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Comparison of Proliferating Cell Nuclear Antigen Immunostaining in Lymph Node Metastases and Primary Prostate Adenocarcinoma after Neoadjuvant Androgen Deprivation Therapy

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Objective: To evaluate the effect of neoadjuvant androgen deprivation therapy (NADT) on the cellular proliferative activity in primary prostate cancer and lymph node metastases using proliferating cell nuclear antigen (PCNA) immunostaining.
Material and Methods: Metastatic pelvic lymph nodes and tumoral prostatic tissue were obtained from 21 patients after radical prostatectomy and pelvic lymphadenectomy. Sixteen patients received NADT for 3 months prior to surgery; five patients did not and were evaluated as a control group. Histopathologic analysis was performed using PCNA immunostaining, and histopathologic findings of primary tumors and lymph node metastases after NADT were reported. Clinical follow-up was performed for a mean of 43.7 months.
Results: Evaluation of PCNA immunostaining of lymph node metastases in the 16 treated patients revealed a mean positivity for metastatic tumor of 4.5% (SD 3.1%); the corresponding value for the five patients who were not treated with NADT was 19.6% (SD 0.94%) ($p < 0.05$). In four of the treated cases the proliferative activity in the lymph node metastases was greater than that in the other 12 (9.3% and 3.0%, respectively) and no histopathologic regressive changes were observed in these four cases. The residual tumoral proliferative activity in lymph nodes was greater than that in primary tumors (4.5% and 1.3%, respectively).
Conclusions: This study shows that the nodal metastases were responsive to hormonal therapy, as assessed by PCNA staining, although a greater residual proliferative activity was observed after NADT in lymph node metastases in comparison with the primary prostatic tumor. This can be attributed to a metastatic phenotype less responsive to hormonal therapy compared to the primary tumor.

Key words: lymph node metastases, neoadjuvant hormonal therapy, proliferating cell nuclear antigen, prostate cancer.

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The use of neoadjuvant androgen deprivation therapy (NADT) given a few months prior to radical prostatectomy has been analyzed (1–3); it induces regressive effects on the tumoral prostatic cells (4–7) and can reduce the incidence of positive surgical margins (8–10). It was also observed that neoadjuvant therapy did not reduce the incidence of lymphatic invasion and lymph node metastases in stage T_2–T_3 tumors, which occur in 5–10% of patients (11, 12).

In our research and that of other authors, the proliferative activity of premalignant and malignant lesions of the prostate gland, either treated or untreated with NADT, was investigated in histologic sections by analyzing proliferating cell nuclear antigen (PCNA) immunostaining (13–31). PCNA has been identified as a cofactor of DNA polymerase delta and its expression occurs during the late G1 and s phases of the cell cycle (14).

To date, the proliferative activity of lymph node metastases in patients with prostatic carcinoma has only been evaluated in a few studies (13–16). The aim of our study is to evaluate the effect of NADT on cellular proliferative activity in lymph node metastases and primary prostate cancer using PCNA immunostaining.

MATERIAL AND METHODS

Patients

The records of 21 patients with prostate cancer and metastatic pelvic lymph nodes were retrospectively

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retrieved from our database of 150 patients who underwent radical retropubic prostatectomy for clinically localized (T_2) and locally advanced (T_3) prostate cancer between 1990 and 1994. The lymphadenectomy procedure was comparable in all cases, and included obturatory and internal and external iliac lymph nodes.

Sixteen patients received NADT with luteinizing hormone-releasing hormone (LH-RH) analogs and flutamide (750 mg/day) for 3 months prior to surgery; five patients did not receive NADT and were evaluated as a control group. The decision whether or not to give NADT did not depend upon a specific protocol (4). Baseline serum prostate-specific antigen (PSA) was 10–45 ng/ml in all the patients.

