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Cell Kinetics as a Prognostic Indicator in Node-negative Breast Cancer

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Abstract—*The consistency of the prognostic role of cell kinetics (evaluated as the* [^{3}H]*thymidine labeling index, LI) over a period of years has been assessed in* 354 *patients with resectable node-negative breast cancer subjected only to Halsted or modified radical mastectomy. The risk of disease recurrence and death was proportional to LI values and the pattern was superimposable, regardless of menopausal status, in the two consecutive case series entered in this retrospective study. In particular, tumors with high LI* (>2.8%) *had a higher* 6*-year probability* (41% *vs.* 25%, $P < 0.0001$) *of manifesting local–regional and distant metastases and of dying* (19% *vs.* 5%, $P = 0.0005$) *as compared to tumors with low LI. In tumors with high LI the risk of relapse within the first* 2 *years from mastectomy was twofold compared to that of tumors with low LI. Multiple regression analysis showed that LI also retained its prognostic significance in both relapse-free and overall survival when tumor size and estrogen receptor status were considered. The present findings confirm that LI can substantially contribute to the selection of high risk node-negative patients who could be candidates for adjuvant chemotherapy.*

INTRODUCTION

DURING the last 10 years, treatment strategies for resectable mammary carcinoma have essentially been based on breast saving procedures for tumors of limited dimensions [1, 2] and on postoperative systemic therapy for high-risk patients [3, 4]. Nodal status has almost always represented the key prognostic indicator to select patients for systemic adjuvant treatment.

Retrospective clinical studies have consistently shown that approximately three-fourths of women with positive axillary lymph nodes present new disease manifestations within 10 years when treated with local–regional therapy. In addition, the number of involved axillary nodes has proven to be inversely related to both the relapse-free and total survival rates [5, 6]. Approximately one out of every four women with histologically negative axillary nodes does relapse within 10 years from surgery alone. Therefore, while nodal status remains an important prognostic factor, other reliable and reproducible variables need to be studied to identify patients at high risk of treatment failure following local–regional modality alone.

Several morphologic and endocrine criteria have been utilized to classify breast cancers. They include histologic, nuclear, and mitotic grading, tumor necrosis, vascular invasion, and steroid receptors. Unfortunately, not all these variables have turned out to be either reproducible or consistent over time [7]. Breast cancer heterogeneity as well as different treatment strategies and methodological approaches may in part be responsible for discrepancies in results from different case series.

Tumor cell kinetics, evaluated by the *in vitro* [^{3}H]thymidine labeling index, has been studied for a long period of time in various case series and has been consistently found to represent a prognostic variable in different pathologic stages of breast cancer [8–13]. However, the potentials and limits of a prognostic variable are best assessed on a large and homogeneously treated series of patients, possibly subjected to local–regional treatment alone. In addition, a rigorous quality control and an assessment of the consistency of the observed findings over a period of years are especially important when dealing with a biologic marker [7].

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The aim of the present report is to update our previous findings [14, 15] on a larger case series with operable breast cancer and histologically negative axillary lymph nodes.

PATIENTS AND METHODS

Patient characteristics

Starting in July 1972 at the Milan Cancer Institute, thymidine labeling index (LI) was assayed in patients with primary resectable breast cancer with an increasing accrual rate over the years.

For the purpose of the present analysis, only patients with resectable breast cancer (T1–T3a) subjected to either radical or modified radical mastectomy, with histologically proven negative axillary nodes following complete dissection (median number of nodes examined, 14) and with the concomitant determination of LI and estrogen receptors (ER) were considered. In addition, at the time of mastectomy there was no evidence of distant metastases by conventional clinical, radiological and radioisotopic methods, and no form of postoperative therapy, either radiological or drug treatment, was administered until primary treatment failure was documented. From November 1974 to December 1983, a total of 354 women were eligible for this analysis, with a median follow-up time of 62 months. Following pathological examination, 175 women or 49% presented primary lesions measuring 2 cm or less in their greatest dimension, whereas 179 women, or 51%, presented tumors measuring more than 2 cm in the largest diameter. As far as menopausal status was concerned, patients who were either actively menstruating or in whom cessation of menses occurred less than 1 year prior to diagnosis were categorized as premenopausal (210 or 59%) whereas all other women were considered as postmenopausal (144 or 41%). Median age for the entire series was 53 years (range 28–79). The distribution of main patient and tumor characteristics as a function of cell kinetics is reported in Table 1.

