Urol Res (1994) 22:171–176 © Springer-Verlag 1994

ORIGINAL PAPER

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Effects of selected chemotherapeutic agents on PCNA expression in prostate carcinoma cell lines

Received: 17 February 1993 / Accepted: 5 January 1994

Abstract Bivariate flow cytometric analysis of proliferating cell nuclear antigen (PCNA) was performed on prostate carcinoma cell lines (PC-3, DU-145). For both cell lines 100% methanol fixation provided optimal fluorescence intensity of PCNA. The ratio of PCNA/DNA increased in late G1 through early S/phase, followed by a decrease in mid- and late S and enhancement in G2/M phase. PCNA expression was increased in G2/M phase cells treated for 48 h with vinblastine. A slight decrease in PCNA expression was observed with cyclohexamide treatment. Hydroxyurea induced an increase in S-phase fraction along with enhanced PCNA expression. Methotrexate and Adriamycin had little effect on the cell cycle compartments of PC-3 or DU-145; however, methotrexate decreased PCNA expression, while Adriamycin enhanced it. Cisplatin increased S-phase in both cell lines, increasing PCNA expression in PC-3 and decreasing it in DU-145 cells. The data on the effects of drug treatment point to a dissociation between PCNA expression and S-phase fraction as calculated from the DNA distribution. In some cases, e.g., the cisplatin studies, different effects were obtained in the two different cell lines treated with the same drugs. Whether changes in PCNA expression will provide more useful information than S-phase fraction for evaluation of potential antitumor drugs is not known.

Key words Prostate cell lines · PCNA · Cell cycle · Chemotherapeutic agents

Prostate carcinoma is the most common malignant tumor of American men [37]. Although it can be stabilized through hormonal treatment, this tumor eventually develops into a hormonally unresponsive neoplasm that is also resistant to current cytotoxic therapies [18, 24]. Proliferative activity has been correlated with progression and overall prognosis in a variety of malignant tumors [22] and may play a role in carcinoma of the prostate. Most prostate carcinomas have low growth fractions [29], and their response to chemotherapeutic agents differs significantly from that of rapidly growing tumors [14].

Proliferating cell nuclear antigen (PCNA) has been identified as an auxillary protein to DNA polymerase delta [17] and functions as a co-factor in DNA synthesis. It is also implicated in unscheduled DNA snythesis [41]. The synthesis and expression of PCNA are enhanced in proliferating cells [5]. Higher percentages of PCNA-positive cells have been observed in prostate carcinomas than in benign prostate hyperplasia (BPH). Moreover, patients with prostate carcinoma whose tumors have lower PCNA content survive significantly longer than patients with carcinomas of higher PCNA content [23].

In the present study we examined by flow cytometry the expression of PCNA in relation to the cell cycle in the human prostate carcinoma cell lines PC-3 and DU-145, and after treatment with commonly used antitumor drugs: cyclohexamide (CHX), vinblastine (VIN), methotrexate (MTX), hydroxyurea (HYD), Adriamycin (ADR), and cisplatin (CIS). The effects of these drugs on PCNA expression during the cell cycle were considered in terms of their mechanism of action as chemotherapeutic agents.

Supported by NIH grants Ro-1-CA 14134

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Materials and methods

Cells

Human prostate carcinoma cell lines PC-3 and DU-145 were obtained from American Type Culture Collection, Rockville, Md. PC-3 cells were maintained in monolayer culture by serial passage in 25 cm^2 flasks with HAM's F12 medium supplemented with 7% fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (all from GIBCO, Grand Island, N.Y.), incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Table 1 Expression of PCNA in PC-3 and DU-145 cells during the cell cycle (means ± SD)

Cell type	Mean PCNA fluorescence/DNA					
	G1A	G1B	Early S	Mid-S	Late S	G2/M
PC-3	1.00	2.00 ± 0.04	1.79 ± 0.05	1.52 ± 0.04	1.35 ± 0.02	1.41 ± 0.06
DU-145	1.00	2.52 ± 0.02	2.04 ± 0.03	1.78 ± 0.07	1.69 ± 0.06	1.97 ± 0.04

DU-145 cells were maintained in MEM (GIBCO) supplemented with 10% FBS under standard culture conditions.

