ORIGINAL ARTICLE

Expression of p120, Ki-67 and PCNA as proliferation biomarkers in imprint smears of prostate carcinoma and their prognostic value

A. Bantis, A. Giannopoulos, M. Gonidi, A. Liossi, E. Aggelonidou, E. Petrakakou, P. Athanassiades and P. Athanassiadou

Cytology Department and Urology Clinic, Pathology Laboratory, Laiko Hospital Medical School, Athens University, and Urology Clinic, Hippocration Hospital, Athens, Greece

Accepted for publication 17 July 2003

A. Bantis, A. Giannopoulos, M. Gonidi, A. Liossi, E. Aggelonidou, E. Petrakakou, P. Athanassiades and P. Athanassiadou

Expression of p120, Ki-67 and PCNA as proliferation biomarkers in imprint smears of prostate carcinoma and their prognostic value

The cell proliferation markers p120, Ki-67 and proliferating cell nuclear antigen (PCNA) recognize nuclear antigens. The expression of these proteins by immunostaining methods was reported to be of value in determining the prognosis of patients with malignant diseases.

In this study, we evaluated the prognostic significance of the expression of nuclear antigens p120, PCNA and Ki-67 in prostate cancer and compared the results with other prognostic factors.

Imprint smear samples obtained from 70 patients immediately after radical prostatectomy for prostatic carcinoma were immunostained with monoclonal antibodies against p120, Ki-67 and PCNA. The immunostaining results were correlated with Gleason score, tumour differentiation, stage and prostatic specific antigen (PSA) levels.

Our findings demonstrate that p120, Ki-67 and PCNA expression in prostatic carcinoma smears, correlated significantly with the degree of Gleason score ($P < 0.001$). When combining p120, Ki-67 and PCNA positivity with tumour differentiation there was a significant association among these parameters ($P < 0.001$). Overexpression of p120, Ki-67 and PCNA, was also associated with increased PSA serum levels (>4 ng/ml) ($P < 0.001$). The distribution of p120, Ki-67 and PCNA expression in prostate carcinomas was not statistically significant for Ki-67 ($P = 0.69$) and p120 ($P = 0.22$) but was significant for PCNA ($P < 0.001$) as far as the histological stage (T2a, T2b, T2c, T3a). P120, Ki-67 and PCNA expression had significant prognostic value for disease-free survival.

Our results conclude that nuclear antigens p120, Ki-67 and PCNA appear to be additional markers in the field of prognosis of prostatic carcinoma.

Keywords: p120, Ki-67, proliferating cell nuclear antigen, immunocytochemistry, prostate carcinoma, prognostic factors

Introduction

Many investigators have been focusing on identifying prognostic parameters that can separate the aggressive tumours from relatively indolent ones. Cell proliferation has been shown to be a significant prognostic factor in several human malignancies.[1–3]

A proliferation-associated nuclear protein p120, which is expressed during late G_1 to S phase of the cell cycle, has been correlated well with nuclear activation and cell proliferation.[4] Moreover, it has been demonstrated in cells of most malignant tumours, with suggested possible prognostic value.[1,3–5] Ki-67 and proliferating cell nuclear antigen (PCNA), the most frequently used cell proliferation markers, recognize

Correspondence:
Pauline Athanassiadou, Assistant Professor, Pathology Department, Medical School, University of Athens, 75 Mikras Asias St. GR-115 27 Athens, Greece.
Tel.: +003210-7462171; Fax: 003210-7462157

nuclear antigens presented throughout all phases of the cell cycle but not at rest (G_0 phase) or in the early G_1 phase.[3,6] Ki-67 nuclear staining has been related to biological aggressiveness and prognosis in several cancers including prostate cancer.[7–10] PCNA, also known as cyclin, is a non-histone nuclear protein, the level of synthesis of which correlates directly with rates of cellular proliferation and DNA synthesis. It has been demonstrated recently, that PCNA is an auxiliary protein of DNA polymerase-delta and plays a critical role in the initiation of cell proliferation.[11,12] Correlation of high levels of PCNA staining with poor prognosis for patients with prostate carcinomas has been reported.[3,13]

In this study, we evaluated the immunocytochemical profile of the above-mentioned proliferation-associated proteins and correlated the results with prognostic parameters.