Follow-up

As previously mentioned, following mastectomy no other anti-tumor therapy was applied until new disease manifestation was documented. Patients were examined in the outpatient clinic of our Institute at 6-month intervals during the first 5 years and at 12-month intervals thereafter; the disease status was assessed through physical examination, chest roentgenogram and skeletal survey. In this series of patients, routine bone scans were not planned during follow-up. Once a new disease manifestation was documented, salvage therapy was left to the discretion of the treating physician.

Determination of labeling index

LI was determined on fresh tumor material immediately following surgery. After removal of fat and necrotic areas, the neoplastic tissue was minced into fragments of a few cubic millimeters. From 15 to 20 fragments were placed in 199 medium with 20% fetal calf serum, antibiotics (100 U penicillin and 100 μg streptomycin per ml), and 6 μCi/ml of [^{3}H]thymidine (sp. act. 5 Ci/mmol), and were incubated for 1 h with agitation at 37°C. Medium components came from Flow Laboratories (Irvine, U.K.), and the radioactive precursor was purchased from the Radiochemical Centre (Amersham, U.K.). At the end of incubation, fragments were fixed in Bouin's solution, dehydrated in alcohol, and embedded in paraffin. Autoradiographs (ARG) were performed on histologic sections according to the stripping film (Kodak AR10) technique. After an exposure time of 10 days at 4°C, autoradiograms were developed in Kodak D-19b for 5 min at 18°C and fixed in Kodak F5. The samples were stained with hematoxylin and eosin at 4°C. The LI was determined by scoring a total of 3000–10,000 cells from different specimens of the same tumor. When the specimens were small enough to allow the radioactive precursor to completely penetrate, counting was performed through all sections. Otherwise, the counting was limited to the periphery of the

Table 1. Main patient and tumor characteristics as a function of [^{3}H]*thymidine LI*

	Low LI		High LI	
	No. of cases	%	No. of cases	%
Menopause				
Premenopausal	115	62.2	95	56.2
Postmenopausal	70	37.8	74	43.8
Tumor size				
≤2 cm	106	57.3	69	40.8
>2 cm	79	42.7	100	59.2
ER status				
Positive	154	83.2	95	56.2
Negative	31	16.8	74	43.8

section. No threshold for considering labeled nuclei was necessary because the background was always less than 1.5 grains per 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 labeled cells was 20 [8, 15].

Determination of cytoplasmic estrogen receptor content

Tumor samples obtained from surgery were immediately placed in plastic vials, frozen at −25°C and stored at −70°C. The ER content was assayed according to the EORTC method [16] by using the dextran-charcoal technique and the evaluation of the receptor concentration was carried out according to Scatchard [17]. Tumors with receptor concentrations below or equal to 10 fmol/mg cytosol protein were considered as receptor negative (ER−) whereas tumors with receptor content above such a value were considered as receptor positive (ER+). According to the aforementioned criterion, 249 (70.3%) and 105 (29.7%) tumors were considered ER+ and ER−, respectively.

Statistical analysis

Relapse-free survival (RFS) and overall survival were computed starting from the date of surgery by means of the Kaplan–Meier product-limit method [18].

When LI was utilized as a continuous variable, estimates of the cumulative hazard at 6 years of tumor relapse or death for each value of LI were computed by means of Cox's model [19]. LI was also utilized as a qualitative variable, and for this analysis the median value of 2.8% determined on 1000 patients with operable breast cancer was used as cutoff to define slowly and rapidly proliferating tumors. According to this criterion, 185 tumors or 52% were classified as slowly proliferating (low LI) and 169 (48%) as rapidly proliferating (high LI) tumors.

The log-rank test was used to assess differences between the various patient subsets [20]. The hazard function was estimated according to the method proposed by Simes and Zelen [21]. Six-month intervals were chosen to calculate the hazard estimates which were smoothed in plots by using medians from three to five branches. To assess the joint effects of LI, tumor size and ER status (all as discrete variables) on RFS and survival Cox's multiple regression model [19] was used.

