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p27^{kip1} and Ki-67 (MIB1) Immunohistochemical Expression in Radical Prostatectomy Specimens of Patients with Clinically Localized Prostate Cancer

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Abstract. *The immunohistochemical expressions (IE) of p27^{kip1} and Ki-67 (MIB-1), both involved in cell cycle regulation and cell proliferation, and their ability to predict biochemical failure, were assessed in patients with clinically localized prostate cancer who had underdone radical prostatectomy of curative intent. In addition, p27^{kip1} and Ki-67 (MIB1) expressions were correlated with several pre-operative and post-operative parameters, such as Gleason score, extracapsular extension, seminal vesicle involvement, pelvic lymph nodes metastasis, positive surgical margins, coexistence of high-grade prostatic intraepithelial neoplasia, tumour size, prostate volume and PSA levels. Our analysis involved 130 consecutive radical prostatectomy specimens. A statistically significant correlation of low p27^{kip1} IE with seminal vesicles involvement, increased tumour volume and high pre-operative PSA values was documented. Low p27^{kip1} IE was significantly correlated with an increased likelihood of biochemical failure after radical prostatectomy. In addition, the increased IE of Ki-67 (MIB1) correlated significantly with metastatic disease in the pelvic lymph nodes and was a significant predictor of biochemical failure. Cox regression analysis, which included p27^{kip1} expression, Ki-67 (MIB1) expression and all the pre-operative and post-operative parameters, showed that pelvic lymph node involvement and Ki-67 (MIB1) IE were independent prognostic markers of biochemical failure after radical prostatectomy.*

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Prostate cancer (PC) is the most frequently diagnosed malignancy in elderly men (1, 2). The widespread introduction of serum prostate specific antigen (PSA) measurements, as a pre-screening tool for the detection of PC in healthy men, has significantly increased the number of men diagnosed with clinically localized PC and the number of patients who undergo radical prostatectomy with curative intent. Disappointingly, biochemical failure is as high as 30-50% in patients with clinically localized PC following radical surgery (3, 4). The Gleason score, tumour stage and pre-operative PSA levels are predictors, to a certain degree, of the biochemical failure. Ideally, clinical decisions should be based on reliable tools capable of assessing the exact stage and aggressiveness of the disease before surgery. Therefore, intensive clinical investigation aims at the development of reliable molecular techniques for the detection of the transcripts of PSA and prostate specific membrane antigen (PSMA) in the peripheral blood and in the bone marrow biopsy specimens of patients with clinically localized PC, with sometimes promising, albeit not always reproducible, data (5-8). Indeed, PC, which is already metastatic at diagnosis or able to produce metastasis as a result of its histological grade, must be distinguished from indolent prostate cancer tumours. Patients with aggressive tumours should be candidates for adjuvant therapy before or after surgery and radiotherapy. For this reason, in prostate cancer (PC) there is an urgent need to develop reliable molecular methods and new prognostic markers capable of predicting disease spread at diagnosis and disease recurrence after surgery of curative intent.

In this study, the prognostic value of tissue markers, such as p27^{kip1} and Ki-67 (MIB-1), both involved in cell cycle regulation and cell proliferation *vis-à-vis* biochemical failure after radical prostatectomy, were addressed. In a variety of

human tumours $p27^{kip1}$ expression is decreased, and low $p27^{kip1}$ expression has been associated with poor outcome in breast, colorectal and lung carcinomas (9-12). In PC specimens, suppressed $p27^{kip1}$ expression is also correlated with advanced pathological features, tumour progression and poor survival (13-18). In addition, some previous studies showed that the proliferation fraction of primary PC, as assessed by Ki-67 (MIB1) expression, was associated with grade or stage of PC and clinical outcome of patients after radical prostatectomy (18-26).

Therefore, the immunohistochemical expression (IE) of $p27^{kip1}$ and Ki-67 (MIB1) was assessed in radical prostatectomy specimens of patients with PC. In addition, multivariate analysis was performed on the IE of these two cell cycle and proliferation regulators and of several clinicopathological parameters, such as Gleason score, extracapsular extension, seminal vesicle involvement, pelvic lymph nodes metastasis, positive surgical margins, coexistence of high-grade prostatic intraepithelial neoplasia (PIN), tumour size, prostate volume and pre-operative PSA levels *versus* biochemical failure after radical prostatectomy.

Materials and Methods

In this study, 130 consecutive radical prostatectomy specimens, from patients who underwent radical prostatectomy for localized PC at the "Evangelismos" General Hospital, Athens, Greece, between 01/1994 and 12/2001, were evaluated. The median age of the patients was 66 years (range 47-76 years). Pre-operative PSA revealed a median PSA of 9.23 (2.5-45.0) ng/ml. Follow-up was available for 94 patients, so the other 36 (referred for surgery to our hospital from rural areas of Greece and followed-up locally) were excluded from the statistical analysis, which concerned biochemical failure. The follow-up period ranged from 1 to 97 months (median: 28 months). A cut-off of 0.2 ng/ml serum PSA, on at least two occasions, with the second elevation measured at least 6 months after the first, established a biochemical failure.