Materials and methods

Samples were obtained from 70 patients who underwent radical prostatectomy for prostatic adenocarcinoma, immediately after prostate removal at the operation theatre. Patients' ages ranged from 59 to 75 years (mean 67.11 years). Imprint smears were taken from different areas of macroscopically estimated prostatic carcinoma. After air-drying, smears were fixed in ethanol/acetone 1 : 1 for 10 minutes and stored at −70 °C until used for an immunocytochemical procedure.

The histopathological diagnoses were made on sections from the same samples that were used for the imprints. The TNM system (based on the staging system of the American Joint Committee on Cancer)[14] was used for pathological staging, and grading of the primary carcinoma was evaluated according to the Gleason score system. None of the patients had been treated with radiation or androgen depletion prior to prostatectomy.

Follow-up after surgery included serum prostatic specific antigen (PSA) at 6 months postoperatively and monthly (mean 33.2 months) thereafter. The data of studied cases of prostate carcinoma are shown in Table 1.

Immunocytochemical staining was performed by the avidin–biotin complex (ABC) immunoperoxidase method.[15] Smears were incubated for 45 minutes with normal rabbit serum (Dakopatts, Glostrup, Denmark) diluted 1 : 50 in phosphate-buffered saline (PBS) and then rinsed in three changes of PBS for 5 minutes. Primary antibodies, PCNA, Ki-67 (Dakopatts) and p120 (BioGenex; San Ramon, USA) were applied in dilution 1 : 200, 1 : 40, 1 : 40, respectively and the smears incubated overnight.

Table 1. Clinical characteristics of 70 patients with prostate adenocarcinoma treated with radical prostatectomy

	n	Percentage
Age (years)		
<65	14	20.0
65–69	35	50.0
>70	21	30.0
Stage		
T2a	41	58.6
T2b	20	28.6
T2c	6	8.6
T3a	3	4.2
Gleason score (grade)		
2–4	19	27.1
5–6	30	42.9
≥7	21	30.0
Differentiation		
Well	11	15.7
Moderate	49	70.0
Poor	10	14.3
Pre-treatment prostatic specific antigen (ng/ml)		
0–4	13	18.6
5–9	36	51.4
≥10	21	30.0

T2a, tumour involves 50% of a lobe or less; T2b, tumour involves more than 50% of a lobe; T2c, tumour involves both lobes; T3a, unilateral extracapsular extension of the tumour.

After washing in PBS, smears were incubated in biotinylated rabbit antimouse antibodies (Dakopatts) at 1 : 300 dilution for 30 minutes at room temperature. They were again washed and incubated in streptavidin–biotin complex (Dakopatts) followed by the ABC complex horseradish peroxidase (HRP; Dakopatts).

Visualization was achieved by a final incubation in diaminobenzidine tetrahydrocloride 0.06% in PBS containing 0.03% hydrogen peroxide (DAB; Sigma, Poole, UK). Smears were counterstained with Mayer's haematoxylin.

Normal human tonsil smear was used as a positive control. For each antibody-negative control, studies were performed in which normal rabbit serum was used instead of the primary antibody.

A total of 500–1000 cells were counted by surveying five to 10 microscopic fields with two observers in every case, to determine the average labelling index.

The field to be counted was chosen under ×400 magnification from the well-labelled area. The labelling index was expressed as the percentage ratio of total labelled cells to the total number of cells counted. To compare the measurements of two observers, a Kendall correlation test was used.

All staining was confined to the nucleus of tumour cells and was shown as partial, diffuse and granular brown pattern (Figures 1–3).

PCNA, Ki-67 and p120 positivities were scored as follows: staining of <20% of cells(−), 21–40%(+), 41–60%(++), 61–100%(+++). Nuclear PCNA, Ki-67 and p120 staining was positive if >21% and negative if <20% of cells stained.

