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ORIGINAL PAPER

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P27^{Kip1} and Ki-67 expression analysis in transitional cell carcinoma of the bladder

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Abstract P27^{Kip1} protein is a cell cycle inhibitor which blocks the transition of cells from G1 to S phase, while Ki-67 is the most specific marker of proliferative activity. Both proteins are independent predictors of clinical outcome in various neoplasms. The aim of the study was to assess the prognostic value of p27^{Kip1} and Ki-67 expression in urothelial bladder tumours. P27^{Kip1} and Ki-67 expressions were evaluated immunohistochemically in archival samples of 45 superficial and 26 invasive transitional cell carcinomas obtained by a transurethral resection. In the patients with superficial tumours, disease-free survival (DFS) was positively influenced by good histological differentiation as well as by concurrent high p27^{Kip1} and low Ki-67 expression. Multivariate analysis has confirmed that tumour grade and p27^{Kip1}/Ki-67 status were independent predictors of DFS ($p = 0.028$ and $p = 0.029$, respectively). P27^{Kip1} or Ki-67 expressions did not influence overall survival. We conclude that a variable combined of p27^{Kip1} and Ki-67 expressions is a better predictor of DFS in superficial bladder tumours than either protein alone.

Keywords Transitional cell carcinoma · Bladder neoplasms · p27 · Ki-67 · Disease-free survival

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Introduction

Transitional cell carcinoma (TCC) of the bladder has two major histopathological and clinical presentations. Eighty percent of all newly diagnosed cases are superficial tumours confined to the urothelium or the lamina propria (pTa and pT1) and the other 20% are invasive tumours. However, the clinical course of the disease is more diverse than could be expected from this classification. Less than 50% of superficial tumours can be durably cured by one transurethral resection (TURT); the other superficial tumours do recur, usually within 3 years after primary treatment. Progression to muscle-invasive disease is observed in 10–13% of patients [12, 15, 17]. Tumour stage, tumour grade, tumour size, multifocality and presence of dysplasia or carcinoma in situ in random biopsy specimens are most important prognostic factors of recurrence or progression in superficial tumours. However, these factors do not help in therapeutic decision-making in an individual patient [12, 15, 17, 24, 31]. In the invasive form of TCC of the bladder, tumour stage and nodal involvement are important clinical prognostic factors of survival for uniformly treated patients [3, 37].

There is a search for novel prognostic factors that would give information independent of classic predictors. In recent years much attention has been focused on significance of cell cycle regulatory proteins in TCC of the bladder. Among the proteins or genes studied in TCC are p53, Rb, MDM2, p16, p21, p27^{Kip1}, Ki-67, cyclins E and D1, etc. Until now, there has been no consensus on their prognostic significance. The most encouraging results have been obtained for p53 as a predictor of recurrence or progression of superficial tumours [28]. As far as p27^{Kip1} and Ki-67 are concerned, there have been few reports on their prognostic significance in the TCC of the bladder. Both proteins were assessed together in only four studies and their results were contradictory [10, 14, 16, 32].

P27^{Kip1} protein is an inhibitor of the cell cycle which blocks its progression by binding and inhibiting the

activity of cyclin E/Cdk2 complex. High $p27^{Kip1}$ expression is found in quiescent cells (G_0 phase) and in the early G_1 phase. In late G_1 phase, $p27^{Kip1}$ is quickly degraded, which enables the transition from G_1 to S phase and the beginning of DNA replication. Mutations in the $p27^{Kip1}$ gene are rare [25]. Differing $p27^{Kip1}$ levels do not result from specific gene aberrations; rather they represent an inherent activity of the $p27^{Kip1}$ in assessed tissues. It has been found that $p27^{Kip1}$ expression is an independent prognostic factor of overall survival and progression in lung, breast, colorectal and prostate cancer [9, 20, 35, 39].