Radical prostatectomy specimens were submitted fresh for routine pathological examination. The specimens were weighed, inked and fixed in buffer-formalin. Routine processing included sectioning and paraffin- embedding of the whole prostate gland. Four-mm-thick paraffin sections were used for haematoxylin-eosin staining and the performance of the immunohistochemical staining. Almost all selected sections contained benign prostatic glands, which could serve as internal positive controls for immunohistochemistry. Grading was established according to the Gleason score (GS) differentiation system (range 2-10) (27). GS 7 carcinomas were stratified to 7a and 7b, according to the predominate grade, as there is increasing evidence that the biological behaviour of Gleason scores 3+4 (7a) and 4+3 (7b) of Gleason score 7 is different with regard to the prognosis (28, 29). A 2-scale grouping was used for the final evaluation of the histological grading, as follows: group I≤7a and group II≥7b. An area with the highest Gleason score was selected for immunohistochemistry. Staging was carried out according to the TNM 2002 classification (30). The following pathological parameters were evaluated: extension of the tumour to an inked specimen margin, extracapsular extension into periprostatic tissue, seminal vesicle and pelvic lymph nodes involvement, prostate weight, tumour volume and coexistence of high-grade PIN.

The immunohistochemical streptavidin biotin peroxidase protocol using the DAKO LSAB+Kit Peroxidase was performed, with commercially available antibodies: the mouse monoclonal antibody against $p27^{kip1}$ (clone SX53G8; DAKO, Glostrup, Denmark), at a dilution of 1:10 and the mouse monoclonal antibody against Ki-67 (clone MIB-1; DAKO), at a dilution of 1:10. Staining procedures included deparaffinization in warm xylene for 5 min with two changes of xylene at room temperature, followed by rehydration by transfer through graded alcohols. Endogenous peroxidase activity was blocked with 0.5% H_20_2 in methanol for 10 min. The sections were pre-treated with 10 mmol/L citrate buffer (pH 6.1) in a microwave for 5 min and incubated overnight at 4˚C with the hK10 primary rabbit polyclonal and mouse monoclonal antibodies in 3% BSA. After two washes of the sections in 50 mM Tris buffer (pH 7.6), the biotinylated Link (DAKO Corporation, USA) was applied for 15 min and a streptavidin-peroxidase conjugate followed for another 15 min. The enzymatic reaction was developed in a freshly prepared solution of 3,3'-diaminobenzidine tetrahydrochloride using DAKO Liquid DAB Substrate-Chromogen Solution for 10 min (brown colour). The sections were then counterstained with haemalum, dehydrated, cleared in xylene and mounted.

Most of the detectable staining was heterogeneous and focal in nature. For $p27^{kip1}$ scoring, an area with the lowest density of positive cells for the protein expression was chosen. In contrast to Ki-67 scoring, the area with maximum immunostaining was selected. Then malignant cells were counted at 400x magnification. The number of cells with positive nuclear staining for $p27^{kip1}$ and Ki-67 (MIB-1) were recorded. Then the results were grouped and divided into low and high expression groups. $p27^{kip1}$ immunoexpression in neoplastic cells was scored as high if more than 30% of the cells were stained and as low if less than 30% of the cells were positive (31, 32). For the proliferation index Ki-67 (MIB-1), a cut-off point of 5% was used to distinguish high and low expression groups.

Patients were subdivided into groups based on different clinical or pathological parameters. In this analysis, $p27^{kip1}$ and Ki-67 (MIB-1) were classified into two categories (positive and negative groups), and associations between gene expression status and other qualitative variables were analyzed using the Fisher's Exact test. Because the distribution of serum PSA, tumor percentage, prostate weight and patients' age were not Gaussian, the analysis of differences in $p27^{kip1}$ and Ki-67 (MIB-1) values between the two groups was performed with the nonparametric Mann-Whitney *U*-test.

Survival analyses were performed by constructing Kaplan-Meier Progression-Free Survival (PFS) curves, where differences between curves were evaluated by the log-rank test, as well as by estimating the relative risks for relapse and death using the Cox proportional hazards regression model. Only patients for whom the status of all variables was known were included in the multivariate regression models, which incorporated $p27^{kip1}$, Ki-67 (MIB-1) and all other variables for which the patients were characterized. In the multivariate analysis, the clinical and pathological variables that may affect progression-free survival, including Gleason score, surgical margins, PIN, extra capsular periprostatic tissue invasion, seminal vesicles' invasion, nodal status and tumor percentage were adjusted. Statistical analysis was performed using the SAS software (SAS Institute, Cary, NC, USA).