Statistical analysis

The relationship of p120, Ki-67 and PCNA expression with all prognostic factors (PSA, Gleason score, tumour differentiation and stage) was assessed by one-way analysis of variance (ANOVA) followed by tests of multiple comparisons, as p120, Ki-67 and PCNA did not deviate from normality (Tamhane test). The simultaneous effect of all variables to the expression of p120, Ki-67 and PCNA was investigated by multiple linear regression.

Figure 1. Prostate carcinoma cells with positive nuclear immunoreactivity for proliferating cell nuclear antigen (×500).

Figure 2. Positive nuclear immunostaining for Ki-67 in imprint smear of prostate carcinoma (×500).

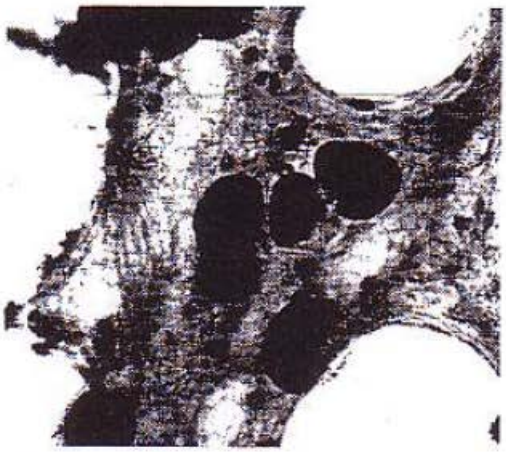

Figure 3. Poorly differentiated prostate carcinoma cells with positive nuclear immunoreaction for p120 protein (×500).

Results

The immunocytochemical expression of the cell proliferation-associated study proteins p120, Ki-67 and PCNA is summarized in Figure 4. Positive expression for p120, Ki-67 and PCNA was detected in 64 (91.4%), 63 (90%) and 60 (85.7%) prostate carcinoma smears, respectively.

The significant correlations ($P < 0.001$) between the degree of Gleason score and p120, Ki-67 and PCNA expression in prostate carcinoma smears are shown in Figure 5. We observed that an increasing Gleason tumour grade was associated with an increase in the percentage of nuclei stained positively for the three biomarkers.

When combining p120, Ki-67 and PCNA positivity with tumour differentiation, there was a significant association between these parameters ($P < 0.001$; Figure 6). It can be deduced that the percentage of positive nuclei increase with the decreasing degree of differentiation.

Overexpression of p120, Ki-67 and PCNA in prostate carcinoma cell smears was associated with an increase in pre-treatment PSA serum levels (>4 ng/ml) ($P < 0.001$) (Figure 7).

Table 2. Distribution of p120, Ki-67 and PCNA expression in prostate carcinomas according to histopathological staging

			No. of p120 (%)		No. of Ki-67 (%)		No. of PCNA (%)		
Stage	*n*	Percentage	(+)	(−)	(+)	(−)	(+)	(−)	Total
T2a	41	59	26	74	38	62	30	70	100.0
T2b	20	29	26	74	33	67	28	72	100.0
T2c	6	8	48	52	54	46	67	33	100.0
T3a	3	4	66	34	74	26	85	15	100.0
Total	70	100							
P-value			0.022		0.069		0.000		

Table 3. Prognostic value for the disease-free survival assessed by multiple linear regression model

			P-values		
	Score	d.f.	p120	Ki-57	PCNA
Stage	0.057	1	0.201	0.314	0.005
Gleason score	0.902	1	0.005	0.000	0.005
Differentiation	0.000	1	0.000	0.002	0.010
Prostatic specific antigen	0.258	1	0.366	0.047	0.542

Residual $\chi^2 = 13.833$ with 4 d.f. significance = 0.054.
PCNA, proliferating cell nuclear antigen.
Dependent variable: p120, Ki-67 and PCNA long rank test.

patients who had low expression (+) compared with those who had high expression (++ or +++).

To date, four patients have died from prostatic adenocarcinoma (three in 1.5 years and one in 13 months). The remaining patients were alive and well at the last follow-up.

