18. SAS User's Guide, Statistics, Version 6.07. Cary, North Carolina, SAS Institute Inc., 1991.
19. Gordon LI, Harrington D, Anderson J, *et al.* Comparison of a second generation combination chemotherapeutic regimen (m-BACOD) with a standard regimen (CHOP) for advanced diffuse non-Hodgkin's lymphoma. *N Engl J Med* 1992, **327**, 1342–1349.
20. Cooper IA, Wolf MM, Robertson TI, *et al.* Randomised comparison of MACOP-B with CHOP in patients with intermediate-grade non-Hodgkin's lymphoma. *J Clin Oncol* 1994, **12**, 769–778.

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Heterogeneity of Intratumour Proliferative Activity in Primary Breast Cancer: Biological and Clinical Aspects

A. Paradiso, A. Mangia, A. Barletta, S. Fusilli, F. Marzullo, F. Schittulli and M. De Lena

The present retrospective study was undertaken to verify whether the extent of intratumour proliferative activity variation or the method of quantifying tumour proliferative activity is related to biological characteristics and clinical outcome in a series of operable node-negative breast cancer patients. For tumour proliferative activity evaluation, the ^{3}H-thymidine autoradiographic assay was used. After incubation of 3–8 samples from different areas of the equatorial section of each tumour for 1 h at 37°C with ^{3}H-thymidine, the following methods were used for evaluation of tumour cell labelling: mean tumour labelling index (LI), the highest labelling value from a specific area (LI-max), and the extent of intratumour labelling variation from several samples (LI–CV). LI-max was related to ER and PgR status, and linearly correlated with LI (c.c. = 0.92, $P < 10^{-6}$) whereas LI–CV was independent of tumour size, grade ER and PgR status, but dependent on the number of tumour samples analysed for each tumour. After 5 years of median follow-up, disease-free survival was only related to tumour size (T1 versus T2: 84 versus 64%, $P < 0.04$ by log rank analysis) and different LI values (low versus high ^{3}H-Tdr-LI: 86 versus 61%, $P < 0.03$ by log rank analysis). LI-max and LI–CV values were not significantly related to clinical outcome. Cox multivariate analysis confirmed the independent prognostic value of LI and tumour size on disease-free survival.

Key words: proliferative activity, breast cancer
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INTRODUCTION

TUMOUR PROLIFERATIVE activity, determined according to various assays, has been demonstrated to be of prognostic relevance in several human cancers [1], and in particular for operable breast cancer patients [2, 3]. One of the major criticisms against these studies is directed at a potential source of error: the variability of site to site tumour proliferative activity [4]. Moreover, this tumour variability has been demonstrated not to be artefactual and is of greater magnitude than the intra-operator counting error [5, 6].

While genetic and non-genetic factors [7, 8] have frequently been suggested to explain the heterogeneity of regional tumour cell kinetics, scarce discussion has been directed to the biological and clinical significance of these intratumour differences.

One of the first methods frequently utilised to predict the breast cancer cell proliferation rate was the *in vitro* uptake of the nucleic acid precursor ^{3}H-thymidine by tumour fragments, and

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this has been demonstrated to be an independent prognostic indicator for node-negative breast carcinoma patients [9–12].

The present retrospective study was undertaken to verify whether heterogeneity of breast cancer proliferative activity as determined by various techniques for quantifying ^{3}H-thymidine labelling, is related to other common tumour characteristics and the clinical outcome in a series of T_{1-2} node-negative primary breast cancer patients. The methods used to evaluate tumour heterogeneity were: the mean tumour labelling index (LI), the highest labelling value (LI-max) with reference to a specific tumour area, and the extent of the labelling variations (LI–CV) among several intratumour samples (i.e. intratumour cell kinetic heterogeneity expressed as coefficient of variation).

MATERIALS AND METHODS

A consecutive series of 179 patients with node-negative primary invasive breast carcinomas ($T_{1-2}N_0M_0$ according to UICC criteria) treated by radical surgery in the Senology Department of the Oncology Institute of Bari, Italy between 1985 and 1991 were admitted to the present study. Patients were classified as postmenopausal when more than 2 years had elapsed from last menses.