Table I. *Pathological characteristics of 130 radical prostatectomies performed on patients with localized prostate cancer.*

Variable	RP[a] specimens (%)
Gleason score	
5	2 (1)
6	27 (21)
7a	32 (25)
7b	43 (33)
8	16 (12)
9	10 (8)
Surgical margins	
Negative	86 (66)
Positive	44 (34)
PIN[b]	
Absent	28 (21)
Present	102 (79)
Periprostatic tissue invasion	
Absent	60 (46)
Present	70 (54)
Seminal vesicles' invasion	
Absent	96 (74)
Present	34 (26)
Nodal status	
Negative	120 (92)
Positive	10 (8)

[a]Radical prostatectomy
[b]Prostatic intraepithelial neoplasia

Results

The clinicopathological characteristics of the 130 patients, who underwent radical prostatectomy, included in this study are presented in Table I. Most of them showed advanced local growth of tumour. The Gleason scores ranged between 6 and 9, with a higher prevalence of score 7 (75 cases, approximately 58%). When the grouped Gleason scores (up to 6, 7 and 8-10) were compared between the preoperative biopsy specimens and the following radical prostatectomies, 47/83 (57%) showed concordance, while the biopsy score was lower in 30 (36%) cases and higher in 6 (7%) cases. The prostate weight ranged between 8 g and 250 g (median: 45 g) and PC tumor volume ranged between 1% and 100% (median: 30%) of the prostate volume.

Among radical prostatectomy specimens, positive surgical margins were identified in 44/130 (34%) cases, seminal vesicles involvement in 34 (26%), extracapsular invasion in 70 (54%) and metastatic disease in the pelvic lymph nodes in 10 (8%) cases. High-grade PIN was observed in 102 (79%) specimens.

Of the 94 patients with follow-up data, 35 (37%) had a biochemical recurrence during this period. Regarding the predictive significance of the examined clinicopathological parameters, univariate statistical analysis showed the following results. Strong predictors for relapse were extracapsular extension of the tumour ($p=0.003$), seminal vesicle involvement ($p<0.001$), tumour percentage ($p<0.001$) and metastatic disease in the pelvic lymph nodes ($p=0.005$), but not the positive surgical margins ($p=0.14$) and Gleason score (0.061). Coexistence of high-grade PIN in the specimen did not carry any prognostic significance ($p=0.33$).

The expression of p27^{kip1} was identified in the nuclei of benign prostatic gland epithelial cells and it was generally high and uniform. In some cases a weak cytoplasmic staining was also noted. In high-grade PIN, p27^{kip1} expression was also evident but to a lesser degree than in normal prostatic tissue. Low levels of p27^{kip1} expression were identified in 32/130 cases of PC (25%) (Figure 1A, B). MIB-1 staining was increased in the prostatic carcinoma in comparison with normal prostatic glands. The proliferation index was generally low. High Ki-67 (MIB-1) expression was found in 10 cases only (8%) (Figure 2).

The relationship between a wide variety of clinicopathological parameters with the IE of p27^{kip1} and MIB-1 was investigated. Low p27^{kip1} expression did not correlate with Gleason score ($p=0.41$). There were statistically significant correlations of low p27^{kip1} expression levels with seminal vesicle invasion ($p=0.009$) and increased tumour volume ($p=0.025$). There was also a strong association with high levels of preoperative serum PSA ($p=0.002$). In contrast, there was no correlation found concerning extracapsular invasion ($p=0.12$), positive surgical margins ($p=0.62$), pelvic lymph nodes involvement ($p=0.24$) and coexistence of high grade PIN ($p=0.28$) (Table II; Figures 3 and 4).

The proliferation index Ki-67 (MIB-1) was not associated with Gleason score ($p=0.26$). A high Ki-67 (MIB-1) IE showed a strong association with the existence of metastatic disease in the pelvic lymph nodes ($p=0.006$) and a weak, not statistically significant, correlation with seminal vesicle involvement ($p=0.074$) and increased tumour volume ($p=0.095$). There was no statistic association with the rest of the examined clinicopathological parameters (Table III).

A statistically significant higher rate for biochemical failure was found in those patients who expressed low levels of p27^{kip1} protein. Among the 94 patients with available follow-up data, 72 (73%) showed high expression and 20 of them suffered recurrent disease (28%). In the low p27^{kip1} expression group (22 cases, 24%), biochemical relapse was identified in 15 cases (68%) (log rank test, $p=0.001$) (Table IV).