Ki-67 antigen is a nuclear protein of unknown function. Its high expression can be observed in all phases of the cell cycle. During G_0 phase the protein is undetectable. It is considered the most specific available proliferative marker [29]. It has been found that Ki-67 expression level is an independent predictor of recurrence and overall survival in breast cancer [13, 30]. Also, in other neoplasms such as in prostate cancer or soft tissue tumours, the relationship between Ki-67 expression and survival has been reported [1, 4, 36].

The aim of this study was to evaluate the prognostic significance of $p27^{Kip1}$ and Ki-67 expression in TCC of the bladder.

Materials and methods

Patients

The group under study consisted of 71 patients (24 women, 47 men) aged 35–90 (mean, 68.3 years), who underwent transurethral resection of a primary bladder tumour (TURT) in the years 1994 and 1995. Solitary tumours were found in 50 patients; 21 patients had multifocal changes. Total resected tissue specimens were collected for histopathological evaluation. Histological tumour type and grade were determined by one pathologist according to the WHO criteria [22]. Tumours other than transitional cell carcinoma were not taken into the study. The staging of the tumours was carried out according to TNM classification [33]. In all cases studied, muscularis propria was available for evaluation. Of the 71 patients, 45 had superficial (Ta, T1) and 26 had invasive tumours (T2–T4). Distribution into grade and stage categories is shown in Table 1. Patients with carcinoma in situ were not qualified for the study.

After primary TURT, the patients with superficial disease underwent control cystoscopies according to standard clinical practice. The patients with invasive tumours were qualified for radical cystectomy (performed in 18 of 26 cases) or less radical form of treatment depending on the patient's clinical condition. None of them had preoperative radiation or chemotherapy. The follow-up period was defined as time from the first TURT to the last contact or death. The follow-up period ranged from 1 to 82 months (median, 64 months) for patients with superficial disease and from 4 to 76 months (median, 13 months) for patients with invasive disease. During the follow-up period, 13 of 45 (30%) patients with superficial tumours and 21 of 26 (80%) patients with invasive tumours died.

Immunohistochemistry

Paraffin embedded samples were cut into 3-μm serial sections. Antigen retrieval was performed by boiling tissue sections in citric acid buffer (pH 6) in a microwave oven for 6×5 min at 700 W.

Table 1 P27 and Ki-67 expression in relation to clinical and pathological parameters of bladder tumours

	No. of patients	p27 expression		Ki-67 expression	
		Low	High	Low	High
Tumour grade					
G1	18	9	9	15*	3*
G2	39	19	20	18*	21 *
G3	14	8	6	2*	12*
Pathological stage					
Ta	25	11	14	20*	5*
T1	20	11	9	9*	11 *
T2	11	5	6	3*	8*
T3	9	5	4	2*	7*
T4	6	4	2	1*	5*
Solitary	50	28	22	22	28
Multifocal	21	8	13	13	8
Ki-67expression					
Low	35	13*	22*		
High	36	23*	13*		

* statistically significant differences in chi-square test ($p < 0.05$).

Nonspecific tissue reactivity was blocked by 10% goat serum. Sections were incubated overnight at 4°C with primary monoclonal antibodies: NCL-p27 (1: 40; Novocastra, Newcastle upon Tyne, UK) for $p27^{Kip1}$, and MIB-1 (1: 50; Immunotech, Marseille, France) for Ki-67. Endogenous peroxidase activity was blocked by 3% H_2O_2. The detecting system consisted of biotinylated anti-mouse IgG (1: 1500, cat. No. 816), peroxidase conjugated streptavidin (1: 500, cat. No. 309; both from Immunotech) and diaminobenzidine. Between incubations, the sections were washed twice for 5 min in pH 7.4 phosphate buffered saline. Inflammatory tonsils served both as a positive (germinal centres) and negative (cortex) control. Normal mouse IgG (1: 25, Dako, Glostrup, Denmark) was also used as a negative control. Lymphocytes and stromal cells were intrinsic positive controls for tissue reactivity.

