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# Proliferating Cell Nuclear Antigen (PCNA) in Prostatic Carcinoma

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**Proliferating cell nuclear antigen (PCNA) is an auxiliary protein of DNA polymerase-∂ that is synthesized during the late G1 phase through the S phase of cell proliferation. The expression of PCNA correlates strongly with cell proliferation. In the present study, employing NC-012, which is an anti-human PCNA monoclonal antibody, we performed immunohistochemical staining on specimens obtained from 71 new prostatic carcinoma patients by needle biopsy. We analyzed the results of PCNA immunoreactivity for correlation with the prognosis of the patients. The positive staining rate was 0~11.4% for prostatic carcinoma, while it was 0~2.6% in 10 cases of benign prostatic hypertrophy. The PCNA-positive rate tended to be high in cases of moderately to poorly differentiated prostatic carcinoma, but it was not significant. With regard to the prognosis, it was found that the prognosis tended to be worse in the PCNA- positive cases compared with the PCNA-negative cases. Further, multivariate survival analysis of six parameters (i. e., the patient's age, histological grade, clinical stage, serum prostatic acid phosphatase, initial treatment and the PCNA-positive rate) revealed that the clinical stage, histological grade and the PCNA-positive rate were the first, second and third most significant prognostic parameters, respectively.**

## Introduction

Not all prostatic carcinomas are clinically apparent, and even when recognized they do not all express the same biologic or malignant potential. This heterogeneity in the expression of prostatic carcinoma has been the source of much confusion and controversy, affecting the selection of appropriate therapy and the evaluation of results. There is a need to establish prognostic parameters which would identify those individuals who are unlikely to respond to conventional treatment and would benefit from alternative therapies from the onset of clinical examination. Various histological grading and clinical staging systems for prostatic carcinoma have been shown to have prognostic value.[1-3]

PCNA is a protein that is localized in the nucleus of proliferating cells. As an auxiliary protein of DNA polymerase-∂, PCNA has a very close relationship with DNA synthesis. PCNA appears in the nucleus in the late G1 phase immediately prior to the start of DNA synthesis, and its amount reaches a maximum in the S phase, when DNA synthesis begins.[4-8] On the other hand, recently, with the objective of judging the malignancy and prognosis of tumors, various studies have been carried out in the field of urology aimed at achieving determination of the presence of proliferating cells and their correlation with malignancy and the prognosis. Representative examples of the techniques that have been applied are $H^3$-thymidine, DNA-flow cytometry, bromodeoxyuridine, Ki-67 and argyrophilic nucleolar organizer regions.[9-12] However, these techniqies require the use of frozen sections of live tissues or fresh specimens, and they are thus especially unsuited for retrospective studies. In contrast, PCNA is an endogenous substance, and it is retained even after formalin fixation and paraffin embedding. Accordingly, the detection of PCNA is suited even for retrospective studies.

## Materials and Methods

### *Patients and Tissues*

We studied 71 new prostatic carcinoma patients, who had been so diagnosed between March 1980 and July 1990 at the Department of Urology of Nagasaki University Hospital. Their average age at the time of diagnosis was 72.4 years (range:50-90 years). Fifteen patients were in stage B ($T_2N_0M_0$;TNM 1987[13]), 19 in stage C ($T_{3\text{-}4}N_0M_0$), and 37 in stage D ($N_{1\text{-}3}$ or $M_1$). As the initial treatment, all had received endocrine therapy; the majority were given estrogen or chlormadinone acetate, with or without bilateral orchiectomy, and the rest received a luteinizing hormone-releasing hormone agonist. In addition, 54 received cytotoxic chemotherapy, the majority having been given cisplatin and adriamycin and/or 5-fluorouracil, while the balance was given other agents.