According to the Gleason grading applied to the prostatic biopsies before treatment, the 16 treated patients were stratified as follows: Gleason score 2–5, $n = 3$; Gleason score 6–7, $n = 3$; and Gleason score 8–10, $n = 10$ (Table I). The definitive histologic examination identified eight cases with a single lymph node metastasis (pN_1) and eight with multiple microscopic metastases (pN_2). The pathologic staging was as follows: pT_2N_1, $n = 2$; pT_3N_1, $n = 5$; pT_3N_2, $n = 6$; pT_4N_1, $n = 1$; pT_4N_2, $n = 2$.

In the five untreated patients, the pathologic examination identified one case with a single metastatic lymph node (pT_3N_1) and four cases with multiple lymph node metastases (pT_3N_2). Of the primary prostatic carcinomas, three cases had a Gleason score of 5–7 and two had a score of 8–10 (Table I).

The surgical margins were positive in 8/16 (50%) of the patients treated with NADT and in 3/5 (60%) of the untreated patients. The seminal vescicles were infiltrated by tumor in 8/16 treated cases (50%) and 3/5 untreated cases (60%).

The mean length of follow-up was 43.7 months. Disease progression was defined as metastatic disease, local recurrence or detectable serum PSA level >0.4 ng/ml.

Histopathology

The fresh prostate specimens were inked with Indian ink for evaluation of margins and dipped in Bouin's solution for 20–30 s. The total prostatectomy specimens were then fixed for 24–48 h in neutral buffered formalin (4%). Sections (0.5-cm thick) were obtained by working from the base towards the apex of the gland for evaluation of the margins and the extent of neoplasia. Each section was embedded in paraffin and, after adequate treatment, sections (0.3-cm thick) were dehydrated in a graded alcohol series, cleared in xylene and then stained with hematoxylin–eosin (H&E). Two pathologists (R.M., I.G.) reviewed all the histologic slides and selected those with well-represented cancer for quantitative evaluation.

Table I. *Patient characteristics, clinical and pathologic features and PCNA positivity values in primary prostatic tumors and lymph node metastases after NADT*

No. of patients	Pathologic stage	Grading of primary carcinoma	No. of lymph nodes dissected; mean (range)	No. of lymph nodes with metastases; mean (range)	PCNA positivity (%); mean (SD)		Follow-up (months); mean (range)	Disease progression[a]; *n* (%)
					Primary carcinoma	Nodal metastases		
Patients treated with NADT								
16	pN1/pN2	G2–5, $n = 3$; G6–7, $n = 3$; G8–10, $n = 10$[b]	23.6 (11–40)	3.7 (1–10)	1.33 (0.37)	4.5 (3.1)	43.7 (17–74)	9/16 (56)
Total			379	60				
Untreated patients								
5	pN1/pN2	G5-7, $n = 3$; G8-10, $n = 2$ [c]	16 (8–32)	4.6 (1–8)	12.2 (0.64)	19.6 (0.94)	40 (15–60)	3/5 (60)
Total			80	23				

[a]Disease progression defined by local recurrence or PSA failure (>0.4 ng/ml). [b]Grading evaluated in biopsy specimens before NADT treatment. [c]Grading evaluated in surgical specimen.

The same procedure was carried out for the histologic evaluation of pelvic lymph nodes, which were macroscopically examined by means of palpation. Suspicious lymph nodes or those with a diameter >1 cm were examined using frozen specimens. The remaining lymph nodes were fixed in formalin and then paraffin, sequentially sectioned at 0.3–0.5 μm and stained with H&E. The section containing the greatest amount of metastatic tissue was chosen for immunohistochemical analysis using PCNA. The frequency and location of nuclear PCNA staining in primary tumors and lymph node metastases were assessed in 5-μm thick sections cut from formalin-fixed, paraffin-embedded material.

