cells, which may result in an increased release into the medium [14]. In cultured prostate cancer cells the levels of tumour-associated antigens, such as the prostate-specific acid phosphatase or epithelial membrane antigen, also increase during the G_1 phase, remain stable during the G_2 phase and drop during or immediately after cytokinesis [15]. The different behaviour of glycoproteins might be explained by the as yet unclear function of CA-125, which is possibly involved in the homeostasis of resting cells.

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Biological Characterisation of Primary and Metachronous Lesions in Breast Cancer Patients

Rosella Silvestrini, Barbara Valentinis, Maria Grazia Daidone, Giovanni Di Fronzo, Danila Coradini and Bruno Salvadori

Proliferative activity, evaluated as [^{3}H]thymidine labelling index ([^{3}H]dT LI), and hormone receptors were determined on 97 primary breast cancers and on metachronous lesions from the same patient. Overall, the [^{3}H]dT LI of metachronous lesions was significantly higher than that of the primary tumour ($P = 0.003$). Hormone receptor profiles of the two lesions were similar in about 75% of the cases; disagreements were mainly due to a disappearance of hormone receptors in metachronous lesions. In contralateral tumours, [^{3}H]dT LI and hormone receptors were unrelated to those of the relative primary lesion. In this series of relapsing patients, [^{3}H]dT LI was unrelated to hormone receptor status in the primary tumour, but it was higher in the metachronous lesions from patients with hormone receptor-negative primary tumours. For patients given no systemic therapy between surgery and relapse, the time to develop local–regional recurrences or contralateral tumours was inversely related to the [^{3}H]dT LI of the metachronous lesions.
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INTRODUCTION

CANCER IS characterised by a marked biological heterogeneity not only among tumours of different patients, but also among the different synchronous or metachronous lesions of the individual patient. In fact, cell clones with a different biological profile in terms of karyotype, antigenicity, metastatic potential, biochemical properties, hormone receptor status, growth behaviour, chemo- and radiosensitivity may co-exist within the same tumour as a balance between natural or induced selective pressure [1–4]. Moreover, with progression of the disease, the overgrowth of

clones with specific phenotypic, functional or molecular features has been widely demonstrated by experimental and clinical evidence [5–7].

We investigated in a previous study the heterogeneity of cell proliferative status between primary breast cancer and its synchronous axillary lymph node metastasis and the possible clinical implications [8]. Only a few studies have investigated the modulation of proliferative rate and hormone-receptor status in primary breast cancers and metachronous lesions [9–13] and on series of patients limited and selected for logistic and ethical limitations of systematically collecting data.

In the present study we focused our attention on cell kinetics and hormonal receptor status, which have consistently been shown to be important prognostic indicators in breast cancer [14–20]. Proliferative activity and oestrogen (ER) and progesterone receptors (PgR) were determined in parallel on the primary tumour and on different types of metachronous lesions from the same patient on a case series larger than those previously reported [9,10].

MATERIALS AND METHODS

Tumour material and patient population

The proliferative activity and hormonal receptor content of the primary breast cancer and of its metachronous metastases were determined for 97 patients (62 in premenopause and 35 in postmenopause) admitted to the Istituto Nazionale Tumori of Milan from September 1978 to November 1989. Determinations on primary tumours were performed at diagnosis before any treatment. The metastatic lesions were biopsied for pathological assessment and biological determinations at the time of relapse, which ranged from 6 to 107 months (median, 24 months). The metachronous lesions studied were: 44 local recurrences, 22 lymph nodes, 6 visceral metastases and 25 contralateral breast cancers. They represented the first histopathologically documented relapse of disease in 100% of the local recurrences, 86% of lymph nodes, 83% of visceral metastases and 83% of contralateral tumours. Between surgery of the primary tumour and the appearance of the metastatic lesion, patients were given chemotherapy in 43 cases, hormone therapy in 11 cases, and miscellaneous in 7 cases and 36 patients had not been treated.

Determination of [^{3}H]thymidine labelling index ([^{3}H]dT LI)

The [^{3}H]dT LI was determined on fresh surgical or biopsy material as previously described [14]. The [^{3}H]dT LI was evaluated by scoring a total of more than 3000 tumour cells on different specimens from the same tumour lesion and defined as the percentage ratio between labelled and total number of tumour cells. No threshold for considering labelled nuclei was necessary, because the background was always less than 1.5 grains for 100 μ^2 and silver grains of the background were therefore only occasionally observed above the nuclei. The minimum number of grains per nucleus of labelled cells was 20.

