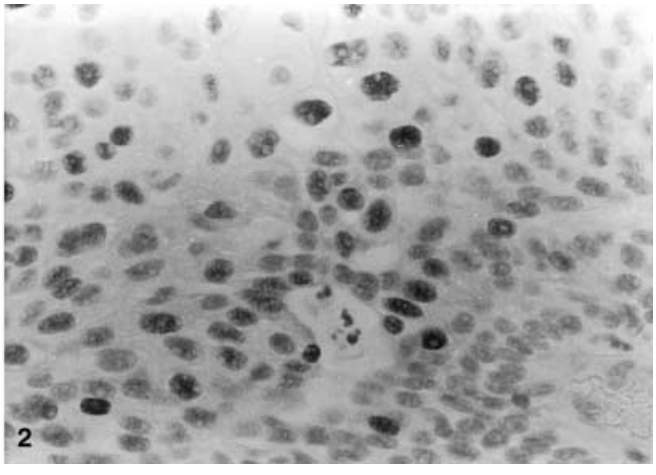

Fig. 2 Nuclear expression of cyclin D_1 in a grade 3 transitional cell bladder cancer. Magnification ×400

Table 2 The relationship between the expression of p21 protein and tumor stage, tumor grade, MIB-1 score as well as the expression of p53 and *bcl-2*. The p21− = <5% of cells positive for p21; p21+ = ≥5% of cells positive for p21; MIB-1− = <15% of cells positive for MIB-1; MIB-1+ = ≥15% of cells positive for MIB-1. The p53− = <20% of cells positive for p53; p53+ = ≥20% of cells positive for p53; *bcl-2* = non basal cells negative for, *bcl-2+* = non basal cells positive for *bcl-2*; χ^2 test and Fisher's exact test (two-tailed *P*-values)

	p21−	p21+	*P*-value	*n*
pTa	49	98	0.542	181
pT1	9	25		
G1	35	61	0.388	181
G2	18	46		
G3	5	16		
MIB-1−[a]	36	82	0.723	171
MIB-1+	18	35		
p53−[a]	41	81	0.612	180
p53+	17	41		
bcl-2−[a]	35	64	0.333	179
bcl-2+	22	58		

[a] Since most of the tumors were small, the amount of tissue for immunohistochemical analysis was limited and the numbers of analyzed parameters are divergent

Table 3 The relationship between p21 and *bcl-2* expression in p53 negative tumors. χ^2 test and Fisher's exact test (two-tailed *P*-value) p21− = <5% of cells positive for p21; p21+ = ≥5% of cells positive for p21; *bcl-2*− = non-basal cells negative for *bcl-2*, *bcl-2+* = non basal cells positive for *bcl-2*. The total number of p53 negative tumors was 133. Since most of the tumors were small, the amount of tissue for immunohistochemical analysis was limited and the numbers of analyzed parameters are divergent. $P = 0.874$

	p21−	p21+	Total
bcl-2−	17	63	80
bcl-2+	8	32	40
Total	25	95	120

Discussion

The Finnbladder III study evaluated the efficacy of a single dose of interferon or epirubicin administered immediately after TUR compared with TUR alone on recurrence of primary superficial (pTa-pT1, grade 1–3) bladder cancer. The three treatment groups were of equal size, evenly balanced and comparable with each other in terms of stage, grade and papillary status distribution. The mean follow-up period was 4.9 years (range 3.7–6.0). A single dose of epirubicin decreased significantly the recurrence risk after TUR but it did not, however, reduce the risk of progression. A single dose of interferon had no significant effect on recurrence or progression [32, 41]. The Finnbladder III study, indeed, made it possible to assess prospectively a series of 207 patients to determine whether the expression of $p21^{WAF1/CIP1}$ or cyclin D_1 proteins can provide additional information on the biological aggressiveness of superficial bladder cancers. This same cohort of patients has been previously analyzed for various biological and clinical factors to reveal new prognostic factors in bladder cancer [30–32].