Discussion

The proliferative activity of malignant tumours has been implicated as a prognostic parameter for the aggressiveness of the tumours and correlated with outcome for several human carcinomas.[2,16,17]

Ki-67 and PCNA have been used extensively as indicators of the proliferative state in cells and p120 protein has been considered to be associated with cell proliferation and has been demonstrated in most tumour cells.[3,5,8,18,19]

PCNA is a nuclear protein expressed by non-neoplastic and neoplastic cells undergoing division.[12,20] The commercially available monoclonal antibody to PCNA may recognize a different epitope on the PCNA molecule.[21] The immunocytochemical evaluation of PCNA labelling index is easy to perform on routinely processed material. Independent of the method used for PCNA scoring, it has been shown that the increasing expression of PCNA labelling index may have a prognostic value in prostatic adenocarcinoma (PAC)[12,22] and in other neoplasms.[2,13]

Several studies have shown that cell proliferative activity, as defined by the Ki-67 index, correlates with the cell growth fraction.[7,8,17] The monoclonal antibody to Ki-67 antigen has been a reliable marker to estimate cellular proliferation. Previous studies of Ki-67 immunostaining in prostate carcinoma have shown a relationship with tumour grade and stage.[17,23] Furthermore, significant association has been found between Ki-67 expression and disease progression.[23,24]

Several investigators have reported that cell proliferation is associated with histological grade but does not correlate with the pathological stage of prostate carcinomas.[25,26]

In the present study, the Ki-67, PCNA and p120 expression values were also increased significantly with increasing Gleason score. In contrast, there was no difference in Ki-67 and p120 expression with the stage of the disease. One of the possible explanations is that these proteins are expressed non-preferentially throughout the cell cycle except in the G_0 phase, so may not be effective in identifying individual tumours with rapid cell turnover but may only be reflective of the overall number of dividing malignant nuclei.[3]

This observation was in agreement with another study which concluded that, with an increasing Gleason tumour score, there is a corresponding increase in the percentage of nuclei stained positively for Ki-67.[26]

However, in prostate carcinoma, the published data are unclear, with two studies reporting an association between the increased Ki-67 level and high-grade carcinoma[27,28] and a separate study reporting no association.[7] Increased Ki-67 levels were also correlated

with advanced clinical stage of prostate carcinoma and a shorter survival time.[22]

Moul *et al.*[10] found that patients with a low Gleason score (2–4) and those with a pre-treatment PSA level (4–10 ng/ml) had a significantly worse 5-year disease-free survival if they exhibited high Ki-67 expression. Overall, however, the Ki-67 expression did not maintain independent significance as a prognostic marker. Perhaps with a larger number of patients, or with longer follow-up, Ki-67 may have clinical use.

Leite *et al.* found that there was no statistical difference in PCNA values when biological parameters such as Gleason score were considered and concluded that proliferative activity is usually low in prostate carcinoma.[19] However, another study has shown that the percentage of PCNA-positive nuclei increases with decreasing degree of differentiation.[25]

P120 protein has been demonstrated in most tumour cells but is absent in normal tissues and benign tumours. The role of p120 in the proliferation of cells was suggested as the reduction in the rate of tumour cell proliferation using monoclonal p120 antibody.[3,4]

Studies of the significance of p120 expression in human carcinoma samples are limited. Ueki *et al.*[29] studied immunohistochemical expression of p120 protein in colorectal tumours and reported a graded increase in p120 overexpression in severe dysplasia and carcinoma. Uchiyama *et al.*[30] reported differences in p120 expression between different types of lung carcinomas. However, neither of these studies reported a correlation between p120 expression and clinicopathological prognostic parameters. To our knowledge, there have been few studies of p120 and prostate carcinomas.[1,3,4] In our study, immunoreactivity for p120 was found to be 91.4% in prostate carcinoma smears. This finding is high compared with other studies which found a p120 positivity of 36–76%.[3,4] Furthermore, we found p120 expression to be correlated with increased tumour aggressiveness and poor prognosis. This is in agreement with other studies.[4,31]

With regard to the disease-free survival after radical prostatectomy, all studied markers of proliferation correlated with recurrence in univariate analysis. The significant co-expression of the proliferation-associated proteins in our study supports their collective role in tumour proliferation and they are independent predictors of disease recurrence after radical prostatectomy. This is not surprising, given that all these markers in the four patients who died of prostate carcinoma correlated with high tumour grade and poor differentiation, possibly reflecting a population of actively dividing cells.