Determination of cytoplasmic hormonal receptor

Immediately after surgery, primary and metastatic tumour samples were placed in plastic vials, frozen at −25°C and stored at −70°C. The ER and PgR contents were assayed by using the dextran-coated-charcoal technique according to the European Organization for Research and Treatment of Cancer method [21,22]. Cut-off values of 10 and 25 fmol/mg cytosol protein were used for ER and PgR, respectively. ER and PgR content information was available for 92 (95%) and 89 (92%) primary tumours, respectively, and both determinations for 91 (94%) metachronous lesions.

Statistical analysis

The relation between the [^{3}H]dT LI of the primary tumour and that of its metachronous lesion was assessed by the Spearman rank correlation coefficient (r_s) on the overall series of cases and on subsets defined by site of relapse. The χ^2 test was used to compare frequency distributions. The Wilcoxon test was used to assess differences in ranked distribution of values.

RESULTS

Proliferative activity

The median [^{3}H]dT LI value of the primary tumour in this selected series of relapsing patients was 4.6% (range, 0.2–20%). It was higher than that previously reported for a consecutive series including both relapsing and non-relapsing patients [23], and it was similar regardless of the site of relapse, except for a higher value for a few patients with visceral relapse (Table 1). The median [^{3}H]dT LI of metachronous lesions (5.6%; range 0.3–26%) was significantly higher than that of the primary tumour ($P = 0.003$), and this finding was mainly evident for lymph node metastases (Table 1).

Moreover, when [^{3}H]dT LI values of primary and metastatic lesions from individual patients were matched, a significant association ($P = 0.0001$) was observed, even though the data were highly scattered (Fig. 1). The relationship was maintained for the different types of metachronous lesions distinctly analysed (r_s: 0.50–0.77), except for contralateral tumours ($r_s = -0.035$).

[^{3}H]dT LI was analysed as a qualitative variable, and the overall median value (5.0%) of primary and secondary lesions of the present series was adopted as a cut-off of slow and rapid proliferation. 40% of patients with slowly proliferating primary tumours presented rapidly proliferating metachronous lesions. The increase in [^{3}H]dT LI was less evident for local recurrences and more frequent for lymph node and visceral metastases, as well as for contralateral cancers (Table 2). Rapidly proliferating primary tumours maintained this kinetic status in a high number of local recurrences, lymph node and visceral metastases (from 76 to 100%). Contralateral cancers showed a lower [^{3}H]dT LI activity than the corresponding primary tumour in about 80% of the cases.

ER and PgR status

In the overall series of 92 patients for whom oestrogen receptor determination on the primary lesion was available, ERs were detected in 76% of primary tumours, and this frequency was not affected by menopausal status (Table 3). The frequency of ER− tumours was slightly higher for patients who relapsed in lymph nodes or viscera. The frequency of ER+ metachronous lesions (63%) was lower but not significantly different from that of primary tumours, and it was not affected by menopausal status or site of relapse. The overall agreement in ER status between primary and metastatic lesions was 76%, with a higher rate for local recurrences and lymph node lesions (80–84%), the most substantial groups, and a lower rate (57%) for contralateral cancers.

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Table 1. Proliferative activity of primary and metachronous lesions as a function of site of relapse

Site of relapse	No. of cases	[³H]dT labelling index (%) Primary lesion Median	Primary lesion Range	Metachronous lesion Median	Metachronous lesion Range
Local	44	4.7	0.2–20.0	5.3	0.5–21.6
Lymph node	22	4.7*	0.9–14.8	7.4*	1.3–18.4
Visceral	6	7.6	2.3–10.4	9.2	0.3–23.6
Contralateral	25	4.0	0.7– 9.3	4.5	0.3–26.0

* $P = 0.01$.

The determination of PgR on primary tumours and their metachronous lesions was available for 89 patients. The frequency of PgR+ tumours was 57%, and it was significantly lower in postmenopausal than in premenopausal subgroups (37 vs. 67%; $P = 0.008$, Table 3). Moreover, a low frequency of PgR+ tumours was observed in patients who relapsed with lymph node metastases. A significant decrease in PgR+ lesions was observed in metachronous metastases with respect to primary tumours (57 vs. 38%; $P < 0.025$). This decrease was limited to premenopausal patients ($P < 0.025$), and it was mainly evident in visceral lesions and still marked in local recurrences and contralateral lesions. The overall agreement in PgR status between primary and metastatic lesions was 72%, with the highest concordance in lymph node lesions (94%).