PC-3 and DU-145 cells were seeded at 2×10^5 cells/ml culture media, as above, 24 h before the experiments were started. Drugs were added to the flasks at various concentrations. Cells were harvested using 0.05% trypsin-EDTA (ethylenediaminetetraacetic acid, from GIBCO). Cell viability was assessed by trypan blue exclusion. Counts were done with a hemocytometer. All experiments were performed in triplicate.

Drugs

All drugs were purchased from Sigma (St. Louis, Mo.). VIN was dissolved in DMSO (dimethyl sulfoxide) and stored at −20°C. A stock solution of 1 mM MTX was made in H_2O and stored at −20°C.

Immunofluorescence staining of PCNA

Several methods of fixation/permeabilization were tested to obtain optimal PCNA binding. Fixation procedures used were as follows [27]: 100% methanol for 10 min at −20°C followed by 0.5% NP-40 for 10 min on ice; 70% ethanol for 10 min at −20°C followed by 0.5% Triton X-100 for 5 min on ice; 100% acetone for 10 min at −20°C followed by 0.5% NP-40 for 3 min on ice; 0.5% paraformaldehyde for 10 min at +4°C followed by 0.5% NP-40 for 3 min on ice; 2% paraformaldehyde for 5 min room temperature (RT; 23°C) followed by 0.5% Triton X-100 for 3 min at +4°C.

The best results, that is maximum PCNA staining for both PC-3 and DU-145 were achieved with the first fixation treatment listed. After fixation, cells were incubated for 60 min at RT in presence of primary antibody against PCNA (PC-100, Oncogene Sciences, Uniondale, N.Y.), diluted 1:50 in PBS containing 1% bovine Serum albumin (BSA). Cells were then washed and incubated with a fluorescein isothiocyanate (FITC) – conjugated goat antimouse IgG antibody (Sigma Immunochemicals) diluted 1:180 in PBS with 1% BSA. Cells were washed again, resuspended in 10 µg/ml propidium iodide (PI) (Polysciences, Warrington, Pa.) and 0.1% RNAse A (Worthington Biochem, Freehold, N.J.) in PBS and incubated at RT for 30 min prior to measurement. Isotype control 1gG2a antibodies (Becton–Dickinson, Mountain View, Calif.) were run in parallel with PCNA.

Flow cytometry

The fluorescence of cells stained with PI and FITC was measured using a FACScan flow cytometer (Becton Dickinson). Red (PI) and green (FITC) emissions from each cell were separated using the standard optical filter packs of the FACScan. PI and FITC fluorescence signals were collected in linear mode. Data were acquired and processed on LYSYS II software (Becton-Dickinson). Cell cycle phases were defined on the basis of nuclear DNA content. The mean PCNA fluorescence was calculated at G1A (early G1), G1B (late G1), early S phase (<33% DNA replicated), mid-S phase (33–66% DNA replicated), late S phase (>66% DNA replicated) and G2/M. Background fluorescence (mean of isotype controls) was subtracted, and the data recalculated per unit of DNA (expressed as DNA index). Details of the gating strategy are described by Bruno et al. [10] and Gorczyca et al. [21]. The mean PCNA/DNA ratio in the G1A phase was set at 1.00, and the values of PCNA/DNA in other phases are related to the G1A phase.

Results

Expression of PCNA in PC-3 and DU-145 cells

The bivariate distribution of PCNA fluorescence and DNA content of PC-3 and DU-145 cells represents the expression of the antigen in relation to the cell's position in the cell cycle (Fig. 1A, 2A). Table 1 shows PCNA distribution (as mean PCNA/DNA) at each cell cycle phase. Gl was heterogeneous, some cells expressing PCNA at levels several fold higher than the others. It appears that PCNA rapidly increases in late Gl through early S phase, followed by a decrease in mid- and late S phases and is then enhanced again at G2/M phase. The PCNA/DNA ratio, however, decreases progressively during cell progression from late Gl through S phase.

Effects of VIN, MTX, HYD, CHX, CIS, and ADR on PCNA expression and cell cycle in PC-3

A stathmokinetic experiment was performed in which cells were arrested in mitosis by the addition of VIN 0.5 µg/ml for 48 h [12]. G2/M phase cells increased from 26% (0 h) to 59% (48 h). These cells displayed a minor increase in PCNA/DNA. The ratio of PCNA/DNA in G2/M rose from 1.41 (untreated) to 1.53 (48 h) (Fig. 1B).