Immunohistochemical evaluation was performed without knowledge of the clinical data. Expression of the proteins was estimated by counting the percentage of stained cells out of 800 cells (200 consecutive cells were counted in four different foci) under 400× magnification. $P27^{Kip1}$ labelling index (LI) was assessed in randomly chosen fields, while Ki-67 LI was evaluated in places with the highest expression, in order to assess the highest proliferative potential of a tumour. Cut-off points were established at median values of Ki-67 and $p27^{Kip1}$ indices (30% and 21%, respectively). Indices below the median were classified as low, while indices equal to or above median were classified as high.

Statistical analysis

The following independent variables were included in statistical analyses: tumour stage, histological grade, multifocality (solitary vs multifocal), $p27^{Kip1}$ LI, Ki-67 LI (low vs high), a variable created by combined evaluation of both proteins' expression (high Ki-67/high $p27^{Kip1}$ vs high Ki-67/low $p27^{Kip1}$ vs low Ki-67/high $p27^{Kip1}$ vs low Ki-67/low $p27^{Kip1}$). The association between the variables studied was calculated using contingency table methods and tested for significance with the Pearson χ^2 test. Survival curves were calculated using the Kaplan-Meier method and differences were tested for significance with use of the log-rank test [21]. Cox proportional hazards regression model was used in multivariate analysis of overall survival and disease-free survival and for calculation of relative risks [6]. The final model was obtained by elimination of variables that had p-value higher than 0.1 in the primary model. The results were considered significant when $p \leq 0.05$. All

calculations were performed with use of commercial software (STATISTICA).

Results

Tumour tissue showed a predominant nuclear staining for $p27^{Kip1}$ and exclusively nuclear staining for Ki-67 antigen. Intensity of $p27^{Kip1}$ reactivity was heterogenous: from very weak to strong. Only moderate or strong nuclear staining for the $p27^{Kip1}$ was considered positive. Cytoplasmic reaction, observed in some cases, did not disturb the evaluation and was not taken into account.

Thirty two percent of the assessed tumours had high expression of Ki-67 and low expression of $p27^{Kip1}$, and nearly the same percentage of samples had a reverse characteristic. The negative association between Ki-67 LI and $p27^{Kip1}$ LI was statistically significant ($p = 0.02$). Associations between Ki-67 LI, $p27^{Kip1}$ LI and clinicopathological tumour characteristics are drawn in Table 1. High Ki-67 LI was associated with higher stage ($p = 0.002$) and higher grade ($p = 0.0005$) of the tumours. High proliferation was seen more often in solitary than in multifocal tumours but the difference was not statistically significant. In contrast to Ki-67 LI, high $p27^{Kip1}$ LI was observed more often in superficial, low-grade and multifocal tumours; however, the differences were not statistically significant.

Disease-free survival analysis

Twenty seven of the 45 patients (60%) with superficial bladder tumours experienced at least one recurrence. In univariate analysis, tumour grade was the only clinicopathological parameter associated with disease-free survival (DFS) ($p = 0.018$; Fig. 1). Tumours with high Ki-67 LI, as well as tumours with low $p27^{Kip1}$ LI had shorter DFS, but the differences were not significant ($p = 0.068$ and $p = 0.085$, respectively). After dividing samples into four groups according to the level of expression of both proteins (i.e. high Ki-67/high $p27^{Kip1}$; high Ki-67/low $p27^{Kip1}$; low Ki-67/high $p27^{Kip1}$; low Ki-67/low $p27^{Kip1}$), it was found that tumours with low Ki-67 expression and high $p27^{Kip1}$ expression had a lower risk of recurrence than tumours with other characteristics ($p = 0.006$; Fig. 2). In the former group, the cumulative proportion of patients surviving without recurrence was 68% at the end of the follow-up period, while it was 17% for the rest of the cases. The differences between other subgroups were insignificant. Multivariate analysis has confirmed independent prognostic value of both grade and Ki-67/$p27^{Kip1}$ status for DFS ($p = 0.028$ and $p = 0.029$ respectively, Table 2).