Relationship between [³H]dT LI and ER or PgR status

In this series of relapsing patients, median [³H]dT LI values of the primary tumor from ER+ and ER− cases were similar (4.5 and 4.9%, respectively), except for tumours which subsequently recurred locally, in which the median [³H]dT LI for ER+ primary tumours was 4.5% and for ER− primary tumours was 8.0% ($P = 0.01$). Among metachronous lesions which had been ER+ at presentation, the median [³H]dT LI was 4.8% compared with 7.5% for cases which had been ER− at presentation ($P = 0.01$). No significant difference was observed between the [³H]dT LI of PgR+ and PgR− primary tumours (4.2 and 4.7%, respectively). Metachronous lesions tended to have a higher [³H]dT LI if the primary tumour had been PgR−.

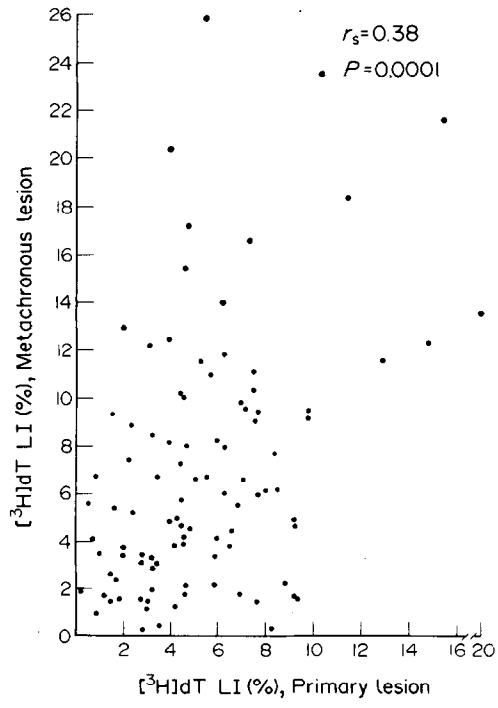

Fig. 1. The [³H]dT LI (%) of the primary tumour and of its metachronous lesion (97 cases).

Relationship between [³H]dT LI and clinical outcome

In order to avoid any possible interference of systemic therapy on subclinical metastatic disease, analysis of the clinical outcome as a function of proliferative activity of the primary or metachronous lesions was performed only in patients who did not undergo any adjuvant treatment between surgery and relapse. Among these, meaningful calculations were possible only for patients with local–regional relapses or contralateral cancers, which were the most substantial groups in the series. Proliferative activity of the primary tumour was not indicative of time to relapse. Conversely, the cell kinetics of metachronous lesions was inversely related to the interval between surgery and appearance of the lesion. In fact, the median [³H]dT LI value of metachronous lesions was higher in patients relapsing before than after 24 months from surgery, and it was respectively 1.5 and 3 times higher for patients who exhibited local–regional relapses (6.8 vs. 4.2%) and contralateral lesions (4.5 vs. 1.7%).

Table 2. Variations in proliferative activity from the primary to the metachronous lesion

	Variation (%)			
Primary →	Low LI*		High LI*	
Metachronous lesion →	Low LI	High LI	Low LI	High LI
Local recurrence	74	26	24	76
Lymph node	50	50	10	90
Visceral	50	50	0	100
Contralateral	50	50	78	22

* LI cut-off value: 5%.

Table 3. Frequency of ER+ and PgR+ primary and metachronous lesions as a function of menopausal status and site of relapse

	Incidence (%)			
	ER+		PgR+	
	Primary lesion	Metachronous lesion	Primary lesion	Metachronous lesion
Overall	76	63	57*	38*
Menopausal status				
Premenopause	78	60	67*†	42*
Postmenopause	72	68	37†	32
Site of relapse				
Local	85	70	61	39
Lymph node	59	53	37	32
Visceral	60	60	60	20
Contralateral	79	58	67	46

* Primary vs. metachronous lesions, $P < 0.025$.
† Pre- vs. postmenopause, $P = 0.008$.