Treatment of PC-3 with HYD, 1 mM for 48 h [38], resulted in the arrest of cells in S phase (Fig. 1C). The PCNA/DNA ratio increased slightly during early, mid- and late S phases.

The stability of this antigen was also evaluated in cells in which protein synthesis was inhibited by CHX. Cells were exposed to 3 µg/ml for up to 48 h [43] (Fig. 1D). A slight decrease in PCNA expression was observed with no modification in the cell cycle.

The mechanisms of action of ADR and MTX are not totally clear. Since these drugs stabilize DNA-topoisomerase cleavable complexes, they are often classified as inhibitors of topoisomerases. Consequently, their pharmacological activity is believed to be a results of these interactions. Neither of the drugs at 1 µg or 2 µg/ml [3, 26] altered the cell cycle of PC-3 at 6, 24, or 48 h (Fig. 1E, 1F). PCNA expression decreased in late G1, S, and G2/M

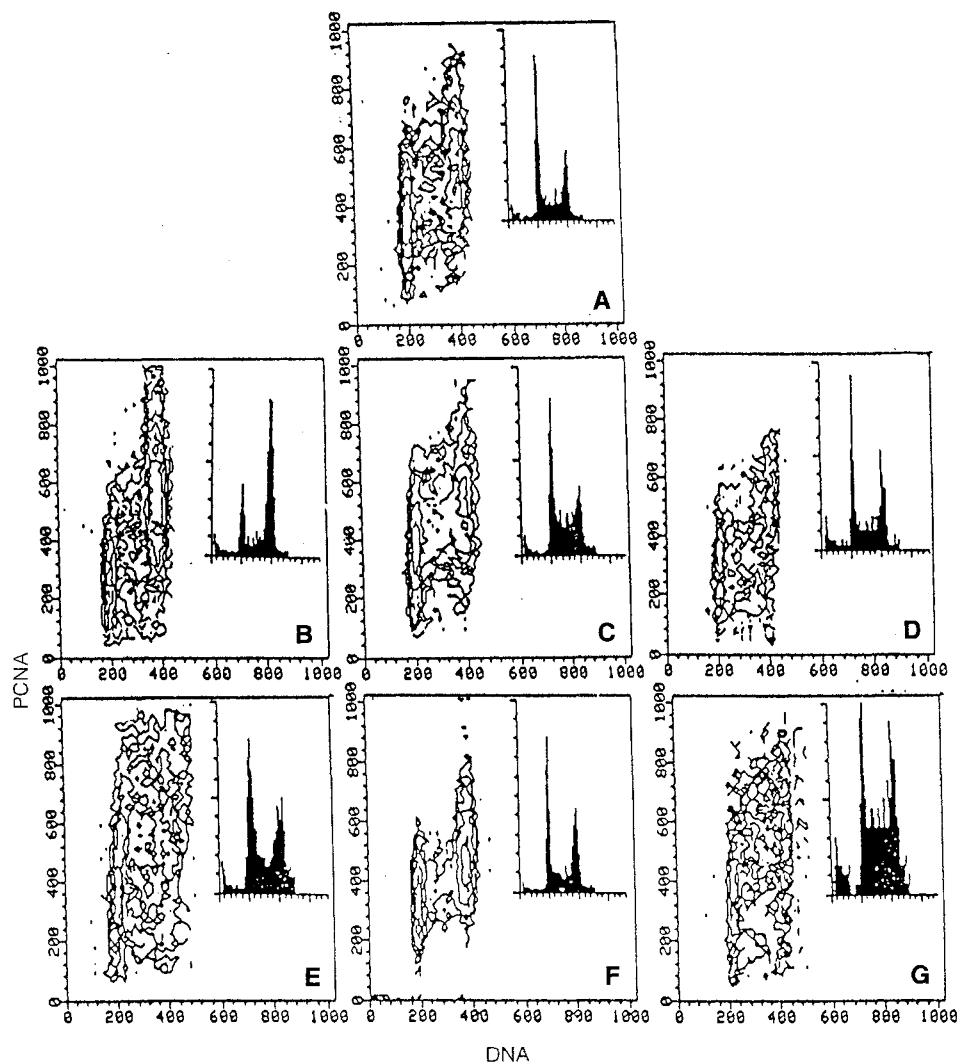

Fig. 1A–G Expression of PCNA in PC-3 cells in relation to their position in the cell cycle. **A** Untreated and exponentially growing cells; **B–G** cells treated for 48 h with **B** VIN 0.05 μg/ml; **C** HYD 1 mM; **D** CHX 3 μg/ml; **E** ADR 2 μg/ml; **F** MTX 1 μg/ml; **G** CIS 25 μM. *Insets*, corresponding DNA frequency histograms