After surgical removal, fresh pathological material was immediately analysed by the pathologist. The primary tumour was isolated from surrounding tissue, and one fragment was used for routine histological analysis and cytohistological tumour differentiation grading according to Fisher [13]. From the remaining primary tumour material, a 3–5 mm thick slice was excised at the equatorial plane (central surface) of the tumour, immersed in salt solution, and transferred to the laboratory for *in vitro* assays of hormone receptor content, and LI and DNA content by flow cytometry.

Sampling procedure and autoradiographic proliferative activity study

A total of 3–8 (mean: 5) samples of approximately 1 mm^3 each were randomly selected from different areas of the equatorial slice of each tumour, and subjected to autoradiography procedures within 1 h after surgery. After pooling, the samples were immersed in tissue culture medium and incubated with ^{3}H-Tdr (specific activity, 5 Ci/mmol) for 1 h at 37°C. At the end of incubation, tumour tissue samples were fixed in Bouin's solution and embedded in paraffin, according to previously reported procedures [14]. Successively, one 5 μm section from each sample was prepared and placed on a glass slide for preparation of autoradiographs. Autoradiographic stripping film (Kodak AR10; Eastman Kodak Co., Rochester, NY, U.S.A.) was then applied on the slide. After an exposure time of 10 days at 4°C, the autoradiograms were developed in Kodak D19b and fixed in Kodak F5 solutions. Finally, the autoradiographic slide (in which each tumour sample is represented by one slice) was prepared and scanned by an expert operator, with an optical microscope under a high and low power field. When the samples were small enough to allow the precursor to completely penetrate, counting was performed throughout all the section; otherwise, the counting was limited to the periphery of the section (up to 80 microns in depth). Subsequently, the following characteristics were evaluated.

(a) the presence of sufficient neoplastic cellularity in the section of each tumour sample; a tumour sample was considered evaluable when at least 200 tumour cells were analysed in autoradiographic section (according to this criteria, 641/907 of all tumour samples were evaluable, with a median number of evaluated neoplastic cells per each sample section of 371);

(b) the number of ^{3}H-Tdr labelled and unlabelled neoplastic cells in each tumour sample. Since ^{3}H-Tdr labelled cells were distributed heterogeneously throughout the various tumour samples, tumour proliferation characteristics were quantified according to the following formulas:

(i) mean tumour proliferation was calculated by counting the ^{3}H-Tdr labelled and unlabelled tumour cells from all samples of the same tumour, and expressed as the percentage of overall labelled tumour cells with respect to the total tumour cell number (LI); each tumour was considered evaluable when at least 2000 tumour cells from all the samples of that tumour were counted;

(ii) after the preliminary evaluation of cell labelling in selected microscope fields (100 ×), the within sample LI with the highest density of ^{3}H-Tdr labelled cells was considered the maximum LI value of each tumour (LI-max);

(iii) the intersample spread of the LI values for each tumour was computed by the calculation of coefficient of variation of LI values (LI–CV = standard deviation mean × 100) and was considered as the direct expression of intratumour cell proliferative heterogeneity.

The laboratory where the autoradiographic procedures were performed participated in the Programme of Quality Control for LI coordinated by the Italian Society of Basic and Applied Cell Kinetics [15].

Hormone receptor dosage

A sample of tumour material was frozen in liquid nitrogen and stored at −80°C for no more than 1 month. Tumour cell cytosol for hormone receptor analysis was obtained by dissolving powdered tumour biopsy tissue with a pH 7.4 buffer containing 1.5 mM EDTA, 10 mM monothioglycerol, 10 mM sodium molybdate; the solution was then centrifuged at 105 000 ***g*** for 60 min and the supernatant fraction was eluted. Oestrogen (ER) and progesterone (PgR) receptors were successively assayed by the DCC method according to the European Organization for Research on Treatment of Cancer [16]. The cut-off value for ER and PgR analyses was 10 fmol/mg cytosol protein. The laboratory, where hormone receptor dosage was performed, participated in the European Programme of Quality Control for Hormone Receptor Assay, coordinated by the Italian *ad hoc* committee [17].

