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Original Paper

Primary Chemotherapy in Locally Advanced Breast Cancer (LABC): Effects on Tumour Proliferative Activity, bcl-2 Expression and the Relationship Between Tumour Regression and Biological Markers

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The rate of tumour cell proliferation evaluated by the [^{3}H]-thymidine labelling index ([^{3}H]-dT-LI) is known to be an independent prognostic factor in patients with operable breast cancer and significantly predicts the response to chemotherapy in patients with advanced disease. In locally advanced breast cancer (LABC), we examined whether chemotherapy induced modifications in [^{3}H]-dt-LI, and bcl-2 expression and their relationship with tumour regression and prognosis. 70 LABC patients received three courses of primary chemotherapy (FEC: 5-fluorouracil 600 mg/m^2, epidoxorubicin 60 mg/m^2, cyclophosphamide 600 mg/m^2, followed by surgery and subsequent adjuvant chemotherapy consisting of three courses of FEC alternated with three courses of CMF (cyclophosphamide 600 mg/m^2, methotrexate 40 mg/m^2, 5-fluorouracil 600 mg/m^2). Tumour biological markers were evaluated on diagnostic biopsy, before primary chemotherapy and at surgery. Tumour cell proliferation was determined by [^{3}H]-dT-LI, whilst bcl-2 expression was examined by immunohistochemical staining. The overall response rate to primary FEC was 74.3% (95% confidence interval 57.6–83.2%). The response rate correlated with high [^{3}H]-dT-LI: 88% (29/33) of patients with high [^{3}H]-dT-LI achieved an objective response compared with 62% (23/37) of patients with low [^{3}H]-dT-LI ($P = 0.014$). The 3 patients achieving a pathological complete response after induction treatment had high proliferative tumours. The highest 2-year relapse free survival (66.6%) was observed in patients with low [^{3}H]-dT-LI after primary chemotherapy. The median bcl-2 expression values before and after primary chemotherapy were 0% (range 0–80) and 30% (range 0–90), respectively ($P = 0.03$). Our data indicate that primary chemotherapy can modulate tumour cell kinetics and apoptosis-related genes. Pretreatment proliferative activity correlated with tumour response, whilst post-treatment [^{3}H]-dT-LI correlated with relapse free survival. © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: locally advanced breast cancer (LABC), cell kinetics, [^{3}H]-thymidine labelling index, apoptosis-related genes, bcl-2
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INTRODUCTION

TUMOUR CELL kinetics evaluated by ^{3}H-thymidine labelling index ([^{3}H]-dT-LI) represents an independent prognostic factor in breast cancer [1–3]. The growth rate of breast cancer depends on a large number of growth-regulating factors, including oncogenes, onco-suppressor genes and their products involved in tumour cell proliferation and death. Mitosis and apoptosis are interrelated and probably controlled by common regulatory mechanisms [4]. New biological indicators, closely linked to cell proliferation and

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apoptosis control, have been investigated to improve the prognostic characterisation of human breast cancer [5, 6]. Several studies indicate that the *bcl-2* proto-oncogene plays a fundamental role in preventing apoptosis induced by different agents. However, the role of *bcl-2* in breast cancer biology is not completely understood. The *bcl-2* gene product is also involved in cell differentiation and the loss of *bcl-2* expression seems to be associated with a poor prognosis in operable breast cancer [5, 7]. Chemotherapy can modify some biological features of breast cancer cells, such as the proliferative rate and DNA ploidy [8], but very few studies have analysed the effects of chemotherapy on *bcl-2* expression *'in vivo'* [6, 9]. Locally advanced breast cancer (LABC) is a good model to evaluate the '*in vivo*' effects of chemotherapy on the biological profile of the tumour and to correlate these modifications with clinical outcome. Within the framework of a randomised trial in LABC, we investigated the modifications induced by primary chemotherapy on [^{3}H]-dt-LI and *bcl-2* expression and their relationship to tumour regression and prognosis.

PATIENTS AND METHODS

Patient population

70 patients with histologically diagnosed (LABC) entered the study; this patient population represents the standard arm of a multicentre randomised trial aimed at evaluating the role of dose intensity of primary chemotherapy in LABC. All patients received primary chemotherapy consisting of three courses of FEC (5-fluorouracil 600 mg/m^2, epidoxorubicin 60 mg/m^2 and cyclophosphamide 600 mg/m^2) on day 1 every 3 weeks. After surgery patients received six additional courses of adjuvant chemotherapy consisting of three courses of FEC alternated with three courses of CMF (cyclophosphamide 600 mg/m^2, methotrexate 40 mg/m^2, 5-fluorouracil 600 mg/m^2). Patients with inflammatory breast disease received radiation treatment within 4 weeks of the completion of adjuvant chemotherapy.

Evaluation of biological markers

Biological markers were determined at diagnosis, on tumour samples obtained with tru-cut biopsy, and at surgery. The feasibility of the biological markers investigated before and after primary chemotherapy was based on priority ranking: histological diagnosis, [^{3}H]-dT-LI and *bcl-2* expression.