For our immunohistochemical study, formalin-fixed, paraffin-embedded tissue specimens of each patient's neoplastic prostatic glands were retrieved from the files of the Pathology Division of the Central Diagnostic Laboratory. All specimens were prepared from transperineal or transrectal needle biopsies that had been conducted prior to treatment. All hematoxylin and eosin-stained sections of these cases were reviewed to assess the histological grade

according to Gleason's grading system For our controls, similarly fixed, paraffin-embedded tissue specimens of benign prostatic hypertrophy (BPH) were stained immunohistochemically, these latter tissue specimens have been obtained from patients with bladder cancer when they were undergoing total cystectomy

*Immunohistochemical Procedure*

Paraffin sections were stained immunohistochemically by a biotin-streptavidin method with horseradish peroxidase according to the following step-by-step procedure

1) deparaffinization in xylene-alcohol,
2) incubation in methanol containing 0 3% hydrogen peroxide for 20min to block endogenous peroxidase,
3) incubation in phosphate-buffered saline (PBS) (pH 7 2) containing 2% bovine serum albumin and 10% goat serum for 60min to reduce nonspecific background staining,
4) incubation with a monoclonal PCNA antibody (NC-012, Novocastra Laboratories, Ltd , Newcastle upon Tyne, UK), at a dilution of 1 100 in PBS (pH 7 2) containing 2% bovine serum albumin, overnight at 4 ℃ ,
5) incubation with biotinylated goat anti-mouse IgG (Zymed Laboratories, Inc , San Francisco), 1 100 in PBS (pH 7 2) containing 2% bovine serum albumin for 60min,
6) incubation with peroxidase-conjugated streptavidin (Zymed Laboratories, Inc , San Francisco), 1 500 in PBS (pH 7 2) containing 2% bovine serum albumin for 30min,
7) subjection to a diaminobenzidine reaction with 0 05% hydrogen peroxide for 5min,
8) counterstaining with hematoxylin, and,
9) mounting in glycerol

The slides were rinsed with PBS after step 2, and again rinsed with PBS after steps 4 through 6, and then rinsed with distilled water after steps 7 and 8

*Assessment of PCNA Staining*

Sections were counted at high power (x 200), and the nuclei of 1000 tumor cells were counted in each cases using an eyepiece graticule The PCNA index was calculated as the percentage of positive tumor cell nuclei The PCNA index of 0% was considered as negative staining and the index of more than 0 1% as positive staining

*Statistical Analysis*

Cancer-specific survival curves were calculated according to the Kaplan-Meier method and statistical significance was determined by the generalized Wilcoxon method In the cancer-specific survival analysis, deaths from causes unrelated to carcinoma of the prostate were treated as withdrawals in the same manner as those lost to follow-up To establish which parameters might influence survival and to estimate the extent of their impact, Cox's proportional hazards regression model was used

The possible prognostic parameters examined were the age, clinical stage, histological grade, serum prostatic acid phosphatase (PAP), PCNA positivity and the initial treatment The correlations between the PCNA positivity and the other parameters were evaluated by the chi-square test

## Results

PCNA-immunolabeled nuclei were clearly and easily identified In both prostatic carcinoma and BPH specimens, immunostaining of PCNA was confined to the nuclei in the tissues, but it showed variable patterns Cribriform tumors often displayed more positivity than did other growth types of prostatic carcinoma (Fig 1) Of the 71 patients, 28 had a PCNA-negative tumor (39 4%) and 43 had a PCNA-positive tumor (60 6%) The parameters and their categories studied by multivariate analysis are shown in Table 1 The correlation between the PCNA immunoreactivity