RESULTS

Labeling index as a continuous variable

In the overall series of 354 patients, the individual LI values and cumulative risk of relapse at 6 years appeared to be proportional (Fig. 1a). This finding held true both for premenopausal and postmenopausal patients (Fig. 1b). Moreover, this relapse pattern was superimposable in the two consecutive series of patients, that is, for the 210 women entered between 1974 and 1980, and for the 144 patients operated on from 1981 (Fig. 2). Similarly, the individual LI values were directly related to the expected cumulative hazard of death, although with a less steeper slope for the small number of events (data not shown).

Labeling index as a discrete variable

Regardless of LI, the 6-year RFS in our case series was about 70%, a finding in agreement with other clinical reports on large series of patients [22, 23]. When the generally adopted criterion of low vs. high LI was used, RFS was significantly different for the two kinetic subgroups (75% vs. 59%, $P < 0.0001$) (Fig. 3a).

The difference between patterns of hazard of relapse in the two subsets of patients, with rapidly and slowly proliferating tumors respectively, is shown in Fig. 4. The first subset showed a peak of hazard within 2 years from mastectomy, which then progressively decreased, reaching the values recorded for patients having slowly proliferating tumors.

The probability of overall survival was significantly different between the two kinetic subsets

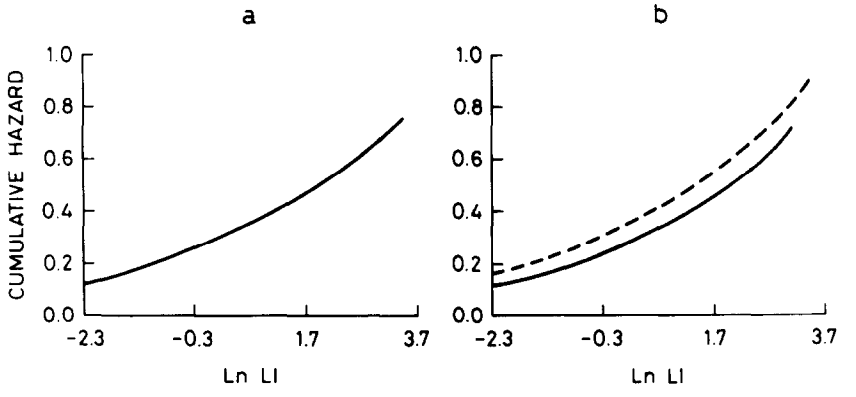

Fig. 1. Relationship between LI values and expected cumulative hazard of relapse at 6 years by Cox's model. a: Overall series; b: ——, premenopausal; -----, postmenopausal.

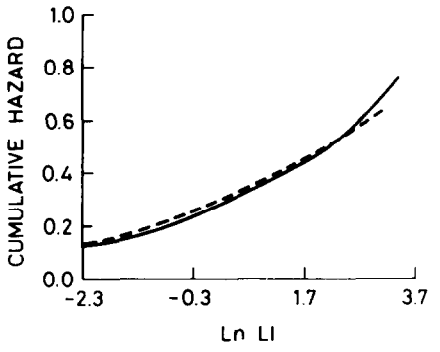

Fig. 2. Relationship between LI values and expected cumulative hazard of relapse at 6 years by Cox's model. ——, Series 1974–1980 (210 cases); -----, series 1981–1983 (144 cases).

(95% vs. 81%, $P = 0.0005$) with seven deaths in 185 patients having slowly proliferating tumors compared to 23 deaths in 169 women with rapidly proliferating cancers (Fig. 3b).

The cumulative hazard of distant metastases was significantly higher in women with rapidly proliferating tumors vs. those with slowly proliferating tumors (0.36 vs. 0.22, $P = 0.0013$) (Fig. 5). As far as site of first failure is concerned, more than half of the women showing distant metastases presented new disease manifestations in visceral sites regardless of whether the primary tumor had slowly (58%) or rapidly proliferating characteristics (60%); in contrast, women having high LI showed a relatively higher frequency of bone involvement (33% vs. 25%). However, when the total series of 354 cases was considered, patients with rapidly proliferating cancer showed a higher risk of recurring in visceral (15% vs. 8%, $P = 0.046$) and bone sites (8% vs. 3%) compared to women with slowly proliferating cancer. No difference was detected when soft tissue metastases were taken into consideration. As far as local–regional relapse was concerned, again rapidly proliferating tumors showed a higher recurrence rate compared to slowly proliferating tumors (14% vs. 5%, $P = 0.003$).