phase with MTX treatment. ADR increased PCNA expression in S phase.

CIS was added at 24 μM for 48 h [2] increasing the S phase to 65% in PC-3 cells (Fig. 1G). PCNA expression was enhanced with CIS treatment.

Effects of VIN, MTX, HYD, CHX, CIS and ADR on the expression of PCNA and cell cycle in DU-145

Figure 2 shows the effects of the drugs on DU-145 cells. Treatment of DU-145 cells with 1 mM HYD for 48 h induced an increase in S phase (Fig. 2C). The expression of PCNA in S phase was also slightly increased. CHX, ADR, MTX had a marginal effect on the cell cycle (Fig. 2D–F), whereas the influence on PCNA expression differed. MTX decreased PCNA expression moderately, while CHX slightly decreased, and ADR incrased PCNA expression. Exposure of CIS for 48 h blocked 70% of cells in S phase and resulted in complete loss of PCNA staining of some cells (Fig. 2G).

VIN blocked cells in M phase, increased PCNA expression in G2/M and decreased it in G1 phase (Fig. 2B).

Discussion

Although hormonal therapy is the mainstay of treatment for prostate carcinomas [33], they inevitably develop into a hormonally unresponsive disease resistant to current therapeutic modalities. A "modern era" of chemotherapy research in prostate cancer was introduced in 1973 by the National Prostate Cancer Project [15].

The human prostate carcinoma cell lines PC-3 and DU-145 are androgen-independent models [25, 39] that are useful for the study of chemotherapeutic agents. In this report PC-3 and DU-145 were treated with six different drugs and examined for changes in expression of PCNA and modulation of the cell cycle. Elevated expression of PCNA has been observed in carcinomas of the breast, lung, kidney, urinary bladder and uterine cervix and