The H&E-stained sections of all primary tumors and lymph node metastases were reviewed in order to examine the effect of NADT. The same parameters employed for the evaluation of the effects of prostatic therapy were used for the examination of the treated lymph node metastases. The following cytoarchitectural criteria were used for the evaluation of neoplasia: presence of a nucleolus; chromatin disposition; nuclear profile regularity; nucleo:cytoplasmatic ratio; cytoplasmatic alterations; and architectural criteria of differentiation (17).

Antibodies

Anti-PCNA antibodies (PC10; Dako) were used at a concentration of 0.5 μg/ml for 10 min at room temperature. Biotinylated goat antimouse antibody was used as the linker molecule and was applied at a dilution of 1:100 for 10 min. After further washing, sections were incubated in streptavidin–horseradish peroxidase complex (Cambridge Biosciences) at a dilution of 1:100 for 10 min. Diaminobenzidine-hydrogen peroxide (Sigma) was used as chromogen and a light Mayer's hematoxylin counterstain was applied. Sections were dehydrated in alcohol, cleared in xylene and mounted in DPX. To ensure the consistency of PCNA staining, a positive control of prostate carcinoma was included. PCNA expression and location were evaluated in histologic sections using a Leitz Orthoplan microscope equipped with an eyepiece graticule, which was used to randomly select an area in which 1000 cells were counted. All identifiable staining was regarded as positive. The percentage of PCNA-stained nuclei was evaluated for 1000 nuclei per case. Areas of normal lymphatic architecture served as internal controls for the staining of the metastatic deposits as germinal centers contained many positively stained nuclei. It was possible to express the average value of nuclear positivity for PCNA for 1000 neoplastic cells in all cases, with the exception of three cases treated with NADT in which we evaluated ≈300 neoplastic nuclei, due to a small amount of metastatic tumor, and in another case with a single microscopic metastasis in which we evaluated 100 nuclei.

Statistical analysis

The data were stored on a Power Apple Macintosh computer. Stat View™ software was used for calculation of the mean, standard variation and standard deviation (SD), as well as for statistical analysis, which was performed using the Mann–Whitney test.

RESULTS

Evaluation of proliferative activity of PCNA in lymph node metastases

A total of 379 lymph nodes (average diameter 0.93 mm; range 0.4–1.5 mm) were removed during pelvic lymphadenectomy in the 16 treated patients with an average of 23.6 per patient (minimum 11; maximum 40); 60 of these were metastatic (mean 3.6 per patient) (Table I).

A total of 80 lymph nodes were removed in the five untreated patients (minimum 8, maximum 32) with an average of 16 lymph nodes per patient; 23 of these were metastatic (4.6 per patient) (Table I).

Evaluation of PCNA immunostaining of lymph node metastases was performed in all 21 patients. In the 16 treated patients (pN_1, $n = 8$; pN_2, $n = 8$), the degree of PCNA positivity of metastatic tumor was 4.5% (minimum 1.0%; maximum 10.8%; SD 3.1%); in the five untreated patients (pN_1, $n = 1$; pN_2, $n = 4$) it was 19.6% (minimum 18.4%; maximum 20.9%; SD 0.92%) and this difference was statistically significant ($p < 0.05$; Table II).

Nuclei that stained positive for PCNA showed a granular pattern, with variable intensity. Nuclei of neoplastic cells with more intense staining occurred behind the marginal sinus of lymph nodes. PCNA was confined to nuclei with the exception of neoplastic cells

Table II. *Histopathologic findings in lymph nodes after 3 months of NADT*

Finding	Treated patients ($n = 16$)	Untreated patients ($n = 5$)
Histiocytosis in medullary sinus	+++	++
Perivascular hyalinosis	++	+
Follicular hyperplasia	−	+
Cortex expansion	−/+	+
Fibrosis	−/+	−
Adipose involution	+	−/+
Apoptosis	++	−
Therapy-induced changes	++/+	−

+++ = greatly increased expression; ++ = moderately increased expression; + = slightly increased expression; +/− = focal expression; − = no expression.