High proliferation index Ki-67 (MIB-1) was also associated with biochemical recurrence of the disease (log rank test, $p=0.005$). A high proliferation index was found only in 9 cases and 7 of them relapsed (78%). In contrast, 85 cases showed low Ki-67 (MIB-1) expression. Recurrent disease was identified in 28 of them (24%). Taking the

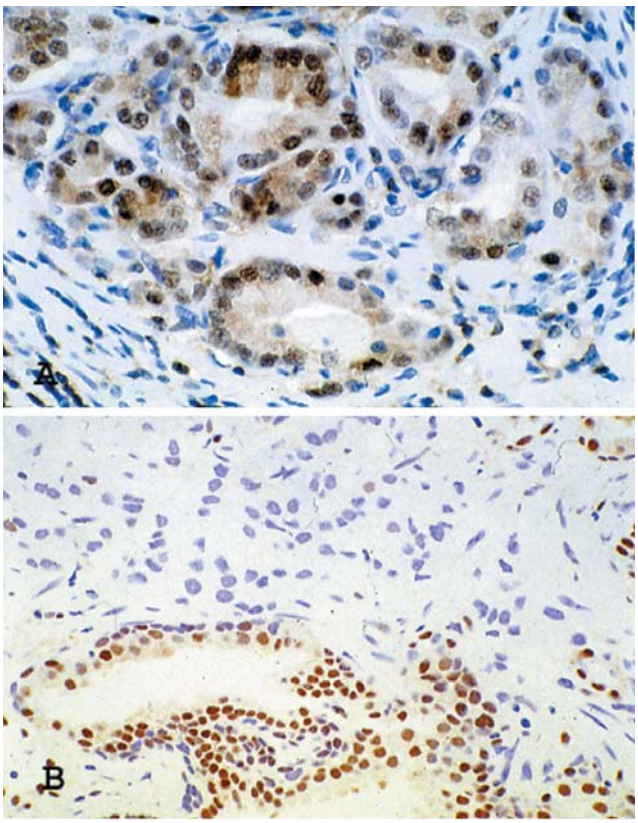

Figure 1. *An example of the immunohistochemical (IE) detections of p27^{kip1} in prostate cancer (PC) specimens. Panel A: High nuclear p27^{kip1} IE in PC, magnification x200. Panel B: Loss of p27^{kip1} IE in PC. Note the positive nuclear staining in adjacent benign prostatic epithelium (magnification x200).*

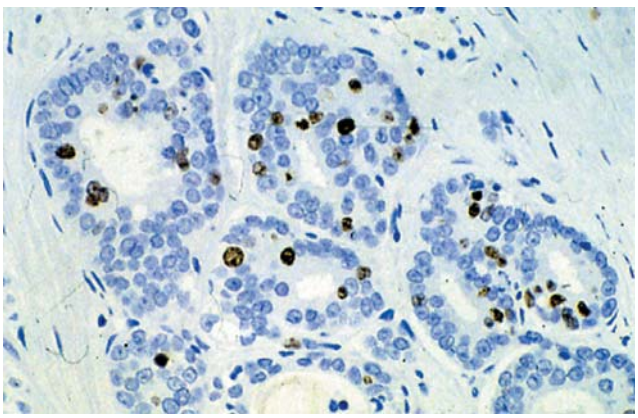

Figure 2. *An example of the immunohistochemical (IE) detections of Ki-67 expression in prostate cancer (PC) specimens. Note the high nuclear Ki-67 (MIB1) IE in PC (magnification x200).*

hazard ratio (HR), estimated from a Cox proportional hazards regression model, equal to 1.00 for patients with negative p27^{kip1}, it was found to be 0.31 for patients with positive p27^{kip1} (p=0.001, 95% CI=0.16-0.61). The HR was found to be 3.31 for patients with positive Ki-67 (MIB1) (p=0.005, 95% CI=1.43-7.64) (Table IV; Figures 5, 6).

Table II. *Relationship between p27^{kip1} immunoexpression and clinicopathological parameters in patients with prostate cancer.*

Variable	Patients	No. of patients (%)		p value[a]
		Low p27^{kip1} expression	High p27^{kip1} expression	
Gleason score				
</=7a	61	13(21)	48(79)	0.41
7b-10	69	19(28)	50(72)	
Extracapsular extension				
–	60	11(18)	49(82)	0.12
+	70	21(30)	49(70)	
Seminal vesicle invasion				
–	96	18(19)	78(81)	0.009
+	34	14(41)	20(59)	
Lymph node invasion				
–	120	28(23)	92(77)	0.24
+	10	4(40)	6(60)	
Surgical margins				
–	86	20(23)	66(77)	0.62
+	44	12(27)	32(73)	
PIN[b]				
–	28	9(32)	19(68)	0.28
+	102	23(23)	79(77)	

[a]Fisher's exact test
[b]Prostatic intraepithelial neoplasia

Multivariate analysis (Cox regression) of these parameters revealed that pelvic lymph nodes metastasis (p=0.040) and high IE of Ki-67 (MIB1) (p=0.043) were independent prognostic markers for biochemical failure (Table V).