Table 4 The relationship between the expression of cyclin D_1 and tumor stage, tumor grade, MIB-1 score as well as the expression of p53, p21 and *bcl-2*. The p21− = <5% of cells positive for p21; p21+ = ≥5% of cells positive for p21; cyclinD_1− = <10% of cells positive for cyclinD_1; cyclinD_1+ = ≥10% of cells positive for cyclinD1; MIB-1− = <15% of cells positive for MIB-1, MIB-1+ = ≥15% of cells positive for MIB-1; p53− = <20% of cells positive for p53, p53+ = ≥20% of cells positive for p53; *bcl-2*− = non basal cells negative for *bcl-2*, *bcl-2+* = non basal cells positive for *bcl-2*. χ^2 test and Fisher's exact test (two-tailed *P*-values)

	CyclinD_1−	CyclinD_1+	*P*-value	*n*
pTa	30	121	0.001	187
pT1	17	19		
G1	18	74	0.006	187
G2	16	54		
G3	13	12		
MIB-1−[a]	21	96	0.014	176
MIB-1+	21	38		
p53−[a]	21	99	0.001	186
p53+	26	40		
p21−[a]	25	30	<0.001	171
p21+	20	96		
bcl-2−[a]	18	83	0.011	185
bcl-2+	29	55		

[a] Since most of the tumors were small, the amount of tissue for immunohistochemical analysis was limited and the numbers of analyzed parameters are divergent

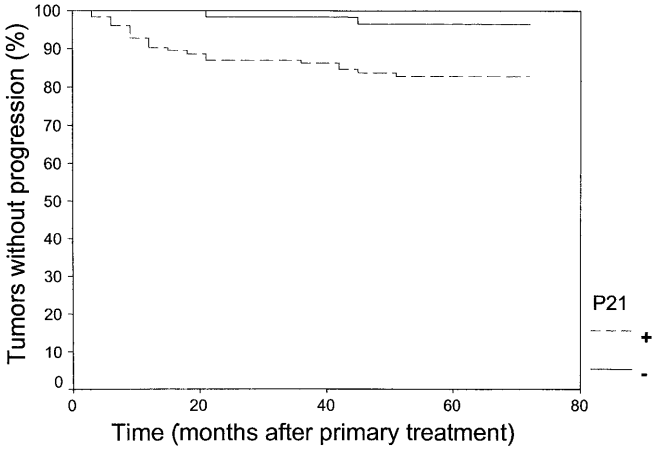

Fig. 3 Kaplan-Meier survival curve comparing the progression rate of 58 (*continuous plot*) and 123 (*interrupted plot*) patients with $p21^{WAF1/CIP1}$ <5% and ≥5%, respectively. $P = 0.0089$

Loss of cell cycle regulation is one of the key issues in the development and progression of malignant tumors [18]. Progression through the cell cycle requires the coordinated activity of cyclin-dependent kinases (CDKs), cyclins and CDK-inhibitors (CKIs) [16]. The $p21^{WAF1/CIP1}$ was one of the first cyclin-dependent kinase inhibitors (CKIs) described. The $p21^{WAF1/CIP1}$ seems to mediate the wild-type p53-dependent cell-cycle arrest but not apoptosis [12]. It is now known that certain growth factors [35, 9] can increase $p21^{WAF1/CIP1}$

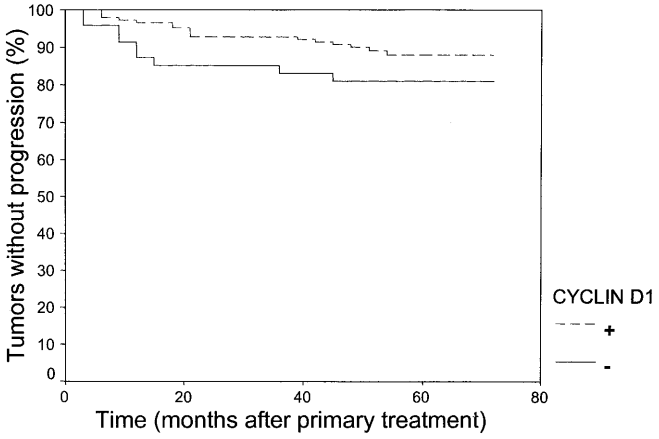

Fig. 4 Kaplan-Meier survival curving comparing the progression rate of 68 (*continuous plot*) and 119 (interrupted plot) patients with cyclin D_1 < 10% and ≥10%, respectively. $P > 0.1$