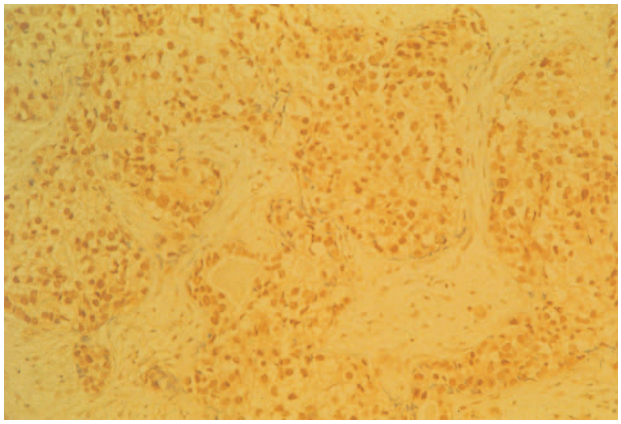

Fig. 1. PCNA expression in a metastatic lymph node of an untreated patient. An area of solid tumor with poor differentiation shows positivity for PCNA immunostaining in most of the nuclei of tumor cells.

in mitosis, which showed faint cytoplasmic staining. In treated lymph node metastases, in which tumor foci appeared that did not show regressive aspects due to hormonal therapy, PCNA showed more intense staining and was more widely expressed; in lesions with moderate regressive effects that showed apoptotic phenomena, PCNA immunostaining was rarely expressed. In untreated lymph node metastases, PCNA expression was higher in large tumor foci with poor differentiation than in small peripheral lesions (Figs 1 and 2; Table I).

Evaluation of proliferative activity of PCNA in corresponding primary prostatic neoplasia

Evaluation of PCNA immunostaining in prostatic adenocarcinomas was performed in 21 patients. In the 16 patients treated with NADT, the degree of PCNA positivity in primary prostatic carcinoma was 1.33% (SD 0.37%); in the five untreated patients, it was 12.2% (SD 0.64%) and this difference was statistically significant ($p < 0.05$). Nuclei with pyknotic chromatin, which were more frequently observed in the treated adenocarcinomas, did not stain positive for PCNA (Table II).

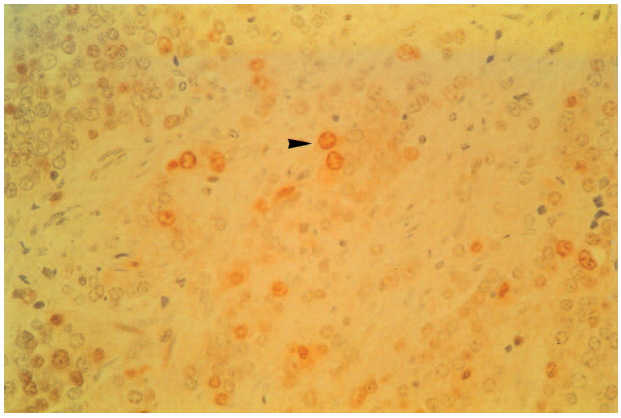

Fig. 2. PCNA expression in a metastatic lymph node of a treated patient. Metastatic tumor nuclei expressing PCNA immunostaining are identifiable as light brown areas (*arrowhead*).

Histopathologic findings in lymph node metastases after NADT

The histopathologic findings observed in the lymph nodes of treated patients were as follows: metastatic cells with increased cellular apoptosis and signs of moderate regressive effects due to therapy, cells with inconspicuous nucleoli, nuclear shrinkage, chromatin condensation and sometimes pyknosis, cytoplasmatic clearing and enlargement by coalescence of vacuoles and rupture of cell membranes. Mitoses were more numerous than in primary prostatic tumors and were observed more frequently in high-grade lesions with poor differentiation. Cellular necrosis was not found in any case (Table II).