Discussion

Prostate cancer is a biologically heterogeneous disease (33) with clinical course, which can vary from that of an indolent disease to a rapidly growing tumours with the ability to produce metastases. A large number of patients who undergo radical prostatectomy have organ- or specimen-confined disease on pathological examination, with a Gleason score mostly of 7. Despite these favourable characteristics, a significant number of patients develop biochemical failure after surgery. Indeed, in our study, 35 out of 94 patients (37%) with available follow-up data presented with biochemical failure after radical prostatectomy. Therefore, our aim was to compare the conventional variables for assessing an individual's risk from PC with p27^{kip1} and Ki-67 expression as prognostic value markers.

Both p27^{kip1} and Ki-67 proteins are involved in cell cycle regulation and cell proliferation. The cell cycle is controlled

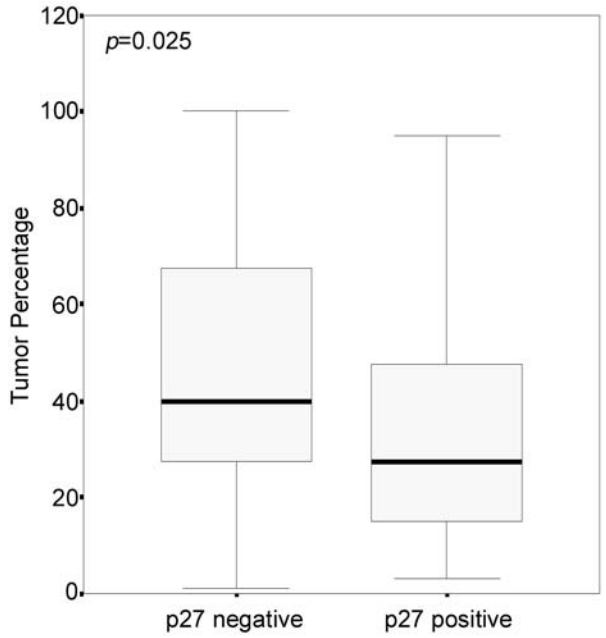

Figure 3. *Correlation between immunohistochemical (IE) detections of p27kip1 expression in 130 radical prostatectomy specimens with PC and tumor volume (percentage of tumor in the surgical specimens). The boxplots display the quartiles (the bold horizontal line being the median). P value was calculated by the Mann-Whitney U-test.*

Table III. *Relationship between Ki-67 (MIB-1) immunoexpression and clinicopathological parameters in patients with prostate cancer.*

Variable	Patients	No. of patients (%)		*p* value[a]
		Low Ki-67 (MIB-1) expression	High Ki-67 (MIB-1) expression	
Gleason score				
</=7a	61	58(95)	3(5)	0.26
7b-10	69	62(90)	7(10)	
Extracapsular extension				
–	60	57(95)	3(5)	0.29
+	70	63(90)	7(10)	
Seminal vesicle invasion				
–	96	91(95)	5(5)	0.074
+	34	29(85)	5(15)	
Lymph node invasion				
–	120	113(94)	7(6)	0.006
+	10	7(70)	3(30)	
Surgical margins				
–	86	81(94)	5(6)	0.26
+	44	39(87)	5(13)	
PIN[b]				
–	28	24(86)	4(14)	0.21
+	102	96(94)	6(6)	

[a]Fisher's exact test
[b]Prostatic intraepithelial neoplasia

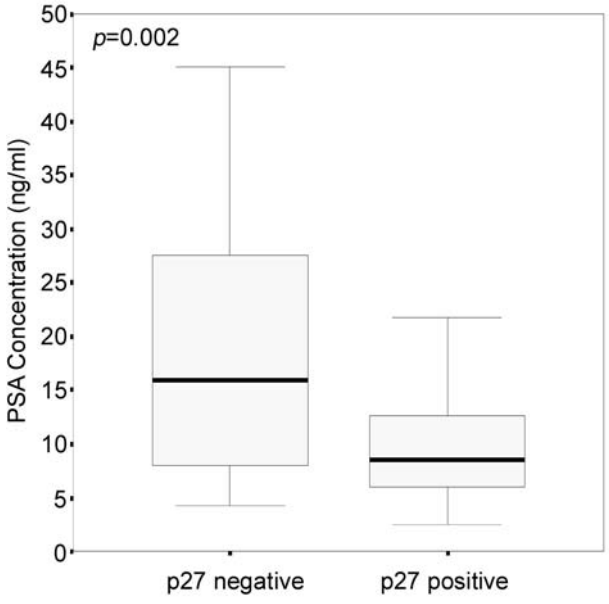

Figure 4. *Correlation between immunohistochemical (IE) detections of p27kip1 expression in 130 radical prostatectomy specimens and pre-operative serum PSA. The boxplots display the quartiles (the bold horizontal line being the median). P value was calculated by the Mann-Whitney U-test.*

