Expression of Ki-67, Cyclin D1 and Apoptosis Markers Correlated with Survival in Prostate Cancer Patients Treated by Radical Prostatectomy

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Abstract. *Objective: The study was designed to analyse the prognostic value of proliferation markers Ki-67 and cyclin D1 and apoptosis in prostate cancer (PC) patients treated by radical prostatectomy. Patients and Methods: Two hundred and eleven patients treated by radical prostatectomy for localised prostate cancer were clinically followed up for a mean of 7.3 years. The primary histopathological specimens were re-analysed to ensure uniform histopathological grading and pT classification. A tissue microarray construction (TMA) was used in immunohistochemisty to assess the expression of Ki-67, cyclin D1 and the apoptosis marker Tag. The results were analysed with light microscopy and the findings were compared to standard histology, pT and clinical follow-up data. Results: The co-expression of Ki-67 and cyclin D1 ($p=0.05$) was common. High fraction of Ki-67 positive cells and a high fraction of apoptotic cells were often present in same tumours ($p=0.05$). High apoptotic rate was related to positive surgical margin status ($p=0.047$). Low expression of Ki-67 was related to a low Gleason score ($p<0.001$), an absence of either capsule penetration ($p=0.029$) or perineural invasion ($p=0.004$). High expression of cyclin D1 was related to perineural growth ($p=0.039$). Prostate specific antigen (PSA) recurrence-free survival (RFS) was predicted by Gleason grade ($p<0.001$) and capsule invasion ($p=0.006$). High expression of Ki-67 ($p=0.03$), as well as high apoptotic rate ($p=0.04$) were related to a high risk of cancer death. In multivariate analysis the seminal vesicle invasion was the only independent predictor of cancer death ($p=0.01$). Conclusion: The expression of Ki-67, cyclin D1 and a high apoptotic rate are related to a malignant phenotype in prostate cancer, but their prognostic value is inferior to standard histological prognostic factors.*

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Prostate cancer (PC) is the most common malignancy in men in most civilised countries. It is diagnosed more often at its early stages and in younger men (1). However, 30% of the cases are presented with capsule penetration at the time of curative therapy despite good prognostic signs pre-operatively (2). In addition 30% of patients treated by radical prostatectomy develop a biochemical relapse. Pre-operative prostate specific antigen (PSA), Gleason score and pT classification are the basis on which the high-risk patients are identified (2). The clinical significance of rising PSA after radical prostatectomy has not been clearly established, since the PSA rise for some men means a lethal disease with metastases, whilst for others never having any symptoms or clinical evidence of the disease. A high Gleason grade and short time to first detectable PSA level are also used as prognostic factors for a high-risk for metastatic disease (3). The prognosis of patients with clinically local tumours cannot be predicted accurately and more precise methods are needed. Proliferation markers are good prognostic markers in advanced PC, but their role in local PC is not well-established in a clinical context.

The proliferation rate can be measured by quantifying the fraction of Ki-67 antigen-positive cells in immuno-histochemically prepared specimens. Ki-67 is a molecule expressed throughout all the points of the cell cycle except in the G0 resting phase. At early stages of PC development the proliferation rate is usually low (4). Proliferation is related to other prognostic factors and to outcome in PC (4-8), though the number of studies on local PC is limited (6, 7, 9).

Cyclin D1 is an amino acid expressed in the G1 phase of the cell cycle which has an important role in regulating the cell cycle and cancer progression (10, 11). In immunohistochemical studies, the overexpression of Cyclin D1 has been related to cancer progression and outcome in mixed cohorts of PC patients (10, 11), but the role of cyclin D1 expression in local PC is unclear.

Apoptosis, or programmed cell death, is essential in the elimination of damaged cells from the tissues and it prevents them from replicating. Apoptosis increases parallel with cell proliferation. If the cell death or proliferation mechanisms become disturbed for some reason, the tumour growth can increase and invasion to local structures occurs. The apoptotic rate, as measured by various methods, is related to several other prognostic parameters and to prognosis in PC (5, 8), but its role in early PC is unclear.

The prognostic value of Ki-67, cyclin D1 and apoptosis was studied in localised PC cases in a cohort treated by radical prostatectomy.