Tumour cell proliferative assay with [^{3}H]*-dT-LI*

Tumour tissue fragments were incubated under sterile conditions for 1 h at 37°C in RPMI 1640 medium (Gibco) with the addition of 20% calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and 6 mCi/ml ^{3}H-thymidine (specific activity 5 μCi/mmol). At the end of the incubation, tumour fragments were rinsed with saline at 4°C, fixed in Bouin's solution and embedded in paraffin. Autoradiography was performed on histological sections using a nuclear track emulsion (Ilford K5). After an exposure time of 6 days at 4°C, autoradiograms were developed at 18°C in Kodak D 19b and fixed in Kodak F5. The slides were stained with haematoxylin and eosin. The [^{3}H]-dT-LI, defined as the percentage ratio between labelled cells and total observed tumour cells, was determined by scoring a total of more than 2000 tumour cells. The median pretreatment [^{3}H]-dT-LI value of 3.1% was used as a cut-off value to define slowly and rapidly proliferating tumours.

bcl-2 *immunohistochemistry*

Analysis of *bcl-2* expression was carried out in 3 μm Bouin-fixed, paraffin-embedded sections. Samples were treated with 2% hydrogen peroxide to inhibit endogenous peroxidase before the addition of normal goat serum. The mouse monoclonal antibody antihuman *bcl-2* oncoprotein (clone 124; Dakopatts, Copenhagen, Denmark) was used at 1:200 dilution in phosphate buffered saline (PBS) supplemented with 1% bovine serum albumin.

The specimens were finally processed using the goat anti-mouse biotinylated antibody and avidin-biotin peroxidase complex (ABC) according to the manufacturer's instructions (Vectastain ABC kit; Vector Laboratories, Burlingame, Califonia, U.S.A.). Both positive and negative controls were added to each experiment. The evaluation of *bcl-2* cytoplasmic immunoreactivity was quantified independently by two investigators counting at least 1000 tumour cells in different fields. The results were expressed as a percentage of tumour cells staining positively for *bcl-2*. For statistical analysis, tumours with less than 5% positive cells were considered *bcl-2* negative.

Statistical analysis

The median pre- and post-treatment values of [^{3}H]-dT-LI and *bcl-2* expression were compared to evaluate the effects of primary chemotherapy. The differences between the means of continuous variables were determined by the analysis of variance and Student's t test. Frequency distributions were tested using the Chi-square test and Yate's correction was applied when appropriate. A significance level of $P = 0.05$ was selected. Relapse free survival was calculated according to the Kaplan–Meier method [10].

RESULTS

The characteristics of the study population are listed in Table 1. The median [^{3}H]-dT-LI value at diagnosis was 3.1% (range 0.01–24.8%) and was reduced to 0.6% (range 0.01–8.1%) after primary chemotherapy ($P = 0.0001$) (Table 2). 3 patients achieving a pathological complete remission after primary chemotherapy were not considered for [^{3}H]-dT-LI or *bcl-2* variations. The median coefficient of variation in [^{3}H]-dT-LI observed in our patients after treatment was 80.6%. After primary FEC a reduction in [^{3}H]-dT-LI ($\geq$ 80.6% of pretreatment values) was observed in 34/67 (51%) patients, an increase ($\geq$ 80.6% of pretreatment values) in 8/67 (12%) patients, while no change was observed in 25/67 (37%). Rapidly proliferating tumours showed a reduction in [^{3}H]-dT-LI in 60% of cases, whilst an increase was only occasionally observed (3%). In slowly proliferating tumours, a decrease in [^{3}H]-dT-LI occurred in 43% of cases and an increase in 19% (Table 3).

Table 1. *Study population*

Number of patients	70
Median age years (range)	52 (26–70)
Median PS (range)	0 (0–1)
Stage at diagnosis	
IIIA	31
IIIB	36
IBC	3

IBC, inflammatory breast cancer; PS, performance status.

Table 2. Changes induced by chemotherapy on tumour ([3H]-dT-LI) and bcl-2 *expression*

	Pretreatment median % (range)	Post-treatment median % (range)	*P* value
[3H]-dt-LI	3.1 (0.01–24.8)	0.6 (0.01–8.1)	0.0001
bcl-2	0 (0–80)	30 (0–90)	0.03

The overall response rate to primary FEC was 52/70 (74.3%, 95% confidence interval (CI) 57.6–83.2%). Tumour regression correlated with pretreatment [3H]-dT-LI, since 88% (29/33) of the patients with rapidly proliferating tumours achieved an objective response compared with 62% (23/37) of the patients with slowly proliferating tumours ($P = 0.014$) (Table 3). The 3 patients achieving a pathological complete remission after primary chemotherapy had highly proliferating tumours.

Using the median pretreatment [3H]-dT-LI as the cut-off, we classified the tumours into four groups according to [3H]-dT-LI values before and after primary chemotherapy. Two-year relapse free survival rates were correlated with [3H]-dT-LI variations: the highest 2-year relapse free survival was observed in patients remaining (66.6%) or achieving (56.5%) a low [3H]-dT-LI after primary chemotherapy (Table 4).