by cyclin and cyclin-dependent kinase (CDK) subunits (34). Several different cyclin-CDK complexes are active at different stages of the cell cycle. CDKs activity is regulated by phosphorylation and by cyclin-kinase inhibitors (CDKIs). These inhibitors, such as p27kip1 protein, disrupt cell division blocking the transition from G1- to S-phase (35). Therefore, the *p27kip1* gene encodes a possible tumour suppressor gene. Genomic alteration of this gene seems to be rare in human malignancies, whereas protein expression is regulated at the post-transcriptional level, through protein translation and degradation (36, 37). Moreover, the indices characterizing an increased rate of proliferation among tumour cells correlate with enhanced metastatic potential of the primary tumour (38). The nuclear Ki-67 protein, which can be identified by the MIB-1 antibody, is expressed in all proliferating cells (G1-S-G2-M0- phase), but not in cells in the quiescent phase (G0) or in the early G1-phase (39). MIB-1 is a monoclonal antibody to recombinant parts of the Ki-67 antigen and has an identical nuclear staining pattern in paraffin sections as Ki-67 antibody in fresh tissues (40).

Reduced expression of p27kip1 has been correlated with adverse pathological features, increased risk of biochemical relapse and poor survival in PC, as previously mentioned. It was supposed that p27kip1 protein expression not only controls cell cycle progression, but might also be associated

Table IV. *Univariate analysis of p27^{kip1}, Ki-67 (MIB1) and other variables with regard to PFS.*

Variable	HR[a]	95% CI[b]	*p* value
Ki-67 (MIB1)			
Negative	1.00		
Positive	3.31	1.43-7.64	0.005
p27^{kip1}			
Negative	1.00		
Positive	0.31	0.16-0.61	0.001
Gleason score			
≤7a	1.00		
7b-10	1.99	0.97-4.11	0.061
Surgical margins			
Negative	1.00		
Positive	1.67	0.85-3.29	0.14
PIN[c]			
Absent	1.00		
Present	0.69	0.33-1.45	0.33
Extracapsular extension			
Absent	1.00		
Present	3.13	1.45-6.71	0.003
Seminal vesicle invasion			
Absent	1.00		
Present	3.42	1.74-6.74	<0.001
Nodal status			
Negative	1.00		
Positive	3.98	1.50-10.57	0.005
Tumor percentage	1.02	1.012-1.034	<0.001

[a]Hazard ratio (HR) estimated from Cox proportional hazards regression model
[b]Confidence interval of the estimated HR
[c]Prostatic intraepithelial neoplasia

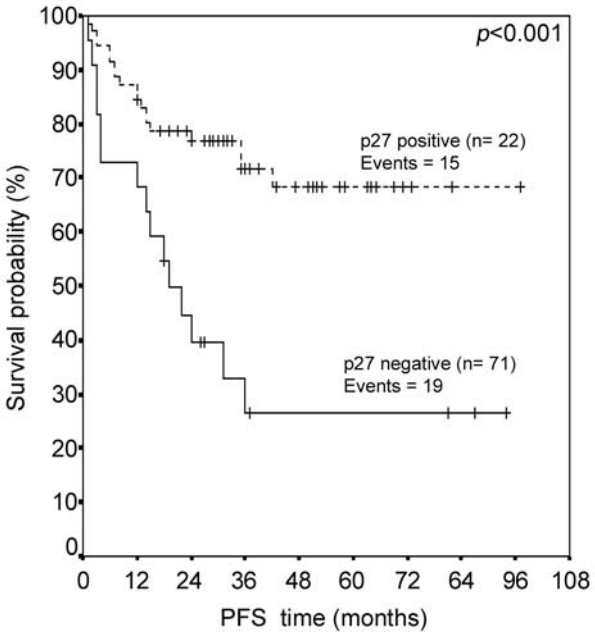

Figure 5. *Correlation between immunohistochemical (IE) detections of p27^{kip1} expression and biochemical failure in prostate cancer patients after radical prostatectomy.*

with other mechanisms responsible for aggressive tumour behaviour. A study in PC cell lines demonstrated that antisense-oligonucleotide mediated the down-regulation of p27^{kip1}, increased proliferation and reduced intercellular adhesion leading to tumour cell invasion, by facilitating individual cell detachment (41). In addition, a recent study showed that the expression of the f-box protein Skp2 (fb11), which is a positive regulator of G1-S transition and promotes ubiquitin-mediated proteolysis of p27^{kip1} protein, was elevated in PC. Furthermore, an inverse correlation of its expression with its biochemical target p27^{kip1} and with its putative negative regulator, PTEN tumour suppressor protein, was observed. Consequently, it was suggested that induction of Skp2 may be causally linked with decreased levels of p27^{kip1} in PC and implicate PTEN in the regulation of Skp2 expression (42).