In conclusion, nuclear proliferation-associated antigens p120, Ki-67 and PCNA appear to be additional markers pointing to aggressive disease in prostate carcinoma.

References

1 Ruschoff J, Bocker T, Buettner R *et al.* In vivo and ex vivo expression of nucleolar proliferation associated antigens (p120, B23) in the prostate. *Verh Dtsch Ges Pathol* 1993;**73**: 103–6.

2 Ioakim-Liossi A, Karakitsos P, Pantazopoulos D, Aroni K, Athanassiadou P. Image cytometric DNA analysis and proliferating cell nuclear antigen (PCNA) expression in transitional cell carcinoma of the bladder. *Cancer Det Prev* 1999;**23**:401–7.

3 Kallakury B, Sheehan C, Rhee S *et al.* The prognostic significance of proliferation-associated nuclear protein p120 expression in prostate adenocarcinoma: a comparison with cyclins A and B1, Ki-67, proliferating cell nuclear antigen and p34cdc2. *Cancer* 1999;**85**: 1569–76.

4 Kallakury B, Sheehan C, Ross J. Co-downregulation of cell adhesion proteins alpha- and beta-catenins, p120 CTN, E-cadherin and CD44 in prostatic adenocarcinomas. *Hum Pathol* 2001;**32**:849–55.

5 Ventura L, Migaldi M, Criscuolo M *et al.* Nucleolar protein p120 expression in oral carcinoma. *Anticancer Res* 1999;**19**: 1423–6.

6 Gerdes J, Schwab V, Lemke H, Stein H. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. *Int J Cancer* 1983;**31**:13–20.

7 Gallee M, Visser-de-John E, TenKate F, Schroeder F, Vander-Kwast T. Monoclonal antibody Ki-67 defined growth fraction in benign prostatic hyperplasia and prostatic cancer. *J Urol* 1989;**142**:1342–6.

8 Battencourt MC, Bauer J, Sesterhenn I *et al.* Ki-67 expression is a prognostic marker of prostate cancer recurrence after radical prostatectomy. *J Urol* 1996;**156**: 1064–8.

9 Keshgegian A, Johnston E, Cnaan A. Bcl-2 oncoprotein positivity and high MIB-1 (Ki-67) proliferate rate are independent predictive markers for recurrence in prostate carcinoma. *Am J Clin Pathol* 1998;**110**:443–9.

10 Moul J, Bettencourt MC, Sesterhenn I *et al.* Protein expression of p53, bcl-2 and Ki-67 (Mib-1) as prognostic biomarkers in patients with surgically treated, clinically localized prostate cancer. *Surgery* 1996;**120**:159–67.

11 Mathews M, Bernstein R, Franza B, Garrels J. Identity of the proliferating cell nuclear antigen and cyclin. *Nature* 1984;**303**:374–8.