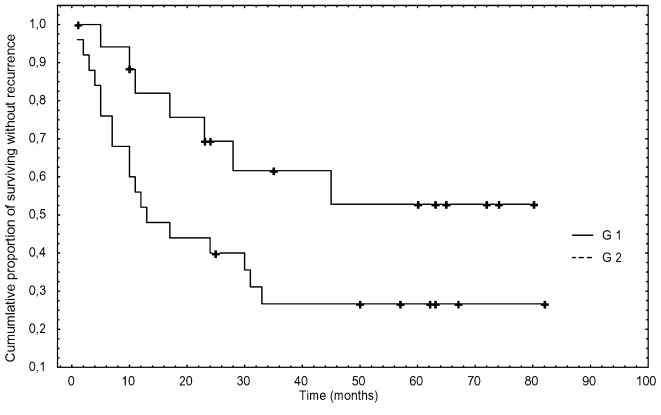

Fig. 1 Disease-free survival in superficial tumours as a function of grade ($p = 0.018$ in log-rank test). Two grade 3 cases were omitted

Overall survival

In univariate analysis of overall survival of all 71 patients, only stage ($p < 0.0001$) and grade ($p = 0.002$) were significant variables. Multivariate analysis identified tumour stage as the only independent significant predictor of overall survival ($p < 0.0001$). In the subgroup of invasive tumours (T2–T4; $n = 26$), stage was also the only significant predictive factor of overall survival ($p = 0.02$). No relationship between Ki-67 or $p27^{Kip1}$ labelling indices and overall survival was found.

Discussion

We have found that the combined $p27^{Kip1}$ and Ki-67 expression level was an independent prognostic factor of

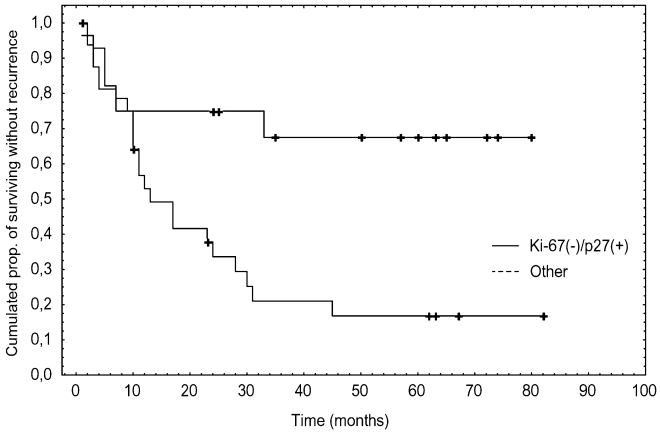

Fig. 2 Disease-free survival as a function of Ki-67/p27 status ($p = 0.006$ in log-rank test)

Table 2 The significant prognostic factors of disease-free survival and their relative risk (RR) by Cox regression analysis in 45 superficial bladder tumours

Variable	RR	95% confidence interval	p
Grade	2.37	1.097–5.12	0.028
Ki-67/p27	3.04	1.12–8.25	0.029

DFS in superficial bladder tumours. The DFS showed similar association with Ki-67/$p27^{Kip1}$ as with tumour grade – an established clinical prognosticator. Simultaneous evaluation of two or more proteins' expression is frequently reported and it may be justified by complex protein–protein interactions. In a recently published study on cyclin D1, $p27^{Kip1}$ and Ki-67 in superficial bladder tumours [32], the most powerful predictor of recurrence was created by a combination of these parameters (low $p27^{Kip1}$/low cyclin D1/high Ki-67 tumours vs other tumours). Important results on simultaneous $p27^{Kip1}$ and Ki-67 evaluation in TCC of the bladder were presented by Korkolopoulou et al. [16], who also assessed a panel of cell cycle regulators. They found that Ki-67/$p27^{Kip1}$ expression status was the only independent prognostic factor of overall survival in the invasive tumours ($p = 0.0019$), although not in the entire group analysed (37 superficial and 83 invasive bladder tumours). In the latter, Ki-67 and pRb expression were independent predictors of overall survival.