The first study on IE of p27^{kip1} in PC was published in 1993. Yang *et al.* (13) found that low p27^{kip1} expression was the strongest predictor of disease progression and was associated with poor survival. There was no relationship between p27^{kip1} staining and pathological stage. Tsihlias *et al.* (14) showed that low p27^{kip1} expression correlated with seminal vesicle involvement and positive surgical margins. Cheville *et al.* (15) found a strong association of low p27^{kip1} with the above two parameters, but also with lymph nodes metastasis and aneuploid cancers. Cote *et al.* (16) demonstrated that low p27^{kip1} levels were correlated with shortened survival in patients with locally advanced disease. Similarly, Cordon-Cardo *et al.* (17) observed that patients with low protein expression had a statistically significant increased risk of disease progression. Vis *et al.* (18) found that reduced p27^{kip1} expression was an independent predictor of poor outcome in surgically treated patients and that it was highly correlated with the Gleason score and pathological stage. In a more recent work, the same team reported that low p27^{kip1} expression in needle biopsy material was a significant predictor of clinically significant disease (43). Thomas *et al.* (31) also reported that preoperative prostate needle biopsy p27^{kip1} correlated with subsequent radical prostatectomy p27^{kip1}, Gleason grade and pathological stage.

In contrast, Erdamar *et al.* (44) did not find any association between p27^{kip1} expression and Gleason score, clinical stage or disease progression after radical prostatectomy. They also demonstrated a dramatically reduced p27^{kip1} labelling in metastatic PC lesions. This last observation concurs with the findings of Fernandez *et al.*

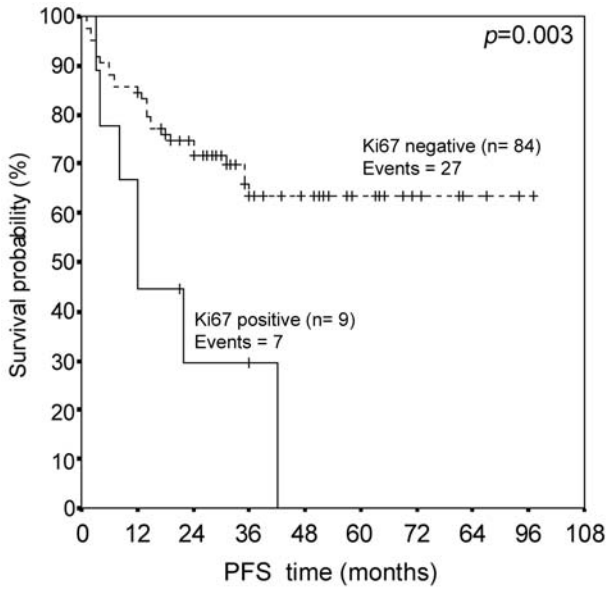

Figure 6. *Correlation between immunohistochemical (IE) detections of Ki-67 (MIB1) expression and biochemical failure in prostate cancer patients after radical prostatectomy.*

Table V. *Multivariate analysis of p27^{kip1}, Ki-67 (MIB1) and other variables with regard to PFS.*

Variable	HR[a]	95% CI[b]	*p* value
Ki-67 (MIB1)			
Negative	1.00		
Positive	2.61	1.03-6.56	0.043
p27^{kip1}			
Negative	1.00		
Positive	0.47	0.21-1.07	0.071
Gleason score			
≤ 7a	1.00		
7b-10	1.42	0.58-3.50	0.44
Surgical margins			
Negative	1.00		
Positive	0.74	0.31-1.82	0.52
PIN[c]			
Absent	1.00		
Present	0.87	0.37-2.02	0.74
Extracapsular extension			
Absent	1.00		
Present	2.71	0.90-8.16	0.076
Seminal vesicle invasion			
Absent	1.00		
Present	1.53	0.47-5.00	0.47
Nodal status			
Negative	1.00		
Positive	3.41	1.06-11.02	0.040
Tumor percentage	1.004	0.98-1.02	0.64

[a]Hazard ratio (HR) estimated from Cox proportional hazards regression model
[b]Confidence interval of the estimated HR
[c]Prostatic intraepithelial neoplasia

(45), who reported down-regulated expression of p27^{kip1} in neoplastic progression from pre-invasive lesions through invasive carcinoma and metastases. So, they concluded that p27^{kip1} down-regulation is a common phenomenon in PC and occurs in the early phases of neoplastic evolution. Cheng *et al.* (46) reported that reduced levels of p27^{kip1} and p21^{waf1} expression were predictive of distant metastasis-free, cancer-specific and all-cause survival for patients selected for salvage prostatectomy for recurrent PC.