expression by a p53-independent pathway. As the quantity of $p21^{WAF1/CIP1}$ in the cell increases, more and more of $p21^{WAF1/CIP1}$ molecules bind to cyclin D-E – CDK-complexes. Unable to phosphorylate their downstream targets, the cyclin-CDKs cannot relieve the cell of late G1 phase checkpoint controls, particularly the R point and the cell arrests in late G1 phase. The capability of $p21^{WAF1/CIP1}$ to bind proliferating cell nuclear antigen (PCNA) along with cyclin-CDK provides a second means by which to inhibit DNA replication [38].

Loss of $p21^{WAF1/CIP1}$ expression should, therefore, contribute to tumor progression. To our surprise 17% of the tumors expressing $p21^{WAF1/CIP1}$ progressed, whereas only 3% of the $p21^{WAF1/CIP1}$ negative ones progressed. However, in the multivariate analysis, the expression of $p21^{WAF1/CIP1}$ did not have any prognostic significance. Aaltomaa et al. [1] suggested that the expression of $p21^{WAF1/CIP1}$ is related to rapid cell proliferation, high tumor grade and poor outcome in prostate cancer patients. Erber et al. [14] reported an increased risk of recurrent disease and shortened survival in patents with squamous cell head and neck cancer over-expressing $p21^{WAF1/CIP1}$. High $p21^{WAF1/CIP1}$ expression also emerged as an important indicator of shortened disease-free survival in glioma patients in a study by Korkopoulou et al. [24]. In a study by Bennett et al. [3] high levels of $p21^{WAF1/CIP1}$ were associated with high tumor grade and predicted short survival in patients with non-small-cell lung cancer. In contrast, Stein et al. [48] reported a statistically significant decreased rate of tumor recurrence and an increased overall survival in patients with $p21^{WAF1/CIP1}$ positive tumors in comparison with those whose tumors had lost $p21^{WAF1/CIP1}$ expression in a cohort of 242 cystectomized patients with an invasive bladder cancer. Lipponen et al. [29] analyzed a mixed cohort of 186 bladder cancer patients with variable treatments and concluded that $p21^{WAF1/CIP1}$ immunohistochemistry offers no better prognostic value over already established prognostic factors. In the current study, we did not find any correlation between the immunoreactivities of p53 and $p21^{WAF1/CIP1}$. This was contrary to the results of Clasen et al. [7]. Furthermore, $p21^{WAF1/CIP1}$ positivity was not related to pT category, tumor grade or tumor proliferation rate (MIB-1-score), whereas Clasen et al. [7] with their rather small amount of data found an inverse correlation between tumor stage, grade and cell proliferative activity.

It is generally accepted that abnormalities in the control of apoptosis play an important role in the development of malignant tumors. The *bcl-2* protein is found to be over-expressed in many types of human tumors and it is a potent inhibitor of apoptosis [31, 20, 21]. Bukholm et al. [5] found a strong association between over-expression of *bcl-2* protein and down-regulation of $p21^{WAF1/CIP1}$ in breast cancers expressing wild-type p53 protein. They suggest that *bcl-2*, during suppression of $p21^{WAF1/CIP1}$, may interfere with the functional properties of p53 protein and thus exercise its oncologic potential. We found no relationship between *bcl-2* and $p21^{WAF1/CIP1}$ immunoreactivities either in p53 positive or negative patients. Taken together, the role of $p21^{WAF1/CIP1}$ in bladder cancer is not clear-cut. Our results are not consistent with previous reports [7, 48], which might be due to the use of different antibodies, reagents and technical procedures. Furthermore, our cohort included only patients with superficial bladder cancer. Based on our results it seems that activated oncogenes override the effects of $p21^{WAF1/CIP1}$ in superficial bladder cancer.