Patients and Methods

Patients. Two hundred and eleven (211) consecutive PC patients were treated by radical prostatectomy in Kuopio University Hospital, Finland, between 1987 and 1999, and in Päijät-Häme Central Hospital, Finland, between 1993 and 1999. The mean (SD) age of the patients was 64.2 (5.5) years and the mean follow-up was 7.3 (2.4) years. All the patients had a clinically local tumour according to clinical TNM classification (12). The presence of distant metastases was excluded by bone scans and chest X-ray examinations. Adequate histopathological samples for immunohistochemistry were available in 184 cases, of which three patients had lymph node metastases on final histological analysis and were, therefore, excluded. Seven percent of patients had adjuvant hormone or radiation therapy.

The follow-up reviews were done at 3-month intervals during the first year, at 6-month intervals during the next year and annually thereafter. Recurrent cancers were screened using laboratory tests (PSA, alkaline phosphatase), digital rectal examination and using different image analysis methods when required.

Histological methods. Radical prostatectomy specimens were used for histological analyses. The specimens were fixed in buffered formalin (pH 7.0), embedded in paraffin, sectioned at 5 μm and stained with haematoxylin and eosin (HE). The specimens were analysed by two pathologists unaware of the clinical data (VK, J-PK). The pT classification was done according the UICC 1997 guidelines UICC (12). Histological grading was done according to Gleason score (13). Capsule invasion, surgical resection margin status, seminal vesicle invasion and perineural infiltration were recorded as absent (0) or present (1).

Immunohistochemistry

Tissue microarray (TMA) construction. Three representative tumour regions of each case were marked on HE stained sections. From these regions, tissue cylinders with diameter of 0.6 mm were obtained and arrayed into a recipient block using a tissue chip microarrayer (Beecher Instruments, Silver Spring, MD, USA). The recipient block was subsequently cut into 5-μm sections on pretreated slides to support adhesion of the tissue samples.

Ki-67 immunohistochemistry. The tissue section was incubated with monoclonal anti-Ki-67 protein (MIB1, Dianova GmbH, Germany) antibody diluted at 1:100 in phosphate-buffered saline (PBS). Several dilutions of the antibody were tested to avoid background staining and to find optimal staining before the entire series was processed. Sections were washed twice for 5 min with PBS, incubated for 20 min with biotinylated secondary antibody (Vector, CA, USA) diluted in 1:200 in PBS. Slides were washed twice in PBS for 10 min and incubated for 20 min in a preformed avidin-biotinylated peroxidase complex (ABC, Vectastain Elite kit, Vector). Sections were washed twice for 5 min with PBS, developed with diaminobenzidine tetrahydrochloride substrate (Sigma, UK), slightly counterstained with Mayer's hematoxylin, dehydrated, cleared and mounted. Normal human tonsil tissue was used as a positive control. The mean fraction of positive nuclei was estimated, and when at least one positive nuclei was present, it was estimated at 1%. For the analysis, the Ki-67 was grouped into two categories 0-5% and >5%, based on previous studies.

Cyclin D1. Paraffin wax-embedded sections from TMA blocks were washed twice in PBS and heated in a microwave oven at 600W for three cycles of 5 min each in 0.001 M EDTA (pH 8.0) for cyclin D1 (NCL-CYCD1-GM, Novocastra, Newcastle-upon-Tyne, UK). Endogenous peroxidase activity was blocked with 5% H_2O_2. After treatment with 1.5% normal horse serum (Zymed, Histostain-plus bulk kit, Zymed Laboratories Inc., San Francisco, CA, USA) anti-cyclin D1 monoclonal antibody was applied to the sections at a dilution of 1:10 in PBS with 1% bovine serum albumin and incubated for 24 h at 4˚C. Then, the sections were washed and biotinylated secondary antibody and avidin-biotin peroxidase reagent (Zymed) were applied to detect bound primary antibody. Diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, MO, USA) was used to demonstrate peroxidase activity. The slides were counterstained with Mayer's haematoxylin, dehydrated, cleared and mounted with DePex (BDH, Poole, Dorset, UK). Samples from the same series without primary antibody served as negative controls. Cyclin D1 positive nuclei were counted from all three sections of each case. The mean fraction of positive nuclei was estimated and, for statistical analysis, the cyclin D1 was grouped into two categories: 0-70% and >70%. The initial grouping was into tertiles, but the two lowest tertiles did not differ in any respect and, hence, were combined.