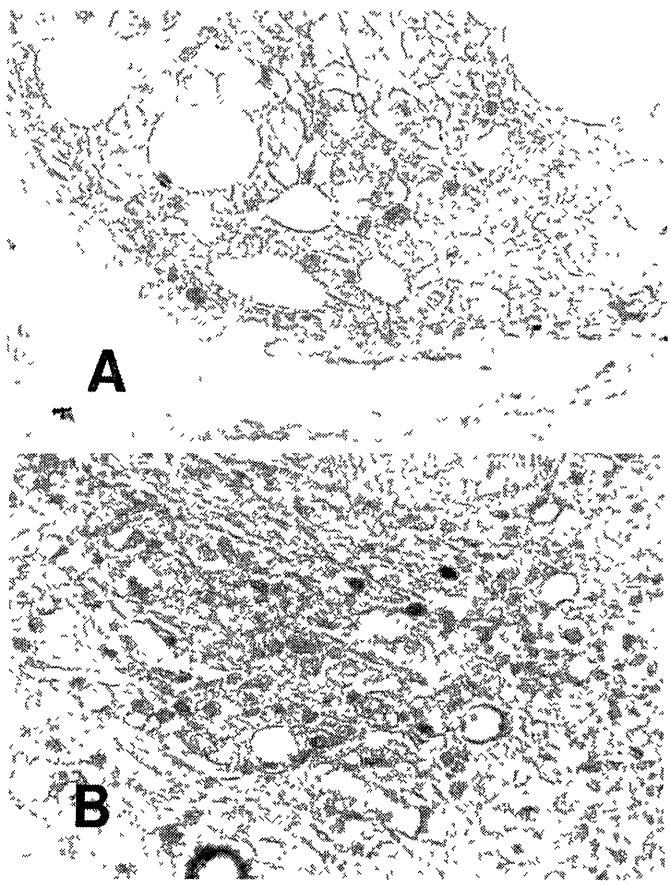


**Fig. 1.** Immunohistochemical distribution of PCNA in the section of prostatic carcinoma Darkly stained nuclei are positive for PCNA A Area of prostatic tumor with a cribriform pattern of growth x 200 B Area of prostatic tumor with a poorly differentiated adenocarcinoma X 200

and the other parameters shown in Table 1 was evaluated by the chi-square test. As a result, the PCNA immunoreactivity showed no significant correlation with the histological grade or with the other four parameters, i. e., the age, clinical stage, serum PAP, and the initial treatment (Table 2). However, as Table 3 shows, the percentage of stained nuclei in well-, moderately- and poorly- differentiated adenocarcinomas were 0.35 ± 0.42% (10 cases), 1.88 ± 2.88% (31 cases), 1.22 ± 2.36% (30 cases), respectively. Thus, the PCNA positivity was much higher in moderately- and poorly-differentiated tumors than in well-differentiated tumors.

The percentage of stained cells in the BPH specimens ranged from 0 to 2.6%, and eight of 10 cases stained negatively for PCNA (Fig. 2).

**Table 1.** Six parameters and thier categories studied in Cox's regression model

| Parameter | Category | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| Age | < 70 (27)* | ≧ 70 (44) | |
| Clinical stage | B (15) | C (19) | D (37) |
| Histological grade | 1 (10) | 2 (32) | 3 (33) |
| Serum PAP | Not elevated (25) | Elevated (46) | |
| PCNA immunoreactivity | Negative (28) | Positive (43) | |
| Initial treatment | Chemo-endocrine (45) | Endocrine (26) | |

* Number of patients.

**Table 2.** Correlation between the PCNA immunostaining score and other parameters

| | PCNA immunoreactivity | | Chi-square test |
|---|---|---|---|
| | Negative | Positive | |
| Age (yrs) | | | |
| < 70 | 10 | 17 | NS* |
| ≧ 70 | 18 | 26 | |
| Clinical stage | | | |
| B | 6 | 9 | NS |
| C | 10 | 9 | |
| D | 12 | 25 | |
| Histological grade | | | |
| 1 | 4 | 6 | NS |
| 2 | 11 | 21 | |
| 3 | 13 | 16 | |
| Serum PNA | | | |
| Not elevated | 11 | 14 | NS |
| Elevated | 17 | 29 | |
| Initial treatment | | | |
| Chemo-endocrine | 16 | 29 | NS |
| Endocrine | 12 | 14 | |

* Not statistical significance.

**Table 3.** Relationship of the histological grade to the PCNA positivity

| Histological grade | PCNA positivity average (%) |
|---|---|
| Well differentiated adenocarcinoma | 0.35 ± 0.42* |
| Moderately differentiated adenocarcinoma | 1.88 ± 2.88 |
| Poorly differentiated adenocarcinoma | 1.22 ± 2.36 |

* Standard deviation.