The univariate analysis of the 6-year clinical outcome as a function of LI, tumor size and ER status is reported in Table 2. When singly tested, LI, ER and tumor size were found to significantly influence RFS and overall survival. The joint prognostic effects of the three variables were investigated by means of a Cox's regression model by resorting to a step-down procedure; all variables together with the first degree interaction terms were inserted in the initial model. Owing to the fact that the contribution to the likelihood ratio tests of the three interaction terms was negligible ($\chi^2 = 2.73$ and $\chi^2 = 0.75$ for RFS and survival respectively), in the second step of the procedure only the regression relative to the main effects was inserted into the model; the results concerning the final model are reported in Table 3. LI retained its importance in predicting both RFS and overall survival also in the presence of tumor size and ER status. Relative to RFS, LI and tumor size were able to significantly influence treatment outcome whereas ER status was not statistically significant. By contrast, when the overall survival was taken into consideration, treatment outcome was significantly affected by LI and ER status and not by tumor size. It is worth mentioning that at the time of treatment failure the presence of initially positive receptor assay has influenced the selection of salvage endocrine therapy.

DISCUSSION

The results of our study confirm in a larger case series than previously reported [14, 15] that cell proliferative activity is a useful index to discriminate aggressive tumor subsets within the node-negative patients. In fact, the higher the proliferative rate of a tumor measured by the thymidine labeling index

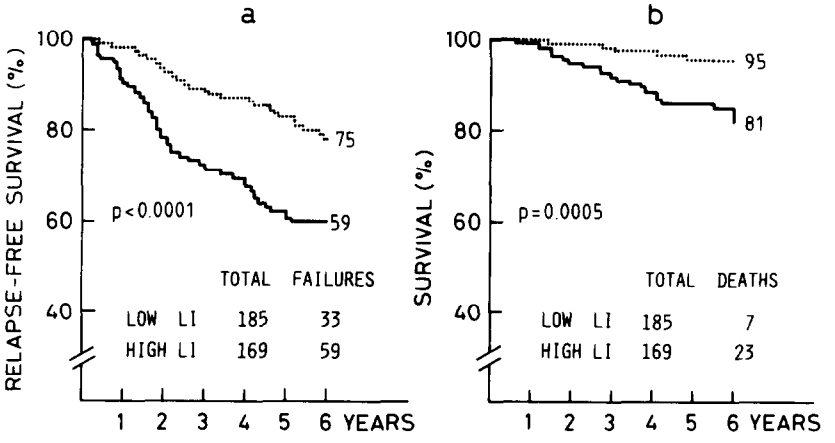

Fig. 3. Probability of relapse-free (a) and total survival (b) in relation to LI of primary tumors in a series of 354 patients with node-negative resectable breast cancer. ·····, Low LI; ——, high LI.

Table 2. Univariate analysis of clinical outcome

	No. of cases	Probability of Relapse-free survival	*P*	Probability of Overall survival	*P*
Labeling index					
Low	185	75%	<0.0001	95%	0.0005
High	169	59%		81%	
Tumor size					
≤2 cm	175	74%	0.007	94%	0.007
>2 cm	179	61%		83%	
ER status					
Positive	249	71%	0.006	92%	0.0004
Negative	105	58%		80%	

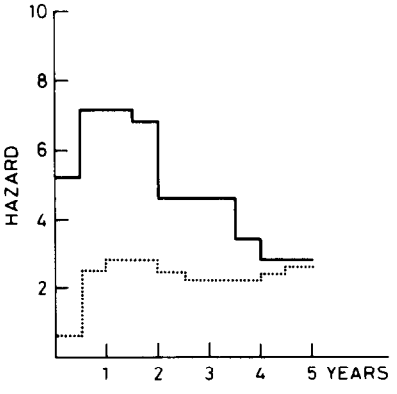

Fig. 4. Estimated hazard function of relapse (per 100 persons per 6 months) in slowly (·····) and rapidly (——) proliferating tumors.