Apoptosis detection. The staining procedures were based on the manufacturer's instructions (Apoptag, *In situ* Apoptosis Detection Kit, Intergen, Oxford, UK). Briefly, after deparaffinisation and dewaxing in xylene sections were treated with proteinase K (20 μg/ml) for 5 min at room temperature, washed twice in 3% hydrogen peroxide for a further 5 min, and then washed in PBS. The kit was then applied and the slides immersed in the TdT solution supplied for 1 h at 37˚C. The slides were then developed using DAB (Sigma) and counterstained with Methyl Green, dehydrated, cleared and mounted with DePex (BDH). Each experiment included a positive control for apoptosis (intestine) and a negative control (omission of terminal transferase). The apoptosis index (AI) was calculated as the ratio of positively stained tumour cells and bodies to all tumour cells

Table I. *The clinical charasteristics of the patients.*

Mean age, years (SD)	64.2 (5.5)
Mean follow-up, years (SD)	7.3 (2.4)
Mean PSA at diagnosis, μg/l (SD)	15.7 (14.6)
PSA μg/ml, n (%)	
<10	69 (40)
10-20	67 (39)
>20	37 (21)
pT category, n (%)	
1-2	122 (67)
3	57 (32)
4	2 (1)
Gleason score, n (%)	
2-6	137 (76)
7	34 (19)
8-10	10 (5)
Capsule invasion, n (%)*	
No	116 (64)
Yes	62 (34)
Surgical margin status, n (%) *	
Negative	113 (62)
Positive	67 (37)
Seminal vesicle involvement, n (%) *	
Negative	143 (79)
Positive	35 (19)
Postoperative PSA < 0.1 μg/l	
Yes	140 (75)
No	41 (25)
Survival, n (%)	
Alive	164 (91)
Dead	17 (9)

*data not available in all cases.

Table II. *The results of Ki-67, apoptosis and Cyclin D1 immunohistochemistry*

Variable (% positively stained)	Number (%)
Ki-67, mean 2%	
0-5%	156 (86)
> 5%	25 (14)
Apoptosis, mean 2%	
0-5%	65 (35)
>5%	116 (64)
Cyclin D1, mean 55%	
0-70%	118 (65)
>70 %	63 (35)

from three biopsies of each case. The cells were counted under x400 magnification. The mean fraction of positive nuclei was estimated and when at least one positive nucleus was present, it was estimated at to 1%. Morphological characteristics of apoptosis were chromatin condensation, nuclear disintegration and formation of crescent caps of condensed chromatin at the nuclear periphery. For the statistical analysis, the apoptotic cells were grouped into two categories: 0-5% and >5%. The initial grouping was into tertiles but the two highest tertiles did not differ from each other and, hence, were combined.

Statistical analysis. For statistical analysis the SPSS-X program package was used. The Chi-square-test was used to analyse the relationship between the groups. Univariate PSA recurrence-free survival (RFS) analysis (log-rank analysis) was based on the Kaplan-Meier method. Multi-variate survival analyses were carried out according to Cox's methods. In RFS analysis, only patients with PSA of zero at the first post-operative analysis were included. A PSA elevation of 0.2 μg/ml or more during the follow-up was considered as a PSA failure event.

Results

The clinical data of the patients are presented in Table I. The results of immunohistochemistry are shown in Table II.

The mean (median; range) fraction of cells positively-stained for Ki-67, cyclin D1 and apoptosis were 4.5% (2.0%; 0-40%), 54.8% (60.0%; 0-95%) and 2.1% (2.0%; 0-10%), respectively. The expression of Ki-67 and cyclin D1 ($p=0.05$), as well as Ki-67 and apoptosis ($p=0.05$) were correlated to each other, but cyclin D1 and apoptosis were not interrelated.

A high fraction of apoptotic cells in tumours was related to a positive surgical margin status ($p=0.047$). A low expression of Ki-67 was related to a low Gleason score ($p<0.0001$), absence of either capsule invasion ($p=0.029$) or perineural invasion (PNI) ($p=0.004$). On the other hand, high expression of cyclin D1 was related to PNI ($p=0.039$).

PSA recurrence was not predicted by cyclin D1, Ki-67 expression or the fraction of apoptotic cells, whereas the Gleason score ($p=0.03$) and capsule invasion ($p=0.01$) were significant predictors of PSA recurrence.

Short PSA recurrence free survival was predicted by a high Gleason grade $p<0.0001$ and capsule invasion ($p=0.01$). The Gleason score ($p=0.05$), perineural invasion ($p=0.03$), capsule invasion ($p=0.006$), positive surgical margin status ($p=0.01$) and seminal vesicle invasion ($p<0.001$) were all significant predictors of cancer specific survival (Table III).