DNA content by flow cytometry

Flow cytometry (FCM) analysis was performed on cell suspensions from 93 primary tumours obtained by mechanical disaggregation of tumour material frozen at −80°C. After staining with 4′–6′-diamidino-2-phenylindole dihydrochloride (1 μg/ml; DAPI, Serva), samples were analysed on a Partec Pas II mercury lamp flow cytometer (Partec, Switzerland). The results were expressed as the frequency distribution of DNA cell content; normal DNA histograms were characterised by a peak corresponding to the DNA content of G_{0-1} diploid cells. Clonal DNA abnormality (aneuploidy) was identified by the presence of an accessory peak generally shifted to the right of the G_{0-1} diploid peak. The coefficient of variation of each DNA histogram was calculated from the G_1 peak according to the formula: CV = the width of the peak of the half maximum × 100 divided by the model channel number of the peak. Mean CV of the series was 4.8%. The percentage of aneuploid cells was defined as the

percentage of cells in the G_{0-1} aneuploid peak with respect to those in the G_{0-1} diploid peak. Diploid tumours were considered as those with 0% aneuploid cells. The laboratory where DNA FCM analyses were performed participated in the quality control study of the Italian Group of Cytometry for DNA content measurement [18].

Clinical study

A total of 101 of 179 patients with the following criteria were entered in the clinical study: radical breast surgery (radical mastectomy or quadrantectomy and axillary dissection plus radiotherapy) for invasive breast carcinoma performed before December 1989, no postsurgical systemic therapy, and available follow-up information from the outpatient clinic with reference to periodical *ad hoc* clinical, haematological and instrumental examinations.

Disease-free interval and cancer-related overall survival of patients were calculated from the date of surgery. Median follow-up of the series was 60 months. Two non-cancer related deaths were observed during the follow-up period, and these data were regarded as censored.

Statistical analysis

The statistical analysis was carried out using BMDP software (Los Angeles, CA, U.S.A.). A univariate analysis was performed to study the distribution curves of LI, LI-max and LI–CV values. Median values of the series have been used to classify each case as having high or low LI, LI-max and LI–CV for comparison with the other biological parameters and clinical follow-up.

Fisher's test and the chi-square test were used to verify the association between two or more categorised variables. The Mann–Whitney non-parametric test for independent groups of data and the Kruskal–Wallis non-parametric test for multiple comparisons were utilised to correlate proliferative activity values to other clinical and biological tumour characteristics.

To quantify the relationships between various continuous biological characteristics, a linear regression model was computed which was tested for significance. In order to analyse the independent contribution of various factors on disease-free survival and cancer-related overall survival, a final Cox multivariate analysis was performed on previously categorised continuous variables. Survival curves for breast carcinoma patients were estimated according to Kaplan-Meier, and significant differences were analysed by the log-rank test (significance limit: ≤ 0.05).

RESULTS

Biological relationships

Of the 179 patients with evaluable autoradiographic assays, 75.2 (42%) were pre/perimenopausal, tumour diameter was less than 1 cm in 4%, between 1 and 2 cm in 60% and larger than 2 cm in 36% of cases. With regard to cytohistological tumour differentiation, 15, 38 and 47% of cases were classified as G_1, G_2 and G_3, respectively. Hormone receptor positive cases were 61% for ER and 58% for PgR; 81% (75/93) of tumours with DNA content evaluated by FCM were aneuploid.

LI had an exponential distribution with a median value of 2.4% (range: 0.01–18.9; mean: 2.9%). The median LI value was not related to tumour size; it was slightly higher in premenopausal than in postmenopausal patients (2.6 versus 2.3%; P = n.s.) in grade 3 tumours when compared with those of grade 1–2 (2.9 versus 2.1%; P = n.s.), and in aneuploid compared with diploid tumours (2.8 versus 2.2%; P = n.s.). Conversely, median LI was significantly lower in ER-positive versus ER-negative cases (2.0 versus 2.9%; $P < 0.003$) and in PgR-positive versus PgR-negative cases (1.9 versus 3.0%; $P < 0.003$).