Cyclin D_1 is a member of the G_1 cyclins involved in regulation of the transition of the cell through the restriction point in late G1 phase [36]. Furthermore, cyclin D_1 is involved in cell cycle regulation through interactions with retinoblastoma protein (pRb) and other cell cycle-related proteins, such as PCNA and $p21^{WAF1/CIP1}$ [19, 53, 11]. Increased cyclin D_1 expression has been demonstrated in a number of primary human tumors and cell lines, and it is associated in most instances with amplification of the cyclin D_1 gene [34, 10, 4]. However, this is not always the case. It has been suggested that other cellular genes may have an influence on the protein expression of cyclin D_1 [10, 17]. Amplification of 11q13 has been demonstrated in between 6% and 21% of the transitional cell cancers of the urinary bladder, although nuclear accumulation of the protein appears in a much greater percentage of cases [10, 4]. Several previous studies have analyzed the prognostic significance of cyclin D_1 in bladder cancer [40, 45, 50, 26].

In the present study, the expression of cyclin D_1 was inversely related to tumor stage, WHO grade and cell proliferation rate, which is at variance with the findings of Osman et al. [40], but in accordance with the results by Bringuier et al. and Lee et al. [4, 26]. The former cohort [40] included, however, squamous cell carcinomas with bilharzia as an etiological factor, and the cohort consisted of mainly muscle infiltrating tumors, whereas the present study included only superficial transitional cell carcinomas. Shin et al. [45] did not find any statistically significant difference between the

expression of cyclin D_1 and tumor grade in a study with 75 transitional cell cancer bladder cancer patients. In our study, cyclin D_1 expression correlated inversely with p53 and *bcl-2* immunostainings, which is in line with the findings of Lee et al. and Bukholm et al. [26, 5]. A positive relationship was found between cyclin D_1 and $p21^{WAF1/CIP1}$ immunostainings. The latter finding is in accordance with the suggestion that intact p53, through $p21^{WAF1/CIP1}$, induces cyclin D_1 synthesis [6].

In the current study, the expression of cyclin D_1 was without prognostic significance in terms of tumor progression, which is in line with the results of Suwa et al. [50]. However, cyclin D_1 immunoreactivity was an independent predictor of tumor recurrence, cyclin D_1 immunopositive tumors being more prone to recur than immunonegative ones. The latter finding concurs with the results of Shin et al. [45]. This further strengthens the hypothesis that two divergent pathways of tumor progression exist. In other words, a tumor type exists that very often recurs but seldomly progresses, and there is also an aggressive tumor type that progresses "without warning" [47].

In conclusion, the results of the current study indicate that in superficial bladder cancer there is no relationship between $p21^{WAF1/CIP1}$ expression and tumor cell proliferation rate, p53 and *bcl-2* immunostainings. The expression of cyclin D_1 is inversely related to tumor stage, grade, cell proliferation rate as well as *bcl-2* and p53 immunoreactivities. A positive relationship has been found between cyclin D_1 and $p21^{WAF1/CIP1}$immunostaining. However, with regard to the risk of progressive disease, $p21^{WAF1/CIP1}$ and cyclin D_1 immunohistochemistry provide no additional prognostic information compared with already established prognostic factors in superficial bladder cancer.

Acknowledgements This study was supported by research grants from Finnish Urological Society, Mikkelin Läänin Maakuntarahasto and special government funding (EVO-funding). The technical assistance of Ms Aija Parkkinen, Mrs Eila Pohjola and Mr Immo Rantala, PhD, is gratefully acknowledged. We wish to thank the referee pathologist Markku Helle, MD, PhD, and Jyrki Nieminen, MD, for the statistical analyses.