DISCUSSION

In human solid tumours, eventual changes in biological characteristics have been occasionally investigated during disease progression and generally limited to selected types of surgically accessible metastases. In breast cancer, many studies have been focused on the modulation of hormone receptor content from primary to metachronous metastases [11–13]. Most data from the literature report a lower frequency of ER+ tumours in relapsing patients than in unselected series of breast cancers as a consequence of the higher incidence of new disease manifestations in the unfavourable receptor-negative subset. The hormone receptor profile of primary and metachronous lesions has been reviewed by Hahnel *et al.* [13], who observed that ER are a rather stable marker over time with a qualitative agreement in receptor status between primary and metastasis in 80% of the cases. Conversely, few studies have analysed the proliferative characteristics of the different lesions during disease progression for individual patients [9,10].

In our experience, on a selected series of relapsed patients for which proliferative activity was also assessed, the frequency of ER+ primary tumours was similar to that observed for the overall series of unselected tumours. This evidence could be ascribed to the high frequency of tumours that developed soft tissue relapses and that are known to be more frequently ER+ than tumours relapsing in viscera [24]. In agreement with data from the literature, an association in ER and PgR profiles between the primary and the relative metachronous metastasis was observed in three out of four of the cases, and the disagreements were mainly due to a disappearance of hormone receptors in metachronous lesions. Moreover, when the different types of metachronous lesions were analysed separately, the agreement was maximum for all the lesions except for contralateral tumours.

The eventual changes in [^{3}H]dT LI from primary to metachronous metastases have been studied by Meyer *et al.* [9,10], who reported an increase in proliferative rate from primary to metachronous lesions. In agreement with their preliminary results, we observed that relapsing primary tumours proliferated faster than primary breast cancers from consecutive, unselected series of patients [23]. A further significant increase in [^{3}H]dT LI with respect to primary tumours was observed in lymph node metastases but not in local recurrences. Conversely, contralateral tumours showed a proliferative activity unrelated to primary tumours. This finding, in association with the lack of an agreement for the matched values in receptor status, supports the hypothesis of a possible functional independence between the primary and metachronous contralateral tumours.

The inverse relation observed between proliferative rate and hormone receptor status, generally reported on large and consecutive series of primary tumours [14,15,25,26], was only partially maintained on the present series of relapsing patients. This finding suggests a prevalence of cell kinetics over hormone receptor status in determining relapse. In addition, the increased proliferative rate observed for metachronous lesions from ER− primary tumours could be ascribed to an increased response to paracrine or autocrine growth factors.

Analysis of clinical outcome as a function of biological markers was not a major object of this study owing to the limited case series, which was heterogeneous in treatment and clinical presentation. However, preliminary evidence showed an inverse relation between time to develop local–regional or contralateral lesions and the proliferative rate of the same metachronous lesions. This latter finding, in association with the evidence of a qualitative proliferation increase in 40% of the initially slowly proliferating cases, is in keeping with the hypothesis that cell kinetics is a functional marker able to parallel and reflect at a biological level the clinical progression of breast cancers.

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Flow Cytometric Characterisation of Proliferating Cell Nuclear Antigen using the Monoclonal Antibody PC10

George D. Wilson, Richard S. Camplejohn, Christine A. Martindale, Adrian Brock, David P. Lane and Diana M. Barnes

Anti-PCNA antibodies have aroused considerable interest recently as potential immunohistochemical markers of proliferation for use on clinical samples. PC10 is a monoclonal antibody which has been shown to recognise its epitope on formalin-fixed, paraffin-embedded, archival material. However, whilst PC10 gives the expected labelling pattern for growth fraction in normal tissues and some tumours, discrepant results have been obtained, for example, in carcinoma of the breast. By means of flow cytometry, we have attempted to characterise the different staining patterns that can be obtained with PC10. Intact fixed cells from proliferative mammalian cultures show 100% labelling, consistent with a growth fraction estimate. In contrast, detergent-extracted nuclei show S-phase specific staining. Nuclei extracted by treatment of fixed cells with pepsin show a different staining pattern again, with many G1 cells weakly stained and staining intensity increasing through S-phase into G2. The results demonstrate that multiparametric flow cytometry can define the cell populations which label with proliferation-related antibodies, such as PC10, under a variety of experimental conditions.
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INTRODUCTION

IDENTIFICATION AND characterisation of the cellular proteins involved in the control of cell proliferation is essential for understanding the mechanisms of growth regulation in both normal and neoplastic tissue. Knowledge of proliferation in human tumours can provide valuable information which may be used prognostically or diagnostically to select appropriate treatments or treatment scheduling, and biologically it may give insight into tumour progression and, in particular, metastatic potential.

Traditionally, proliferation in human tumours, has been assessed using *in vitro* incorporation of tritiated thymidine