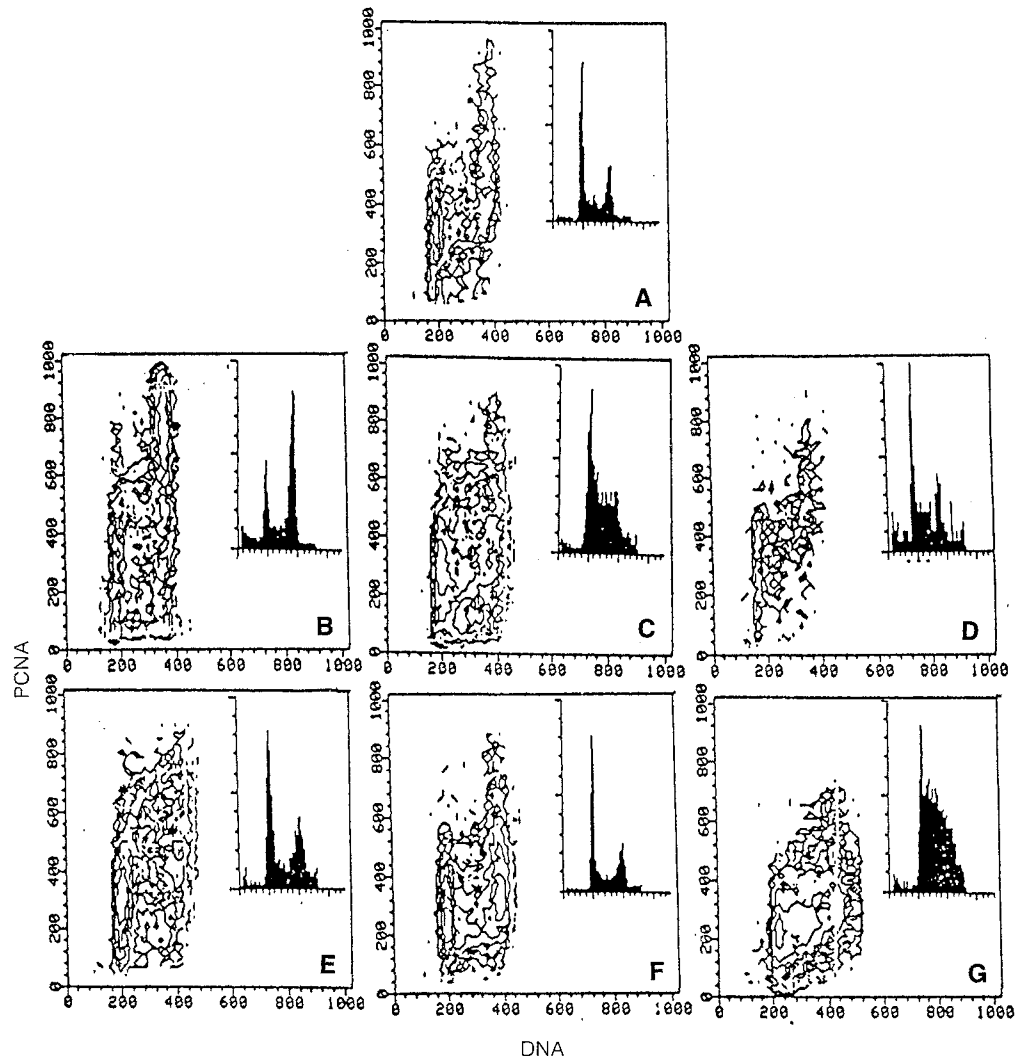

Fig. 2A–G Expression of PCNA in DU-145 cells in correlated with their position in the cell cycle. **A** untreated exponentially growing cells; **B–G**, cells treated for 48 h with **B** VIN 0.05 μg/ml; **C** HYD 1 mM; **D** CHX 3 μg/ml; **E** ADR 2 μg/ml; **F** MTX 1 μg/ml; **G** CIS 25 μM. *Insets*, corresponding DNA frequency histograms

leukemia [16, 18, 20, 22] as well as prostate carcinoma [20]. Several studies have shown that PCNA levels increased to threefold between early G1 and early S and tend to plateau through G2, irrespective of the synchrony technique utilized [5]. The use of anti-sense oligonucleotides suggests that PCNA is an essential requirement for DNA synthesis [34].

Optimal staining characteristics are heavily influenced by fixation/permeabilization methods, which will differ with the nature of the antigen and cells under study. Several methods of fixation have routinely worked well in DNA flow cytometry [36]. Extensive preliminary experiments were conducted to establish the fixation/permeabilization procedure that would provide: specific and quantifiable immunofluorescence staining patterns; high-resolution DNA profiles; minimal cell clumping [20]. Schimenti and Jacobberger [36] showed that methanol fixation, with or without paraformaldehyde pretreatment, gave better DNA % CV than paraformaldehyde with Triton-X-100. An unfixed and washless method provided the best expression of Ki-67 in the bladder carcinoma T24 cell line (Kusuda et al., in preparation) and fresh tissues [32]. The present study found the best expression of PCNA (PC-10 in PC-3 and DU-145 cells) to be with 100% methanol fixation, followed by 0.5% NP-40, which coincided with the results of Landberg and Roos in human hematopoietic cell lines [27]. An additional consideration is that quantitation by indirect immunofluorescence could depend on modification in the PCNA/cyclin protein. Since no cell cycle-dependent modifications of PCNA have been detected [6], a more likely possibility is altered immunogenicity resulting from association with other cellular components. In particular, immunofluorescence studies have revealed two sites of DNA replication and another that is homogeneously diffuse in the nucleoplasm that is extracted by non-ionic detergents [6]. Using the monoclonal antibody PC-10, we found that detergent treatment with 0.5% NP-40 for 10 min on ice extracted the main bulk of PCNA, whereas the S-phase-associated form is resistant. Fluorescence staining was nuclear, not in the cytoplasm (data not shown).