References

1. Aaltomaa S, Lipponen P, Eskelinen M, Ala-Opas M, Kosma VM (1999) Prognostic value and expression of p21(waf1/cip1) protein in prostate cancer. Prostate 39: 8–15
2. Abel PD, Hall RR, Williams G (1988) Should pT1 transitional cell cancers of the bladder still be classified as superficial. Br J Urol 62: 235–239
3. Bennett WP, El-Deiry WS, Rush WL, Guinee Jr DG, Freedman AN, Caporaso NE, Welsh JA, Jones RT, Borkowski A, Travis WT, Fleming MV, Trastek V, Pairolero PC, Tazelaar HD, Midthun D, Jett JR, Liotta LA, Harris CC (1998) $p21^{WAF1/CIP1}$ and transforming growth factor B1 protein expression correlate with survival in non-small cell lung cancer. Clin Cancer Res 4: 1499–1506
4. Bringuier PP, Tamimi Y, Schuuring E, Schalken J (1996) Expression of cyclin D1 and EMS1 in bladder tumours; relationship with chromosome 11q13 amplification. Oncogene 12: 1747–1753
5. Bukholm IK, Nesland JM, Kåresen R, Jacobsen U, Borresen-Dale A-L (1997) Interaction between *bcl-2* and p21 (WAF1/CIP1) in breast carcinomas with wild-type p53. Int J Cancer 73: 38–415
6. Chen X, Bargonetti J, Prives C (1995) p53, through p21(WAF1/CIP1), induces cyclin D1 synthesis. Cancer Res 55: 4257–4263
7. Clasen S, Schulz WA, Gerhards C-D, Grimm MO, Christoph F, Schmitz-Dräger BJ (1998) Frequent and heterogenous expression of cyclin-dependent kinase inhibitor WAF1/p21 protein and mRNA in urothelial carcinoma. Br J Cancer 77: 515–521
8. Cordon-Cardo C (1995) Mutation of cell cycle regulators. Biological and clinical implications of human neoplasia. Am J Pathol 147: 545
9. Datto MB, Li Y, Panus JF, Howe DJ, Xiong Y, Wang XF (1995) Transforming growth factor b induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. Proc Natl Acad Sci USA 92: 5545–5549
10. Donnellan R, Chetty R (1998) Cyclin D1 and human neoplasia. J Clin Pathol: Mol Pathol 51: 1–7
11. Dowdy SF, Hinds PW, Louie K, Reed SI, Arnold A, Weiberg RA (1993) Physical interaction of the retinoplastoma protein with human D cyclins. Cell 73: 499–511
12. El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B (1993) WAF1, a potential mediator of *p53* tumor suppression. Cell 75: 817–825
13. El-Deiry WS, Tokino R, Waldman T, Oliner VE, Burrell M, Hill DE, Healy E, Rees JL, Hamilton SR, Kinzler KW, Vogelstein B (1995) Topological control of $p21^{WAF1/CIP1}$ expression in normal and neoplastic tissues. Cancer Res 55: 2910
14. Erber R, Klein W, Andl T, Enders C, Born AI, Conradt C, Bartek J, Bosch FX (1997) Aberrant $p21^{CIP1/WAF1}$ protein accumulation in head- and neck cancer. Int J Cancer 74: 383–389
15. Gillett CE, Smith P, Gregory WM, Richards MA, Millis RR, Peters G, Barnes DM (1996) Cyclin D1 and prognosis in breast cancer. Int J Cancer 69: 92–99
16. Grana X, Reddy EP (1995) Cell cycle control in mammalian cells: role of cyclins, cyclin-dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CKIs). Oncogene 11: 211
17. Hall M, Peters G (1996) Genetic alterations of cyclins, cyclin-dependent kinases, and cdk inhibitors in human cancer. Adv Cancer Res 68: 67–108
18. Hartwell LH, Kastan MB (1994) Cell cycle control and cancer. Science 16: 1821
19. Hinds PW, Mittnacht S, Dulic V, Arnold A, Reed SI, Weinberg RA (1992) Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. Cell 70: 993–1006
20. Hockenberry D, Nunez G, Milliman C, Schreiber RD, Korsmeyer SJ (1990) *bcl-2* is an inner mitochondrial membrane protein that blocks programmed cell death. Nature 348: 334–336
21. Joensuu H, Pylkkänen L, Toikkanen S (1994) *bcl-2* protein expression and long-term survival in breast cancer. Am J Pathol 145: 1191–1198
22. Kawasaki T, Tomita Y, Bilim V, Takeda M, Takahashi K, Kumanishi T (1996) Abrogation of apoptosis induced by DNA-damaging agents in human bladder-cancer cell lines with p21(waf1/cip1) and/or p53 gene alterations. Int J Cancer 68: 501–505
23. Komiya T, Hosono Y, Hirashima T, Masuda N, Yasumitsu T, Nakagawa K, Kikui M, Ohno A, Fukuoka M, Kawase I (1997) p21 Expression as a predictor for favorable prognosis in squamous cell carcinoma of the lung. Clin Cancer Res 3: 1831–1835
24. Korkolopulou P, Kouzelis K, Christodoulou P, Papanikolau A, Thomas-Tsagli E (1998) Expression of retinoblastoma gene product and p21 (WAF1/Cip1) protein in gliomas: correlations