The frequency distribution of the LI values from the tumour samples with the highest ^{3}H-thymidine labelling (LI-max) demonstrated a mean value of 5.2%. Median value of the series was 4.3%, which was significantly lower in ER-positive (LI-max of ER+ versus ER− − 3.7% versus 5.5%; $P < 0.02$) and in PgR-positive tumours (3.7% for PgR+ versus 5.5% for PgR−; $P < 0.008$). There was no significant correlation between LI and tumour size (median LI-max in T_1 versus T_2 tumours = 4.3 versus 4.3%; P = n.s.) or G characteristics (median LI-max in G_1, G_2 and G_3 subgroups = 4.0 versus 4.3 versus 4.7%, respectively; P = n.s.). Lastly, LI-max did not statistically vary in tumours for which a varying number of tumour samples was evaluated (3–8 for each tumour). A statistical significance between the LI and LI-max values (overall agreement: 91%, $P < 0.001$) with a linear correlation (correlation coefficient = 0.92, $P < 10^{-6}$) was also demonstrated (Figure 1).

With regard to LI–CV, the frequency distribution was near normal (mean = 49, median = 49, mode = 35, SD = 27.4). The LI–CV was independent of tumour size, grade, ploidy, ER and PgR status, low and high LI categories and low and high median number of tumour cells evaluated per sample (cut-off, the median value of the overall series: 371 cells per sample section). Concerning technical aspects, the mean number of cells evaluated per section was not significantly different in tumours with high or low LI–CV values while LI–CV was directly dependent on the number of tumour samples analysed for each tumour (mean LI–CV in cases with $\leq 3, 4, 5$ and > 5 samples evaluated: 32, 49, 68 and 72%, respectively, $P < 0.001$). Furthermore, an inverse linear correlation was evident between LI and LI–CV values (coefficient of correlation = −0.410; $P > 0.001$; Figure 2), but no association was noted with ER or PgR levels. Finally, the LI–CV was independent both of the percentage of aneuploid cells present in the tumour and the presence of within-tumour multiclonality, as evidenced by analysis of DNA content by flow cytometry (data not shown).

Clinical study

The group of 101 node-negative breast cancer patients entered in the clinical study (the criteria for exclusion of 78 patients from clinical analysis has been already reported in the Methods Section) was composed of 93 cases of radical mastectomy and 8

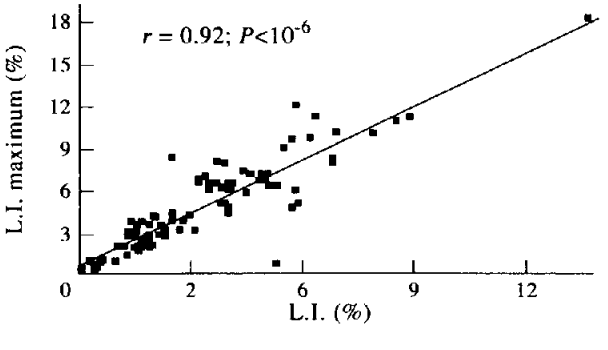

Figure 1. Correlation between LI and LI-max values in a series of 179 node-negative breast cancer patients (correlation coefficient = 0.92, $P < 10^{-6}$).

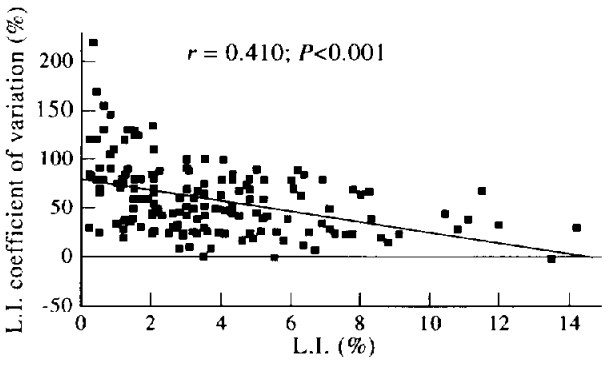

Figure 2. Correlation between LI and LI-CV values in a series of 179 node-negative breast cancer patients.

cases of quadrantectomy+axillary dissection+local radiotherapy; the mean number of removed axillary lymph nodes was 17.5 (range 9–37). A total of 21 relapses (5 local relapses and 16 distant metastases, of which 9 were in bone, 5 in the viscera and 2 in multiple sites) and 11 cancer-related deaths were observed after 80 months of follow-up.