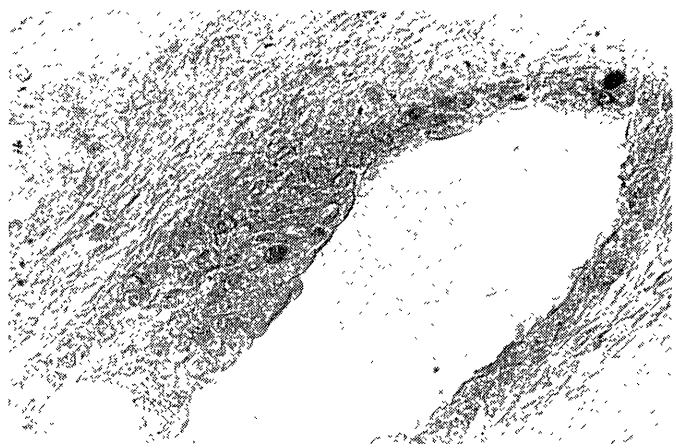

**Fig. 2.** Immunohistochemical staining of PCNA in the section of BPH X 200.

Of the 71 prostatic carcinoma patients studied, 26 died due to their prostatic carcinoma. The cancer-specific survival curves, calculated according to the difference in the PCNA immunoreactivity, is shown in Fig. 3. The patients in the PCNA-positive group seemed to have a slightly worse prognosis than those in the PCNA-negative group, but this was not significant.

Table 4 shows the results of the multivariate analysis. The clinical stage (p = 0.002) and the histological grade (p = 0.01) were found to have a significant effect on the cancer-specific survival. Although the PCNA immuno-

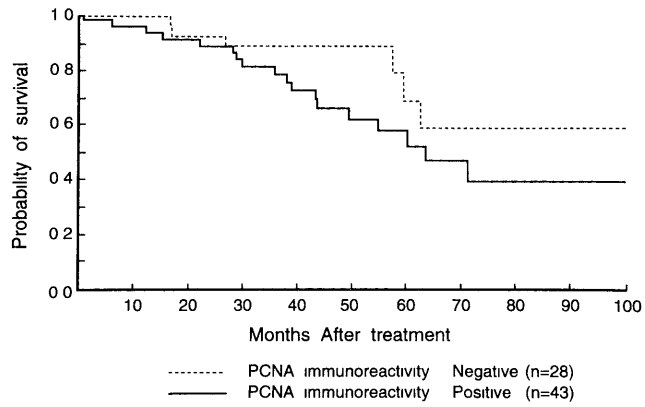


**Fig. 3.** Cancer-specific survival curves according to the PCNA immunoreactivity.

**Table 4.** Multivariate analysis of possible prognotic parameters in Cox's regression model for patients with prostatic carcinoma

| Parameter | Regression coefficient | Standard error | t value* | P value |
|---|---|---|---|---|
| Age (yrs) | 0.405 | 0.517 | 0.784 | 0.44 |
| Clinical stage | 1.315 | 0.405 | 3.245 | 0.002 |
| Histological grade | 1.012 | 0.392 | 2.582 | 0.01 |
| Serum PAP | –0.029 | 0.528 | –0.054 | 0.96 |
| PCNA immunoreactivity | 0.669 | 0.522 | 1.282 | 0.20 |
| Initial treatment | –0.143 | 0.492 | –0.291 | 0.77 |

*t = Regression coefficient / standard error.

reactivity ($p = 0.2$) was the third most significant prognostic parameter, it failed to reach statistical significance.