Negative association between $p27^{Kip1}$ LI and Ki-67 LI observed in our and in other studies does not reach a high level of significance ($p = 0.074$–0.02 [10, 14, 16, 32]). We evaluated KI-67 LI in foci selected for high growth fraction, while $p27^{Kip1}$ LI was evaluated in random fields. Thus, simultaneous high $p27^{Kip1}$ LI and high Ki-67 LI can be explained by the presence of areas with both high and low proliferation in one specimen.

In our study, in about one-third of the cases both proteins had simultaneously either low or high labelling indices and it coincided with a high rate of recurrence. In tumours with low LIs of both proteins, proliferation is probably less intensive, but they maintain the potential to proliferate (lack of inhibition by $p27^{Kip1}$). Similar conclusions were derived by Endl et al. [8], who evaluated expression of Ki-67 and $p27^{Kip1}$ in human tonsil. They found the population of cells that did not express Ki-67 and $p27^{Kip1}$. These cells were localized in the intermediate layer of the epithelium, between proliferating and nonproliferating areas, and could either re-enter the cell cycle or differentiate. Such a population of cells undetermined for proliferation may exist in Ki-67 (–) /$p27^{Kip1}$ (–) specimens and may behave like Ki-67 expressing cells. It seems that the level of $p27^{Kip1}$ LI provides complementary information on physiological activity of cells in Ki-67 low-expressing superficial bladder tumours.

In our study, $p27^{Kip1}$ LI or Ki-67 LI, as separate variables, showed a trend toward association with disease-free survival, but it did not reach statistical significance. Kamai et al. [14] presented a report on 86 superficial bladder tumours, in which high Ki-67 expression conferred 3.6-fold higher relative risk of recurrence; low $p27^{Kip1}$ expression had equally high prognostic value, while tumour stage and grade did not influence disease-free survival. For Ki-67, the same results were obtained even in larger populations [2, 23, 27]. In other studies concerning Ki-67 and $p27^{Kip1}$, their prognostic value for disease-free survival in superficial bladder tumours, when proved, was lower than the value of clinicopathological parameters [11, 26, 31, 38]. Moreover, there are studies that deny that Ki-67 or $p27^{Kip1}$ expression can predict clinical outcome [5, 10, 16, 18, 24]: e.g. Pfister et al. [24] evaluated 308 patients with superficial bladder tumours and found no correlation between Ki-67 expression and disease-free survival.

Studies on expression of cell cycle regulators in invasive bladder tumours and their impact on overall survival (OS) analysis are few. Apart from the above-mentioned study by Korkolopoulou et al. [16], significant results were achieved for $p27^{Kip1}$ by Del Pizzo et al. [7] (although their group was smaller than ours, 29 superficial and 18 invasive tumours) and for Ki-67 by Suwa et al. [34]. $P27^{Kip1}$ and Ki-67 were also recognized as independent predictors of overall survival in the Kamai et al. [14] study, in which they showed higher impact on OS than clinicopathological parameters. In our group, tumour stage was the only independent prognostic factor of OS. $P27^{Kip1}$ and Ki-67 LIs were not associated with OS, neither evaluated separately nor simultaneously.

In summary, we have found that the combined value of Ki-67 LI/$p27^{Kip1}$ LI is more accurate than separate expression of both proteins for prediction of disease-free survival in superficial bladder tumours. Confirmation of this result by large and prospective studies would make its use possible in qualification for additional treatment of superficial tumours (e.g. with intravesical chemotherapy) and for more strict follow-up.

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