Bravo and MacDonald-Bravo reported that synthesis of cyclin (PCNA) in mouse 3T3 cells, induced by growth factors (PDGF; FGF) preceded and was closely correlated

Table 2 Effect of drugs on PCNA/DNA ratio according to cell cycle

Drug	G1A	G1B	Ealry S	Mid S	Late S	G2/M
Vinblastine	↓	↓				↑↑
Hydroxyurea			↑	↑	↑	
Cyclohexamide	↓↓	↓↓	↓↓	↓↓	↓↓	↓↓
Methotrexate		↓↓	↓↓	↓↓	↓↓	↓↓
Adriamycin			↑↑	↑↑	↑↑	
Cisplatin[a]		↑↓	↑↓	↑↓	↑↓	

[a] Cisplatin treatment resulted in decreased expression or complete loss of PCNA in DU-145 cells, but increased expression in PC-3 cells

with DNA synthesis [7]. Detection of PCNA combined with measurements of cellular DNA, followed by bivariate analysis of the correlated data, made it possible to study the expression of this protein in PC-3 and DU-145 prostate cancer cell lines in relation to cell position in the cell cycle. PCNA expression in G1 cells was heterogeneous with respect to DNA content. PCNA levels then rapidly increased in G1 through early S phase, followed by a decrease in the ratio of PCNA/DNA until sometime in late S, and finally enhanced expression at G2/M. The increase in PCNA during early S phase was of a lesser magnitude than the rate of replication, so that the PCNA/DNA ratio declined from early to late S phase. Since all cell constituents double during the cycle, the ratio of a particular constituent per unit DNA [7] or per total protein content [1] is a more accurate representation of specificity of its expression at a given point in the cycle.

A number of chemotherapeutic agents have been identified that may benefit men with metastatic hormone-refractory disease [19]. In the present study several commonly used drugs, VIN, MTX, ADR, CAM, CHX, HYD, and CIS were examined by bivariate flow cytometry to determine the drug effects on cycling PC-3 and DU-145 cells (Table 2).

CHX inhibits RNA and protein synthesis. Recently Bruno et al. [9] and Littleton et al. [28] described the loss of Ki-67 antigen in cells treated with CHX. L. Qiao et al. (in preparation) noted no significant change in p 105 expression in HL-60 cells after CHX treatment. The present study shows a slight decrease in PCNA expression in PC-3 and DU-145 cells after 3 μg/ml CHX treatment for 48 h, without any influence on the cell cycle.

Our data show G2/M prolonged after adding VIN as expected. Since p 105 expression is increased in M phase [30] (L. Qiao et al., in preparation) and p 145 expression decreased in all phases in HL-60 cells [31], PCNA, p 145 and p 105 may provide useful or even essential complementary approaches for studying cell proliferation.

Treatment of cycling cells with HYD blocks further DNA synthesis [44] with an apparent increase in S-phase fraction. Despite the inhibitory effect on DNA, PCNA continued to be expressed in these cells. In fact, HYD-treated cells showed enhanced PCNA levels.

CIS forms bifunctional DNA adducts, including DNA interstrand and DNA protein cross-links [35]. The cells were blocked in S phase in both cell lines after treatment with CIS. CIS had diverse effects on PCNA expression in the two cell lines, it was decreased in DU-145, and increased in PC-3 cells. This difference may be due to availability of epitopes after induced conformational change in chromatin.

MTX and ADR are both intercalating drugs. The mechanism of their cytostatic and/or cytotoxic activity is not entirely clear. They are also considered to be inhibitors of topoisomerase II [40]. Data published by numerous authors [2, 42] indicate that, regardless of cell type and dose of ADR or MTX, G2 arrest is the most conspicuous cell cycle effect of these drugs. Our data show that MTX exerts little effect on the cell cycle but decreases the level of PCNA in both cell lines. ADR increases PCNA expression of S-phase cells. The difference may be related to variations in the stability of the drug-induced complexes of topoisomerase II with DNA, as described by DelBino and Darzynkiewicz [13]. These drugs are able to stabilize topoisomerase II-DNA II-DNA cleavable complexes [4].

PC-3 and DU-145 cells are hormone-independent. The treatment of choice for patients with hormone-refractory metastatic prostatic carcinoma is chemotherapy supplemented by irradiation. In general, the response to drugs is poor. Whether expression of proliferation-associated nuclear markers, such as PCNA, can identify the patients with carcinomas likely to respond to cytostatic drugs is speculative at best.

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