with proliferation markers, p53 expression and survival. Acta Neuropathol (Berl) 95: 617–624
25. Lacombe L, Orlow I, Silver D, Gerald WL, Fair WR, Reuter VE, Cordon-Cardo C (1996) Analysis of $p21^{WAF1/CIP1}$ in primary bladder tumors. Oncol Res 8: 409–414
26. Lee CCR, Yamamoto S, Morimura K, Wanibuchi H, Nishisaka N, Ikemoto S, Nakatani T, Wada S, Kishimoto T, Fukushima S (1997) Significance of cyclin D1 overexpression in transitional cell carcinomas of the urinary bladder and its correlation with histopathologic features. Cancer 79: 780–789
27. Lipponen PK (1993) Over-expression of p53 nuclear oncoprotein in transitional-cell bladder cancer and its prognostic value. Int J Cancer 53: 365–370
28. Lipponen PK, Aaltomaa S, Eskelinen M (1996) Expression of the apoptosis suppressing *bcl-2* protein in transitional cell bladder tumours. Histopathology 28: 135–140
29. Lipponen P, Aaltomaa S, Eskelinen M, Ala-Opas M, Kosma VM (1998) Expression of p21(waf1/cip1) Protein in transitional cell bladder tumours and its prognostic value. Eur Urol 34: 237–243
30. Liukkonen TJO, Lipponen PK, Helle M, Haapasalo HK, Nordling S, Rajala P, the Finnbladder Group (1996) Expression of MIB-1, mitotic index and S-phase fraction as indicators of cell proliferation in superficial bladder cancer. Urol Res 24: 61–66
31. Liukkonen TJO, Lipponen PK, Helle M, Jauhiainen KE, the Finnbladder III Group (1997) Immunoreactivity of *bcl-2*, p53 and EGFr is associated with tumor stage, grade and cell proliferation in superficial bladder cancer. Urol Res 25: 1–8
32. Liukkonen T, Rajala P, Raitanen M, Rintala E, Kaasinen E, Lipponen P, the Finnbladder Group (1999) The prognostic value of MIB-1 score, p53, EGFr, mitotic index and papillary status in primary superficial (stage pTa/T1) bladder cancer: a prospective comparative study. Eur Urol 36: 393–400
33. Makri D, Schulz WA, Grimm MO, Clasen S, Bojar H, Schmitz-Dräger BJ (1998) WAF1/p21 regulates proliferation, but does not mediate p53-dependent apoptosis in urothelial carcinoma cell lines. Int J Oncol 12: 621–628
34. Michalides R, Hageman P, van Tinteren H, Houben L, Wientjens E, Klompmaker R, Peterse J (1996) A clinicopathological study on overexpression of cyclin D1 and of p53 in a series of 248 patients with operable breast cancer. Br J Cancer 73: 728–734
35. Michieli P, Chedid M, Lin D, Pierce JH, Mercer WE, Givol D (1994) Induction of waf1/CIP1 by a p53-independent pathway. Cancer Res 54: 3391–3395
36. Motokura T, Bloom T, Kim HG, Jüppner H, Ruderman JV, Kronenberg HM, Arnold A (1991) A novel cyclin encoded by a bcl-1 linked candidate oncogene. Nature 350: 512–515
37. Mostofi FK, Sobin LH, Torloni H (1973) International histological classification of tumours. In: Mostofi FL, Sobin LH, Torloni H (eds) Histological typing of urinary bladder tumours, no 10. WHO, Geneva
38. Musunuru K, Hinds PW (1997) Cell cycle regulators in cancer. Karger, Basel, pp 76–80
39. Ooms ECM, Andersson WAD, Alons CL, Veldhuisen RW, Boon ME (1983) An analysis of the performance of pathologists in grading bladder tumors. Hum Pathol 14: 140–143