The frequency of disease relapse and probability of disease-free survival in relation to clinical-pathological and tumour proliferative characteristics is reported in Table 1 (median values of the series were used to classify patients as low or high LI, and identify LI-max and LI-CV). Disease-free survival was significantly different in patients with T_1 versus T_2 tumours (84 versus 64%, $P < 0.04$ by log rank analysis, Figure 3b) and with low or high LI (86 versus 61%, $P < 0.03$ by log rank analysis, Figure 3a), but not in patients with different menopausal status, oestrogen or progesterone receptor status, low or high LI-max value (81 versus 69%, respectively) or low or high primary tumour LI–CV (75 versus 77%, respectively). Cox multivariate analysis (Table 2) confirmed the independent prognostic value of LI (relative risk: 1.877; confidence interval (CI) 1.134–5.560) and tumour size (relative risk: 3.868; CI: 1.166–12.837) on disease-free survival.

Table 1. Univariate analysis of influence of prognostic factors on disease free survival

	Number of cases	Actuarial DFS (80 mos)	*P* value
Menopause status			
Pre-	35	73%	
Post-	66	93%	n.s.
Tumour size			
T_1	46	84%	
Receptor status			
T_2	55	64%	0.04
ER−	36	78%	
ER+	65	71%	n.s.
PgR−	38	73%	
PgR+	63	80%	n.s.
^{3}H-Tdr-LI			
Low	51	86%	
High	50	61%	0.03
^{3}H-Tdr-LI-max			
Low	51	81%	
High	50	69%	n.s.
^{3}H-Tdr-LI-CV			
Low	51	75%	
High	50	77%	n.s.

Cancer-related overall survival at 80 months of follow-up was statistically related to LI (92 versus 78% for patients with low or high LI tumours, respectively; $P < 0.05$; Figure 4a) and tumour size (94 versus 76% for patients with small or large tumours, respectively; $P < 0.05$; Figure 4b) but not to LI-max value (89 versus 84% in patients with high or low LI-max, respectively) or LI–CV (86 versus 86% in patients with low or high LI–CV, respectively). The relatively short period of follow-up did not permit a multivariate analysis of cancer-related overall survival.

DISCUSSION

Proliferative activity heterogeneity of human breast cancer has been demonstrated to be of substantial and appreciable magnitude with different laboratory techniques, such as flow cytometry [19], immunohistochemistry [20] and ^{3}H-thymidine autoradiography [11]. Nevertheless, the quantification of this tumour characteristic is routinely performed only on one tumour sample and subsequently related to clinical outcome of breast cancer patients in clinical studies without any comment of how inaccurate or unrepresentative it might be.

In particular, the cell counts and intratumour variability of tumour proliferative activity as determined by ^{3}H-thymidine incorporation assay has been discussed by Lambert [5] who solicited further studies to determine whether mean tumour proliferative activity or the highest value from a specific tumour area might be better indicators of growth rate and clinical behaviour of the disease. Only a few studies have focused their attention on the biological and clinical significance of tumour cell kinetics variability.

Meyer and Wittliff [21], performing ^{3}H-Tdr assay on multiple samples from large primary breast tumours, classified the majority of cases considered as heterogeneous. In their study, the heterogeneity rate did not differ significantly in patients with respect to age or tumour size, whereas heterogeneity tended to be more frequent in cases with higher LI and in cases with lower ER and PgR levels. Ferno and associates [22], analysing two fragments from each primary breast tumour, verified that the prognostic value of DNA measurement might be enhanced by a larger sampling procedure. In a small clinically heterogeneous breast cancer series, the author concluded that patients whose within-tumour S-phase fraction varied significantly seemed to have a poor prognosis. In a series of 52 human breast carcinomas, Going and associates [23] utilised different methods for counting bromodeoxyuridine immunostained breast cancer cells, and found that the ratio between the labelling of 'high labelling' fields and 'random' fields was extremely variable. Wintzer and coworkers [24], focusing their interest on tumour growth as determined by Ki-67 nuclear antigen expression, investigated whether the method of quantifying Ki-67 positive cells influenced the correlation with prognosis. By evaluating the mean Ki-67 labelling of the tumour and the area with the highest labelling density, they demonstrated that both quantifying procedures were highly correlated, but did not demonstrate an independent clinical effect for the highest Ki-67 value with multivariate analysis. Finally, Aaltomaa and associates [25] determined proliferating nuclear cell antigen (PCNA) immunolabelling in a heterogeneous series of breast carcinomas, verifying that the best clinical method to evaluate the antigen expression was that which considered the percentage of nuclei with intense