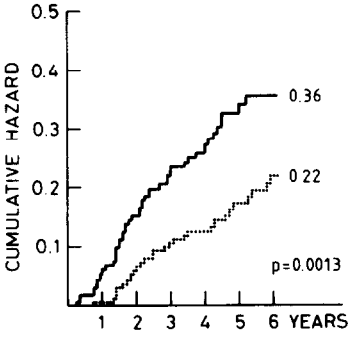

Fig. 5. Cumulative hazard of distant metastases in relation to LI of primary tumors. ·····, Low LI; ——, high LI.

is, the more likely a patient is to have early breast cancer recurrence and short-term survival.

Following all types of surgery, the classical parameters to discriminate between high- vs. low-risk patients are still represented by anatomic findings, i.e. tumor size and nodal status. During the past two decades, the number of histologically positive axillary lymph nodes was confirmed all over the world to be inversely related to treatment outcome

Table 3. Maximum likelihood estimate (β) obtained by final model of Cox's regression analysis, their standard error and significance level of Wald's statistics

Variable	β	Standard error	*P* value
Relative to relapse-free			
Labeling index			
Low	0		
High	0.7366	0.2208	0.0009
Tumor size			
≤2 cm	0		
>2 cm	0.4558	0.2175	0.0361
Relative to survival			
Labeling index			
Low	0		
High	1.1482	0.4446	0.0098
ER status			
Positive	0		
Negative	0.9633	0.3791	0.0111

regardless of whether therapy was only local–regional [22, 23] or combined with systemic adjuvant programs [5, 6].

Aside from tumor grade and steroid receptors, what are the other prognostic indicators that can enable research clinicians to refine the selection of high-risk patients within the node-negative subset? The most important indicators are currently represented by tumor cell proliferative activity, either expressed by the thymidine labeling index or flow cytometric S-phase fraction. As reported in previous studies by Tubiana *et al.* [10] and Meyer [9, 24], and particularly by our own case series on node-negative patients [15], the thymidine labeling index proved to be a strong prognostic indicator independent of tumor size and ER status. In the present

study the predictive capacity of the thymidine labeling index is confirmed on a large series of patients. It is, however, worth mentioning that, in our present series, the multiple regression analysis revealed a somewhat different influence of tumor size and ER status when relapse-free and overall survival were examined as response variables. In fact, ER status was not statistically significant when relapse-free survival was considered, but it was able to significantly influence overall survival. One possible explanation for this different result may be due to the fact that an ER-positive assay on the primary tumor contributed to select salvage endocrine therapy at the time of treatment failure. Flow cytometric analysis on frozen pulverized breast tumors to measure the proliferative rate as the percentage of cells in S-phase, and also determine ploidy, was actively investigated by the San Antonio group [25–27]. On 513 node-negative patients, Clark *et al.* [27] observed that diploid tumors with high S-phase fractions had a high risk of recurrence compared to diploid tumors with low S-phase fractions. S-Phase was not predictive of relapse in patients with aneuploid tumors.

More recent investigations also called attention to the possible prognostic relevance of oncogene amplification. The overexpression of the HER-2/*neu* oncogene [28, 29] was reported to predict short relapse-free survival and survival in stage II breast cancer patients. No data are as yet available from this research group in stage I breast cancer. Recent results [30] have also shown that monoclonal antibodies are able to detect micrometastases in the bone marrow of breast cancer patients with local disease only on staging by conventional methods and that both relapse-free survival and overall survival were significantly shorter for patients with micrometastases.

In conclusion, the results of our study with the thymidine labeling index confirm that the measure of tumor cell proliferative activity can be effectively utilized in the selection of node-negative patients who are at high risk of early relapse. To more accurately select patients to be treated with adjuvant therapies, clinicians should take into consideration a different threshold value from the generally adopted median value.

Although we do not know at present the precise interrelationship of the constellation of various prognostic discriminants, clinicians should take into consideration the fact that biological indicators are gradually replacing the old prognostic factors based only on anatomic findings, i.e. tumor size and nodal status. Further clinical studies will help to define the relative merits of the new discriminants to improve patient selection for adjuvant systemic therapy.

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