Modifications in *bcl-2* expression were evaluable in 30 patients. Of the tumour samples, 63.3% (19/30) had low *bcl-2* expression or were *bcl-2* negative before chemotherapy. The median values of *bcl-2* expression before and after primary chemotherapy were 0% (range 0–80) and 30% (range 0–90), respectively ($P = 0.03$) (Table 2). *bcl-2* expression was not modified by primary chemotherapy in 70% (21/30) of the cases, was increased in 27% (8/30) of the cases and was decreased in 3% (1/30) of the cases. All tumours showing an increased *bcl-2* expression after primary chemotherapy had a low post-treatment proliferative activity.

Table 4. 3H-thymidine labelling index ([3H]-dT-LI) changes and 2-year relapse free survival

[3H]-dT-LI			
Before CT	After CT	No. of patients	2-year relapse free survival
Low	Low	24	16 (66.6%)
High	Low	23	13 (56.5%)
Low	High	8	3 (37.5%)
High	High	12	4 (33.3)

CT, chemotherapy.

DISCUSSION

In LABC very few studies have evaluated the modifications induced by neoadjuvant chemotherapy on biological tumour markers and their relationship with tumour regression and clinical outcome. Previous reports have shown a higher overall response rate to primary chemotherapy in rapidly proliferating tumours and have demonstrated a relationship between pretreatment tumour proliferative rate and variations in [3H]-dT-LI induced by primary chemotherapy [9, 11–13].

The results obtained in the present study in 70 LABC patients confirm that primary chemotherapy can modify, *in vivo*, the proliferative activity of breast cancer: overall, primary FEC induced a greater than 80% reduction in the proliferative rate in 51% of the patients and this decrease was correlated with pretreatment tumour cell kinetics (Table 3). Moreover, pretreatment tumour cell kinetics was a predictor of response to chemotherapy in our series of LABC patients: the overall response rate to primary FEC was 88% in rapidly proliferating tumours and 62% in slowly proliferating ones; this difference was statistically significant ($P = 0.014$).

The prognostic significance of pretreatment [3H]-dT-LI, in terms of disease free and overall survival, remains controversial. Previous reports [14, 15] have shown that LABC with a high proliferative rate has a poor prognosis, but others have not observed this relationship [16]. This discrepancy can be explained by the fact that primary chemotherapy significantly modified the baseline tumour proliferative rate by selecting tumour cell clones with different kinetics. As a consequence, after primary chemotherapy, we frequently observed a residual tumour with a kinetic pattern which was completely different from the pretreatment pattern. This kinetic modulation might influence the subsequent clinical behaviour of the disease. In our series, in spite of the short follow-up, the baseline proliferative activity did not correlate with clinical outcome: on the contrary, we observed that patients with a low [3H]-dT-LI after chemotherapy had a higher 2-year relapse free survival in comparison with those with a highly proliferating tumour after induction treatment. Therefore, post-treatment [3H]-dT-LI, rather than baseline proliferation, should be taken into consideration as a possible prognostic parameter.

Owing to the extensive use of primary chemotherapy even in operable mammary carcinoma, the variation of the kinetic activity of the tumour after chemotherapy could be an important parameter to identify patients to be submitted to differentiated adjuvant treatment.

Several authors have investigated the impact of chemotherapy on apoptosis and on apoptosis-related oncoproteins [8, 17, 18]. In operable breast cancer, the loss of *bcl-2* expression is associated with a poor prognosis and high *bcl-2* expression correlates with low proliferative activity [6, 8]. In our study, the majority of tumour samples had low *bcl-2* expression or were *bcl-2* negative before chemotherapy; this

Table 3. 3H-thymidine labelling index ([3H-dT-LI) modifications and objective responses induced by primary chemotherapy

	Post-treatment* [3H]-dT-LI			Response rate			
	Decrease (%)	Increase (%)	No change (%)	CR no. (%)	PR no. (%)	SD no. (%)	*P* value
Pretreatment [3H]-dT-LI†							
High	18 (60)	1 (3)	11 (37)	3 (9)	26 (79)	4 (12)	0.014
Low	16 (43)	7 (19)	14 (38)	0 (0)	23 (62)	14 (38)	

*Patients with complete response were not included in this analysis. †[3H]-dT-LI cut-off value was 3.1%. SD, stable disease; PR, partial response; CR, complete response.

finding is in agreement with the observation that low *bcl-2* expression characterises mammary tumours with poor prognosis [19]. Primary FEC induced a significant increase in *bcl-2* expression which was not related to baseline values; in addition, despite the limited number of patients, it was interesting to observe that the increase in *bcl-2* expression was observed in those patients in whom chemotherapy maintained or induced a low proliferative rate: all these patients are alive and disease free 2 years after surgery. The overall reduction in proliferative rate and the increased expression in *bcl-2* observed in our study, suggests that chemotherapy kills more aggressive tumour clones while sparing the cells with more favourable biological characteristics.

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