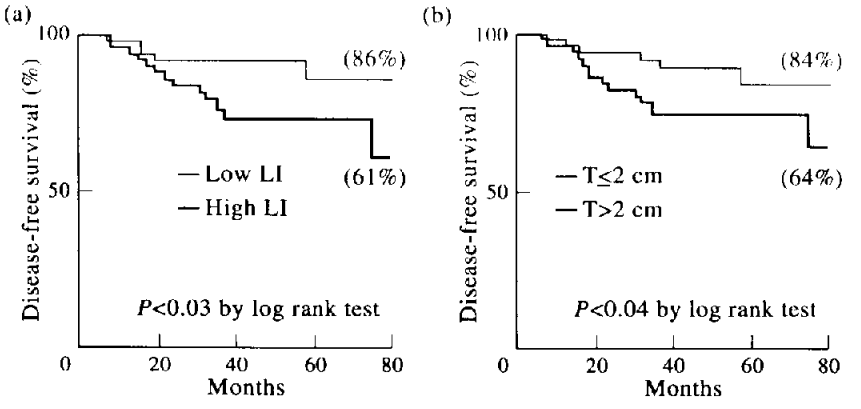

Figure 3. Disease free survival in a series of 101 node-negative breast cancer patients according to tumour proliferative activity (a) and tumour size (b).

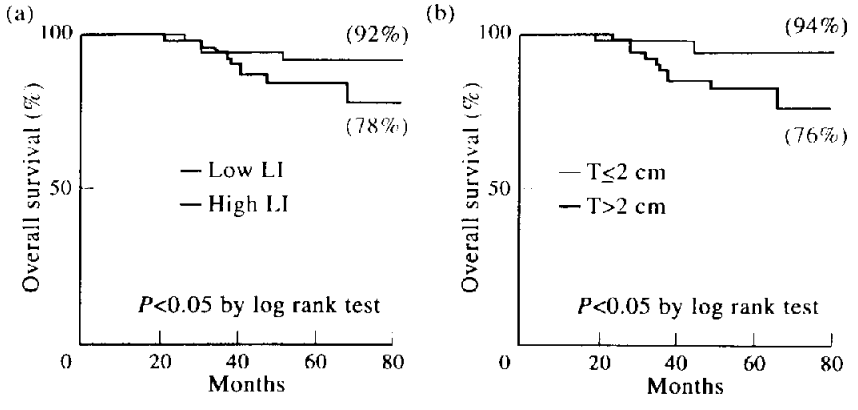

Figure 4. Overall survival in a series of 101 node-negative breast cancer patients according to tumour proliferative activity (a) and tumour size (b).

Table 2. Cox multivariate analysis influence of prognostic factors on disease free survival

	Relative risk	Confidence interval	*P* value
Menopausal status			
pre- versus post-	0.518	0.184–1.458	0.21
T category			
T>2 cm versus *T*≤2 cm	3.868	1.166–12.837	0.017
Cytohistological grade			
G1 versus G2 versus G3	1.057	0.543–2.057	0.87
^{3}H-Tdr-LI			
high versus low	1.877	1.134–5.560	0.046
ER			
negative versus positive	1.236	0.634–2.850	0.38

positivity in a sufficient number of consecutive microscopic fields from the tumour area of highest labelling.

Our study considered an homogeneous and sufficiently large group of T_{1-2} N_0 M_0 patients treated with radical surgery; the clinically followed patients did not receive further systemic therapy after surgery. Our series showed a similar LI median value, hormone receptor status and tumour size to that of previously reported studies [9–12].

The evaluation of the LI-max value from the tumour area with the highest labelling density was strictly related to the mean tumour proliferative activity value (Figure 1) and inversely associated with common markers of tumour differentiation (ER/PgR status, nuclear differentiation grade). It must be noted that LI-max was independent of technical aspects, such as number of samples and/or cells evaluated for each tumour. In conclusion, the mean LI value and the LI-max value from the tumour sample with the highest tumour proliferative activity seem to be part of the same biological phenomenon which is, in turn, related to the hormonosensitivity of the tumour. It might be hypothesised that tumour growth is sensitive to the potential action of regional control factors able to modulate the overall tumour cell kinetics. These biological laboratory data seem to be confirmed by the clinical results of our study; patients with a high LI-max value demonstrated the same tendency for a poor prognosis as tumours with a high LI, but the prognostic role seems to be better correlated to the mean LI value as determined by counting at least 2000 cancer cells from the various tumour samples of each tumour.