PC related survival time was predicted by Ki-67 expression. The mean PC survival time was 15.6 years in patients with a low Ki-67 expression as compared to 10.7 years among patients with a high Ki-67 expression ($p=0.03$, Figure 1). No PC deaths occurred in patients with low apoptosis rates as compared to a PC survival time of 14.1 years among men whose tumours showed a high apoptosis rate ($p=0.04$, Figure 2).

Cox's multivariate survival analysis was carried out including the following prognostic parameters: pT-category, pre-operative PSA value, Gleason score, capsule invasion, surgical margin status, seminal vesicle invasion and PNI,

Table III. *The significant parameters related to prostate cancer specific survival (Kaplan-Meier).*

	Mean time (SE)	95% CI	*p*-value
Gleason grade			0.05
2-6	15.6 (0.45)	14.7-16.2	
7	9.1 (0.28)	8.6-9.7	
8-10	9.1 (0.54)	8.0-10.1	
pT			0.001
<2	no PC deaths		
3-4	11.9 (0.60)	10.7-13.1	
Ki-67			0.003
0-5%	15.6 (0.40)	14.8-16.4	
>5%	10.7 (0.50)	9.7-11.7	
Apoptosis			0.04
0-5%	no PC deaths		
>5%	14.1 (0.54)	13.1-15.2	
PNI			0.03
No	no PC deaths		
Yes	14.9 (0.56)	13.8-16.1	
Capsule invasion			0.006
No	16.1 (0.17)	15.8-16.5	
Yes	12.3 (0.51)	11.3-13.3	
Margin status			0.01
No	16.1 (0.17)	15.8-16.5	
Yes	13.5 (0.69)	12.4-14.8	
Seminal vesicle invasion			<0.001
Yes	16.2 (0.13)	16.0-16.5	
No	9.1 (0.34)	8.5-9.8	

PC: prostate cancer.

Cyclin D1, Ki-67 and apoptosis rate. The only independent predictor of PC survival was seminal vesicle invasion (β=–2.830, SE 1.096, p=0.010, exp (β) =0.059; 95% confidence interval for Exp (β) =0.007-0.506).

Discussion

About 30% of radically treated patients will have a biochemical relapse, which is the first sign of threatening local or systemic relapse (2). Due to the earlier diagnosis and younger age of PC patients, the criteria of adjuvant therapy are under debate. The most important pre-operatively available prognostic parameter is the Gleason score (13), but the Gleason score in biopsy specimens is under- or over-graded in about 30% of cases as compared to radical prostatectomy specimen analysis. Therefore, new accurate prognostic markers are urgently needed.

The proliferative activity of PC is on low average compared to some other tumours and local PCs are particularly slow proliferators (4, 8, 14, 15). Although this series included only 40% of patients with a pre-operative PSA value under 10, the mean fraction of proliferating cells was only 2%, which is in line with the previous reports (14). The expression of cyclin D1 and Ki-67 were

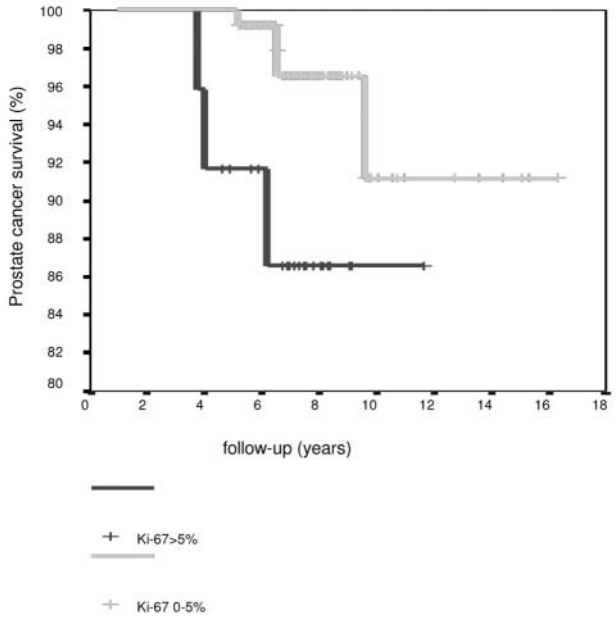

Figure 1. *The cancer related survival categorised according to the fraction of Ki-67 positive cells (p=0.03). Ki-67 0-5%, n=156; Ki-67> 5%, n=25.*