12 Nemoto R, Kawamura H, Miyakawa I *et al.* Immunohistochemical detection of proliferating cell nuclear antigen (PCNA) cyclin in human prostate adenocarcinoma. *J Urol* 1993;**149**:165–9.
13 Visakorpi T. Proliferative activity determined by DNA flow cytometry and proliferating cell nuclear antigen (PCNA) immunohistochemistry as a prognostic factor in prognostic carcinoma. *J Pathol* 1992;**168**:7–13.
14 Fleming I, Cooper J, Henson D *et al.* (eds). *AJCC Cancer Staging Handbook*, 5th edn. Philadelphia: Lippincott, Williams & Wilkins; 1998:203 pp.
15 Hsu S, Raine L, Fanger H. Use of avidin–biotin peroxidase complex (ABC) in immunoperoxidase techniques. *J Histochem Cytochem* 1981;**29**:577–80.
16 Freeman J, McGrath P, Bondata V *et al.* Prognostic significance of proliferation associated nucleolar antigen p120 in human breast carcinoma. *Cancer Res* 1999;**51**:1973–8.
17 McLoughlin J, Foster CS, Price P, Williams G, Abel PD. Evaluation of Ki-67 monoclonal antibody as prognostic indicator for prostatic carcinoma. *Br J Urol* 1993;**72**:92–7.
18 Gerdes J. Kj-67 and other proliferation markers useful for immunohistological diagnostic and prognostic evaluations in human malignancies. *Sem Cancer Biol* 1990;1: 199–205.
19 Leite K, Srougi M, Nesralhah L, Camara-Lopes L. Analysis of p53 expression and proliferative assessment using PCNA in localized prostate carcinoma. *Braz J Med Biol Res* 1999;**32**:283–8.
20 Celis JE, Bravo R, Larsen PM, Fey SJ. Cyclins: a nuclear protein whose levels correlates directly with the proliferative state of normal as well as transformed cells. *Leuk Res* 1984;**8**:143–57.
21 Ogata K, Kurki P, Celis JE *et al.* Monoclonal antibody to a nuclear protein (PCNA) associated with DNA replication. *Exp Cell Res* 1987;**168**:475–86.
22 Cher ML, Chew K, Rosenou W, Carroll PR. Cellular proliferation in prostatic adenocarcinoma as assessed by bromodeoxyuridine uptake and Ki-67 and PCNA. *Prostate* 1995;**26**:87–93.
23 Stapleton A, Zbell P, Kattan M *et al.* Assessment of the biologic markers p53, Ki-67 and apoptotic index as predictive indicators of prostate carcinoma recurrence after surgery. *Cancer* 1998;**82**:168–75.
24 Harper M, Goddard L, Wilson D *et al.* Pathological and clinical associations of Ki-67 defined growth fractions in human prostatic carcinoma. *Prostate* 1992;**21**:75–84.
25 Montironi R, Galluzzi C, Diamanti L, Giannulis I, Scarpelli M, Nictolis M. Proliferating cell nuclear antigen (PCNA) in prostatic invasive adenocarcinoma. Is the proliferation state in the marginal zone of the tumour higher than in the central part? *Anticancer Res* 1993;**13**:129–32.
26 Raymond WE, Leong ASY, Bolt JW, Milios Y, Jose JS. Growth fractions in human prostatic carcinoma determined by Ki-67 immunostaining. *J Pathol* 1988;**156**: 161–7.
27 Brown C, Sauvageot J, Kahane H. Cell proliferation and apoptosis in prostate cancer-correlation with pathologic stage. *Mod Pathol* 1996;**9**:205–9.
28 Tsuji M, Murakami Y, Kanayama H, Sano T, Kagawa S. Immunohistochemical analysis of Ki-67 antigen and bcl-2 protein expression in prostate cancer: effect of neoadjuvant hormonal therapy. *Br J Urol* 1998;**81**:116–21.
29 Ueki T, Nakayama Y, Sugao Y *et al.* Significance of the expression of proliferation associated nuclear antigen p120 in human colorectal tumors. *Hum Pathol* 1997;**27**: 74–79.
30 Uchiyama B, Saijo Y, Kumano N *et al.* Expression of nucleolar protein p120 in human lung cancer: difference in histological type as a marker for proliferation. *Clin Cancer Res* 1997;**3**:1873–7.
31 Botticelli AR, Casali AM, Botticelli L, Zaffe D. Immunohiostochemical detection of cell-cycle associated markers on paraffin embedded and formalin fixed needle biopsies of prostate cancer: correlation of p120 protein expression with AgNOR, PCNA/cyclin, Ki-67/MIB1 proliferation-scores and Gleason gradings. *Eur J Histochem* 1998;**42**: 41–48.