The LI–CV values demonstrated a near-normal frequency distribution with a mean value slightly higher than that reported by other authors [15] and were clearly unrelated to the tumour biological characteristics considered; in fact, LI–CV seemed to be influenced only by technical aspects, such as number of tumour fragments evaluated. In addition, the DNA tumour multiclonality as determined by FCM ploidy and the presence of different percentages of aneuploid cells within the same tumour were not related to primary tumour cell kinetic heterogeneity.

In contrast to the data of Meyer and Wittliff [21] who, in a series of larger tumours, utilised a different cell kinetic quantification concept to classify cases as heterogeneous, our data showed that the tendency of primary breast cancer for a higher regional cell kinetic variability is inversely related to its LI value which concurs with the data of Lambert [5]. Slow proliferating tumours are frequently confirmed to be a heterogeneous entity in which clones with different biological aggressiveness coexist; conversely, fast cell proliferation within the primary tumour seems to represent an obstacle for the coexistence of kinetically different cell clones. From the clinical point of view, high or low tumour LI–CV values were not able to individualise women with different DFS and cancer-related overall survival. However, before drawing definitive conclusions about the clinical and biological role of LI–CV, our data indicate that the number of samples selected from primary tumours should be increased in order to reach a more stabilised and representative LI–CV value.

In conclusion, the tendency of breast cancer to be highly heterogeneous in terms of proliferative activity must still be biologically and clinically interpreted, and future efforts should be addressed to studying genetic factors which are important in the early phases of the carcinogenesis process. In our series of node-negative breast cancer patients, mean tumour proliferative activity characteristics (i.e. ^{3}H-Tdr-LI) seem to be more important for clinical outcome than the presence of a small cell clone with a high proliferative rate or the tendency of each tumour to be kinetically heterogeneous. This observation is consistent with the well-established prognostic role of LI, calculated as the mean value from several tumour samples, which emerges from the literature [9–12]; an impressive similarity has been reported concerning the capability of this biological variable to identify patients with node-negative breast cancer and significantly different disease-free and overall survival.

These data constitute the basis of a prospective clinical trial concerning node-negative breast cancer patients, recently activated in our institute [26], in which patients with a high risk of relapse who are prospective candidates for adjuvant therapy are properly selected according to their LI primary tumour characteristics.