40. Osman I, Scher H, Zhang Z-F, Soos TJ, Hamza R, Eissa S, Khaled H, Koff A, Cordon-Cardo C (1997) Expression of Cyclin D1, but not cyclins E and A, is related to progression in bilharzial bladder cancer. Clin Cancer Res 3: 2247–2251
41. Rajala P, Liukkonen T, Raitanen M, Rintala E, Kaasinen E, Helle M, Lukkarinen O, the Finnbladder Group (1999) Transurethral resection with perioperative instillation of interferon-a or epirubicin for the prophylaxis of recurrent primary superficial bladder cancer: a prospective randomized multicenter study – Finnbladder III. J Urol 161: 1133–1136
42. Sallinen PK, Haapasalo HK, Kerttula T, Rantala I, Kalimo H, Collan Y, Isola J, Helin H (1994) Sources of variation in the assessment of cell proliferation using proliferating cell nuclear antigen immunohistochemistry. Anal Quant Cytol Histol 16: 261–268
43. Sallinen PK, Haapasalo HK, Visakorpi T, Helen PT, Rantala I, Isola JJ, Helin HJ (1994) Prognostication of astrocytoma patient survival byKi-67 (MIB-1), PCNA and S-phase fraction using archival paraffin-embedded samples. J Pathol 174: 275–282
44. Sarkis AS, Dalbagni G, Cordon-Cardo C, Zhang Z, Sheinfeld J, Fair WR, Herr HW, Reuter VE (1993) Nuclear overexpression of p53 protein in transitional cell bladder carcinoma.: a marker for disease progression. J Natl Cancer Inst 85: 53–59
45. Shin KY, Kong G, Kim WS, Lee TY, Woo YN, Lee JD (1997) Overexpression of cyclin D1 correlates with early recurrence in superficial bladder cancers. Br J Cancer 75: 1788–1792
46. Sinicrope FA, Roddey R, Lemoine M, Ruan S, Stephens LC, Frazier ML, Shen Y, Zhang W (1998) Loss of $p21^{WAF/CIP1}$ Protein expression accmpanies progression of sporadic colorectal neoplasms but not hereditary nonpolyposis colorectal cancers. Clin Cancer Res 4: 1251–1261
47. Spruck CH 3rd, Ohneseit PF, Gonzales-Zulueta M, Esrig D, Miyao N, Tsai YC, Lerner SP, Schmutte C, Yang AS, Cote R, et al (1994) Two molecular pathways to transitional cell carcinoma of the bladder. Cancer Res 54: 784–788
48. Stein JP, Ginsberg DA, Grossfeld GD, Chatterjee SJ, Esrig D, Dickinson MG, Groshen S, Taylor CR, Jones PA, Skinner DG, Cote RJ (1998) Effect of $p21^{WAF1/CIP1}$ expression on tumor progression in bladder cancer. J Natl Cancer Inst 14: 1072–1079
49. Stein JP, Grossfeld GD, Ginsberg DA, Esrig D, Freeman JA, Figueroa AJ, Skinner DG, Cote RJ (1998) Prognostic markers in bladder cancer: a contemporary review of the literature. J Urol 160: 645–659
50. Suwa Y, Takano Y, Iki M, Takeda M, Asakura T, Noguchi S, Masuda M (1998) Cyclin D1 protein overexpression is related to tumor differentiation, but not to tumor progression or proliferative activity, in transitional cell carcinoma of the bladder. J Urol 160: 897–900
51. UICC International Union Against Cancer (1978) TNM classification of malignant tumours. UICC, Geneva
52. Wakasugi E, Kobayashi T, Tamaki Y, Ito Y, Miyashiro I, Komoike Y, Takeda T, Shin E, Takatsuka Y, Kikkawa N, Monden T, Monden M (1997) p21(Waf1/Cip1) and p53 Protein expression in breast cancer. Am J Clin Pathol 107: 684–691
53. Xiong Y, Zhang H, Beach D (1992b) D type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. Cell 71: 505–514