## Discussion

Using the immunohistochemical staining method for PCNA, studies were carried out with regard to various kinds of malignant tumors to elucidate the relationships between their stage, grade, prognosis and their cell proliferation. Louis *et al.* reported that, using fresh frozen specimens of brain tumors, detection of PCNA expression was inferior to the Ki-67 technique for judging cell proliferation. However, they demonstrated the usefulness of the PCNA immunohistochemical staining method since it can employ even paraffin-embedded specimens, and they also showed that the positive rate for PCNA expression increases in direct proportion to the histological grade of the malignancy.[14] Woods *et al.*[15] investigated gastrointestinal lymphoma cases and found that PCNA expression correlated well with the histological grade. They demonstrated that the prognosis became worse as the positive rate for PCNA expression increased, and the findings for PCNA immunoreactivity correlated well with the findings of DNA flow cytometry. Yu *et al.*[16] reported that PCNA expression correlated well with the histological grade in cases of hemangiopericytoma, and that there was a tendency for the prognosis to become worse as the positive rate for PCNA expression increased. Jain *et al.*[17] studied gastric carcinoma and reported that PCNA expression showed almost no correlation with the histological grade, clinical stage, lymph node metastasis, etc., but they found that patients with a high PCNA index seemed to have a slightly worse prognosis.

Harper *et al.*[18] reported on their studies of PCNA expression in prostatic carcinoma. Using the PC-10 monoclonal antibody, they performed immunohistochemical staining on specimens from 102 cases of prostatic cacinoma and 20 cases of BPH, and they demonstrated a PCNA index of 1%~58% for prostatic carcinoma and an index of 0%~10% for BPH. In our present study employing the NC-012 monoclonal antibody, we showed slightly lower ranges for the PCNA index: 0%~11.4% for 71 cases of prostatic carcinoma and 0%~2.6% for 10 cases of BPH.

In addition, Harper and colleagues referred to the relationships between the PCNA index and the histological grade, clinical stage, metastatic status and age, and they reported a significant correlation between the PCNA index and the histological grade. They reported that there were no correlations between the PCNA index and the other parameters. In our study, although the PCNA index showed a tendency to be high in cases of moderately to poorly differentiated malignancies, there was no statistical significance. Furthermore, no significant correlation was noted between the PCNA index and the other parameters, i. e., the clinical stage, age, histological grade, serum PAP or initial treatment.

Moreover, Harper investigated the relationship between the PCNA index and the prognosis in 65 cases of prostatic carcinoma, and they reported that the prognosis was significantly ($p < 0.04$) better in the patient group having a PCNA index of 10% or higher compared with the patient group having an index of less than 10%. In our study, although the difference was not statistically significant ($p = 0.2$), we found that the prognosis tended to be worse in the patient group that stained positively for PCNA in comparison with the PCNA-negative group.

This discrepancy in the PCNA immunoreactive distribution findings may have been caused by several factors. The difference in the monoclonal antibodies employed may be the primary reason. Second, the type of specimens evaluated was different. That is, in the Harper study, the specimens were obtained by transurethral resection (TUR), whereas we obtained our specimens by needle biopsy. Therefore, since a biopsy specimen or a TUR specimen does not always reflect the entirety of a tumor, a bias may have occurred in the tissue sampling. The third and final possible reason is the difference in tissue fixation; previous studies have indicated that PCNA is both heat and formaldehyde sensitive.[19] The method of collection of specimens, the handling of specimens until the time of fixation, the fixation procedure, and the time passed from fixation until staining may be factors that influence the results of PCNA immunohistochemical staining. In the present study, in fact, specimens that had been fixed more than 10 years earlier showed a tendency to have a lower rate of positive staining for PCNA than specimens fixed less than 10 years earlier.

Generally, the prognosis for patients with prostatic carci-

noma depends on the histological grade and clinical stage. In the present study, application of multivariate analysis to the six parameters of age, histological grade, clinical stage, serum PAP, the initial treatment and the PCNA immunoreactivity indicated the clinical stage to be the most important prognostic parameter, followed by the histological grade. The PCNA immunoreactivity was demonstrated to be the third most statistically significant prognostic factor.

In conclusion, our study demonstrated that PCNA immunohistochemical staining is easy to perform on routinely-processed materials and permits retrospective studies. We also elucidated that the PCNA index shows a relationship with the histological grade. We surmise that positive PCNA immunoreactivity may be useful in clinical practice for identifying prostatic carcinoma patients with a poor prognosis.

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