1. Camplejohn RS, Brock A, Barnes DM, *et al.* Ki-S1, a novel proliferative marker: flow cytometric assessment of staining in human breast carcinoma cells. *Br J Cancer* 1993, **67**(4), 657–662.
2. Narita T, Funahashi H, Satoh Y, Takagi H. Proliferating cell nuclear antigen immunostaining in breast cancer and its relation to prognosis. *Jpn J Clin Oncol* 1993, **23**(1), 20–25.
3. Allred CD, Clark GM, Elledge R, *et al.* Association of p53 protein expression with tumor cell proliferation rate and clinical outcome in node-negative breast cancer. *J Natl Cancer Inst* 1993, **85**(3), 200–206.
4. Smallwood JA, Cooper A, Taylor I. The errors of thymidine labeling in breast cancer. *Clin Oncol* 1983, **9**, 331–335.
5. Lambert M. Tritiated thymidine labelling *in vitro* of human cancer of the breast: counting error and sampling error. *Eur J Cancer Clin Oncol* 1986, **22**(7), 781–785.
6. Meyer JS, McDivitt RW. Reliability and stability of the thymidine labeling index of breast carcinoma. *Lab Invest* 1986, **54**(2), 160–164.
7. Chen LC, Kurisu W, Ljung BM, *et al.* Heterogeneity for allelic loss in human breast cancer. *J Natl Cancer Inst* 1992, **84**(7), 506–510.
8. Schmidt UR, Lin PS, Mikkelsen RB, Monroe MMT. Proliferative rates of cloned malignant mammary epithelial cells as a measure of clonal heterogeneity in human breast carcinomas. *J Natl Cancer Inst* 1986, **77**(5), 1001–1007.
9. Silvestrini R, Daidone MG, Valagussa P, DiFronzo G, Mezzanotte G, Bonadonna G. Cell kinetics as a prognostic indicator in node-negative breast cancer. *Eur J Cancer Clin Oncol* 1989, **25**(8), 1165–1171.
10. Meyer JS, Province M. Proliferative index of breast carcinoma by thymidine labeling: prognostic power independent of stage, estrogen and progesterone receptors. *Breast Cancer Res Treat* 1988, **12**, 191–204.
11. Hery M, Gioanni J, Lalanne CM, Namer M, Courdi A. The DNA labeling index: a prognostic factor in node-negative breast cancer. *Breast Cancer Res Treat* 1987, **9**, 207–211.
12. Tubiana M, Pejovic MH, Koscielny S, Chavaudra M, Malaise E. Growth rate, kinetics of tumour cell proliferation and long-term outcome in human breast cancer. *Int J Cancer* 1989, **44**, 17–22.
13. Fisher ER, Redmond C, Fisher B. Histologic grading of breast cancer. *Pathol Ann* 1980, **1**(15), 239–251.
14. Paradiso A, Tommasi S, Mangia A, Lorusso V, Simone G, De Lena M. Tumor-proliferative activity, progesterone receptor status, estrogen receptor level and clinical outcome of estrogen receptor-positive advanced breast cancer. *Cancer Res* 1990, **50**, 2958–2962.
15. Silvestrini R, De Lena M, Paradiso A, on behalf of SICCAB. Feasibility and reproducibility of the ^{3}H-thymidine labeling index in breast cancer. *Cell Proliferation* 1991, **24**, 437–445.
16. EORTC Breast Cooperative Group—Revision of the Standards for the assessment of hormone receptors in human breast cancer. *Eur J Cancer* 1980, **16**, 1513–1515.
17. Piffanelli A, Pellizzola D, Giovannini G, Catozzi L, Faggioli L, Giganti M. Characterization of laboratory working standards for quality control of immunometric and radiometric estrogen receptor assays. Clinical evaluation on breast cancer biopsies. *Tumori* 1989, **75**, 550–556.
18. Tirindelli Danesi D, Spanò M, Altavista P. Quality control of the Italian group of cytometry on flow cytometry cellular DNA content measurements. *Cytometry* 1993, **14**, 576–583.
19. Askensten GU, von Rosen AK, Nilsson RS, Auer GU. Intratumoral variations in DNA distribution patterns in mammary adenocarcinomas. *Cytometry* 1989, **10**, 326–333.
20. Marzullo F, Simone G, Labriola A, Paradiso A, Rella C, Petroni S. Il Ki67 come marker istochimico dell'attivita' proliferativa cellulare nel carcinoma mammario. *Pathologica* 1988, **80**, 279–285.
21. Meyer JS, Wittliff JL. Regional heterogeneity in breast carcinoma: thymidine labelling index, steroid hormone receptors, DNA ploidy. *Int J Cancer* 1991, **47**, 213–220.
22. Ferno M, Baldetorp BO, Ewers SB, *et al.* One or multiple samplings for flow cytometric DNA analyses in breast cancer—prognostic implication? *Cytometry* 1992, **13**, 241–249.
23. Going JJ, Stanton PD, Cooke TG. Influences on measurement of cellular proliferation by histology and flow cytometry in mammary carcinomas labeled *in vivo* with bromodeoxyuridine. *Anat Pathol* 1993, **100**(3), 218–222.
24. Wintzer HO, Zipfel I, Schulte-Monting J, *et al.* Ki-67 immunostaining in human breast tumors and its relationship to prognosis. *Cancer* 1991, **67**(2), 421–428.
25. Aaltomaa S, Lipponen P, Papinaho S, Syrjanen K. Proliferating-cell nuclear antigen (PC10) immunolabelling and other proliferation indices as prognostic factors in breast cancer. *J Cancer Res Clin Oncol* 1993, **294**, 288–294.
26. Paradiso A, Mangia A, Barletta A, *et al.* Randomized clinical trial of adjuvant chemotherapy in node negative patients with fast proliferating breast cancer. *Drugs* 1993, **45**(2), 68–74.

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