Moderate regressive aspects due to NADT were observed in only four of the treated patients (25%), slight regressive aspects in eight (50%) and no regressive effects in four (25%). In two patients with multiple lymph node metastases there was a heterogeneous response to hormonal therapy, with the presence of moderate regressive effects in some lymph nodes while others exhibited little response.

The histopathologic findings in the untreated lymph nodes showed that the metastatic lesions were larger than in treated subjects and often invaded the cortical zone and extranodal tissue, with total substitution of the lymph node. All the neoplastic foci were characterized by a high degree of cellular anaplasia with poor architectural differentiation. The lymph node metastases occurred more frequently near the marginal sinus in cases of microscopic foci.

Histopathologic findings in corresponding primary prostatic neoplasia after NADT

The histopathologic changes were characterized by marked regressive and involutive aspects of the tumor, a reduction in tumor gland size and an increase in the interglandular connective tissue. The tumor cells showed inconspicuous nucleoli, nuclear shrinkage, chromatin condensation and pyknosis, cytoplasmatic clearing and enlargement by coalescence of vacuoles and rupture of cell membranes. Mitoses were rarely found in the treated cases.

Follow-up

The average follow-up period of the 16 treated patients was 43.7 months (minimum 22 months; maximum 74 months) (Table I). Disease progression was observed in

nine of them (56%): in seven cases there was biochemical failure 33 months postoperatively, in one case local recurrence and in another local recurrence and bone metastases. All patients with disease progression were treated with LH-RH analogs and antiandrogens. Serum PSA reduction to undetectable levels was observed in all cases, with the exception of one patient, in whom no regressive aspects after 3 months of hormonal therapy were observed in the four metastatic lymph nodes; in these lesions the mean PCNA positivity was 8.1%, while in the primary prostatic tumor, wide involutive aspects were observed and the PCNA positivity was 1.8%.

Of the 16 treated patients, it was possible to observe minor regressive signs and high proliferative activity after NADT in four. In fact, in these patients the average PCNA positivity on primitive prostatic neoplasia was 1.8% (minimum 1.6%; maximum 2.3%), while the average lymph nodal PCNA positivity was 9.3% (minimum 8.1%; maximum 10.8%) ($p < 0.05$); all of these patients had disease progression. In the remaining 12 patients it was possible to observe more evident regressive changes and a lower proliferative activity after NADT; in these patients the average PCNA positivity on primitive prostatic neoplasia was 1.3% (minimum 1.0%; maximum 1.6%), while the average lymph nodal PCNA positivity was 3.0% (minimum 1.0%; maximum 4.9%) ($p < 0.05$). Only five of these patients (40%) had disease progression.

The average follow-up period of the five untreated patients was 40 months (Table I), three of them (60%) had disease progression after a mean time of 25 months.

DISCUSSION

In patients with lymph node metastases the overall survival rates after radical prostatectomy vary from 20% to 66% at 5 years and from 29% to 66% at 10 years (32–34). In pN+ patients treated at the moment of progression, the 5-year mortality rate is 35–50% due to a hormone-independent tumor (32–35). Our data showed heterogeneous regressive effects due to NADT and residual high proliferative activity after androgen blockade in metastatic nodes.

Some authors have focused their attention on grading (18), number and volume of lymph node metastases (19–21), ploidy (22–25), proliferative activity (14), androgenic receptor expression (26) and chromosomal anomalies (27) in metastatic foci in order to identify which was the most influential biologic factor determining prognosis. The originality of our investigation lies in the detection of proliferative activity and histologic features of the tumoral cells of lymph node metastases following neoadjuvant therapy. The aim was to highlight the possible differences between primary and metastatic tumors following NADT.

The results of the PCNA evaluation on lymph node metastases in treated patients showed an interesting reduction in proliferative activity of metastatic tumoral cells induced by hormonal therapy. It is evident that hormonal therapy was effective in at least 12/16 treated patients (average PCNA positivity 3.0%); in the four treated patients with an average PCNA positivity of 9.3% the therapy induced only a partial reduction in proliferative activity.