In our study, there was no association of p27^{kip1} expression with Gleason score. Strong statistically significant correlations of low p27^{kip1} levels with seminal vesicles involvement, increased tumour volume and high preoperative serum PSA values were found. In univariate analysis, reduced p27^{kip1} immunoexpression strongly correlated with an increased likelihood of biochemical recurrence after radical prostatectomy for clinically localized PC, and this relationship was statistically significant.

Although it is not a molecular genetic marker, Ki-67 expression is being studied in radical prostatectomy and needle biopsy specimens. The growth of PC can be variable and rates of progression may be different among tumours of similar stage and grade. The MIB-1 proliferation index was lowest in benign prostatic hyperplasia followed by adenosis, low-grade PIN, low-grade cancer, high-grade PIN and high-grade cancer (47). The findings concerning the relationship between proliferation index and Gleason score or pathological stage vary greatly. In a number of studies, a significant association of a high proliferation index with a higher Gleason score and/or advanced pathological stage was observed (18, 20-23). However, other studies did not find any correlation between Ki-67 (MIB-1) expression and the above parameters (2, 25). The same controversial results have been found regarding the predictive value of the proliferation index in PC. Ki-67 (MIB-1) staining was an independent predictor of recurrence in many studies, either in radical prostatectomy specimens or needle biopsy materials (20, 22, 23, 25, 26, 48, 49). A significant prognostic value of Ki-67 has been detected by univariate, however, not by multivariate, analysis containing the conventional clinicopathological parameters or other tissue markers (21, 24). In contrast, no association between proliferation index and disease recurrence after radical prostatectomy has been reported by other studies (18, 50, 51). Dunsmuir *et al.* (23), although they found that MIB-1 provided prognostic information, questioned its true clinical value since it was expressed mainly in the most

advanced lesions. Cher *et al.* (38) reported that the cellular proliferative fraction of metastatic lymph nodes predicted survival in patients with stage D1 prostatic carcinoma. Matsuura *et al.* (52) found that high Ki-67 was an independent predictor of biochemical relapse in patients treated with androgen ablation for stage C or D PC. Similarly, Ki-67 (MIB-1) was a strong independent prognostic marker of biochemical failure in prostatic cancers treated with radiotherapy (53).

In our study, the proliferation index Ki-67 (MIB-1) did not correlate with the Gleason score in the 2-scale grouping. A statistically significant higher incidence of metastatic disease in the pelvic lymph nodes was identified in patients with high proliferation index. Moreover, there were weak, not statistically significant, associations with seminal vesicle involvement and tumour volume. There was no relationship between Ki-67 (MIB-1) expression and the rest of the clinicopathological parameters examined. On univariate analysis, a statistically significant higher biochemical failure rate was observed in patients with high proliferation index Ki-67 (MIB-1). The ynchronous expression of low levels of $p27^{kip1}$ protein and increased proliferation index was also statistically significant in univariate analysis, but the number of cases was low. Multivariate analysis (Cox regression), which included the two studied markers and the full range of clinicopathological parameters, showed that independent prognostic markers for biochemical relapse of the disease after radical prostatectomy were pelvic lymph nodes involvement and Ki-67 (MIB-1) IE.

In spite these promising findings, there are factors of concern about Ki-67's labelling index clinical application. These are inter-observer variability in the estimation of the expression and different ways of data categorization in previous studies. Also, there has been a considerable variation in the cut-off points used to assess failure risk. Ki-67 labelling index (LI) cut-off points have ranged from 1% (50) to 25% (22), and are most probably reflective of median Ki-67-LI differences. Moreover, a number of other technical variables might contribute to controversies, such as specimen fixation time and storage and variation in the monoclonal antibodies used (53). Also, for $p27^{kip1}$ expression, a recent study showed that inadequate fixation decreased the reliability of its immunohistochemical staining and formalin injection to the specimen was proposed, in order to produce improvement in staining (54).

In conclusion, the results of the present study indicated that measurement of the cellular proliferative fraction, as determined by the IE of Ki-67 (MIB-1), may be a useful marker for biochemical failure prediction and the frequency of the follow-up management in such patients after radical prostatectomy. Apparently, larger prospective clinical trials with longer follow-up are needed to evaluate the prognostic value of the Ki-67 (MIB1) IE in PC specimens.

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