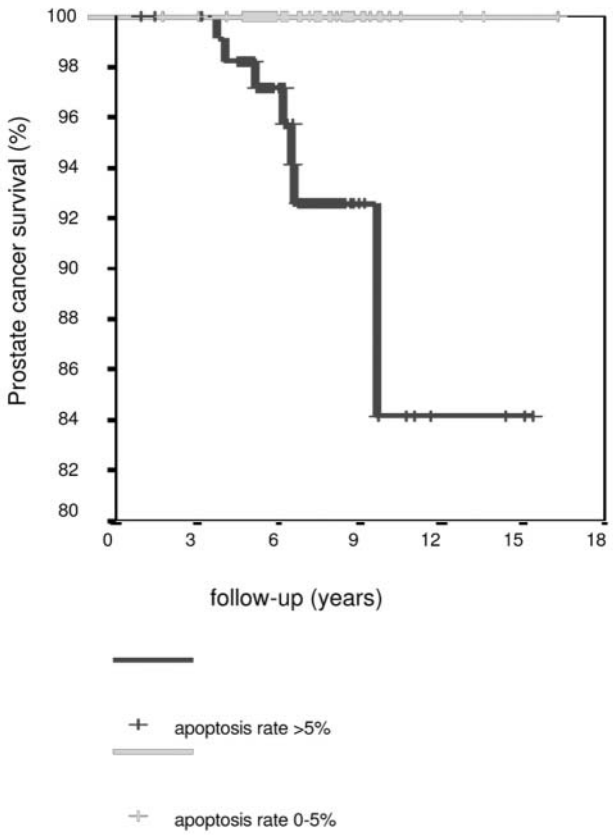

Figure 2. *The cancer-related survival categorised according to the fraction of apoptotic cells (p=0.04). Apoptosis rate 0-5%, n=65; apoptosis rate > 5%, n=116.*

significantly interrelated, which confirms the findings by Drobnjak *et al.* (10). There was a significant interrelationship between high Ki-67 and strong apoptotic activity, which is also in line with previous reports (5). Vesalainen *et al.* found a correlation between a high apoptosis rate and a high Gleason score in a series including all stages of PC (16), whereas in our series including mainly local PCs, Gleason score and apoptosis were not significantly interrelated. Apoptotic index seems to be different (17) in local and disseminated cancers, which most probably explains the different results. Considerable variation also exists, depending on the method of apoptosis detection used.

The higher the proliferation rate, the higher the proportion of cells referring to apoptotic cell death (5). Both Ki-67 and the proportion of apoptotic cells predicted PC specific survival. No deaths from PC occurred in the group of patients with low apoptotic activity and deaths were also rare in the group patients of tumours with a low proliferation rate. Similar survival data in mixed cohorts and even independent prognostic value for Ki-67 expression (4, 7, 9, 14) and apoptosis (18) have been reported in literature previously.

In a mixed cohort, including a large number of PC patients, the AI based on morphological criteria alone had prognostic value (16). The higher the AI, the higher was the probability of cancer death. Pollack *et al.* found prognostic value for Ki-67 using the cut-off value of 7.1% of proliferating cells. Ki-67 expression predicted biochemical failure, distant metastasis, and cancer-specific death in patients treated by radiotherapy (14). All these results are in line with the current survival data.

The Gleason grade is a well-established prognostic factor in PC, whereas the role of PNI is questioned in the literature (16). We found a positive correlation between Ki-67, pT and differentiation of tumours, which has been established previously (4). A positive correlation with PNI and cyclin D1 was also found. So it would seem that a high expression of Ki-67 and cyclin D1 are related to other malignant histological features. The co-expression of cyclin D1 and Ki-67 has been reported previously (10) and confirms our current results. In contrast to disseminated cancers (11), in local tumours cyclin D1 expression seems to have no prognostic value.

This study is based on TMA construction. The tissue block in final analysis is small compared to that used in standard immunohistochemistry, where the entire tissue section is available for analysis. However, in TMA the staining results are more standardised, which is an advantage although information may be missed due to small amounts of tissue. These methodological facts must be taken into account when comparing current results to previous data.

Conclusion

Ki-67, cyclin D and apoptotic markers are statistically significant prognostic factors in an univariate analysis, but in multivariate analysis only seminal vesicle invasion remains statistically significant.

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