Concerning the histologic aspects of metastatic foci, it is interesting to note that cellular apoptosis and chromatin thickening were more evident in the 12/16 patients with an average PCNA positivity of 3.0%, corresponding to the group of patients that showed a greater response to endocrine therapy. In the 4/16 patients with an average PCNA positivity of 9.3% we observed scarce effects and modest cellular apoptosis.

In the untreated cases, histology showed invasive and poorly differentiated carcinoma. Neoplastic foci in lymph nodes of untreated cases were larger than in treated ones and gross lymph node substitution with extranodal tumor extension was often observed. The morphologic aspects observed in untreated patients were similar to those discovered in the 4/16 treated patients who were considered unresponsive to hormonal therapy.

It is interesting to observe that in 2/16 of the treated patients a heterogeneous response to hormonal therapy was discovered in different metastatic lymph nodes, expressed as variable PCNA positivity values and non-uniform regressive histologic aspects. This may therefore indicate the existence of heterogeneous metastatic phenotypes with different hormone sensitivities in the same individual.

The biologic sensitivity of metastatic tumoral cells to NADT was significantly lower with respect to the involutive response of the tumoral prostatic cells: the PCNA positivity in the lymph nodes was 4.5% (SD 3.0%), while that in primitive prostatic neoplasia was 1.3% ($p < 0.05$).

The histomorphologic effects observed in non-metastatic lymph nodes were only slightly different between treated and untreated patients: a greater degree of perivascular hyalinosis and sinus histiocytosis was observed in treated than in untreated patients and the follicular hyperplasia was reduced; in treated cases, the lymph nodal metastases were often surrounded by a thin layer of hyaline connective tissue, which was not observed in untreated cases.

In the current study of lymph node metastases the use of PCNA immunostaining required particular attention and precision, both in the execution of the

method and in its interpretation. In some cases a low number of neoplastic cells were present, which approached the lowest possible limits for reading: in three treated pN_1 patients the number of evaluable tumoral cells was <1000, i.e. 289, 230 and 100 cells, respectively (26).

In recent studies it has been observed that NADT does not reduce the number of metastatic lymph nodes (36). We wish to stress the importance of the technique of histologic sampling. A careful inspection of the whole lymph node, i.e. by performing numerous sections, is important, especially in those cases treated with NADT, as it is able to produce regressive tumor changes and can decrease the metastatic tumor volume. Therefore accurate sampling by means of sequential sections is mandatory in order to avoid missing cancer metastasis in lymph nodes after NADT.

The mean follow-up of our patients is not sufficient for an adequate evaluation of disease progression or for a complete statistical analysis; other authors have estimated that at the same stage progression develops within 2 years in most cases (17, 28). In our study, all four patients with high proliferative activity after NADT in metastatic foci (PCNA positivity 9.3%) showed disease progression, while only 5/12 (40%) patients with reduced proliferative activity after NADT (PCNA positivity 3.0%) had disease progression. In the light of this observation, pN+ patients could be considered to have a worse prognosis because of the presence of phenotypes which are totally or partially resistant to hormono-suppressive therapy. However, the relevance of this study in terms of clinical practice is restricted to those populations in whom extensive PSA screening is not performed.

In conclusion, our study was conducted using the PCNA technique and had the aim of observing the proliferative activity of metastatic cells and primary prostatic tumor cells after NADT. The results revealed that residual proliferative activity in lymph node metastases was greater that that in primitive tumor: 4.5% vs 1.33%, respectively. In four cases the proliferative activity in the lymph nodes was greater in comparison to the remaining 12 cases: 9.3% vs 3.0%, respectively. Such an observation confirms the hypothesis that the lymph node metastases were composed of a metastatic phenotype which is less responsive to hormonal therapy, given the greater residual proliferative activity observed in metastases compared to the primary tumor.

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