Breast Cancer Research and Treatment **38**: 289–297, 1996.

Report

Tumor proliferative activity and response to first-line chemotherapy in advanced breast carcinoma

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Key words: advanced breast cancer, chemotherapy, proliferative activity, Ki-67 immunostaining

Summary

The relationship between tumor proliferative activity and response to first-line chemotherapy and survival was investigated in 76 advanced breast cancer patients. Proliferative activity was determined by means of Ki-67 immunohistologic staining on primary tumors (55 patients) or at the relapse site (21 patients), and was classified as low (≤ 25% of stained cells) or high (> 25% of stained cells). The usual WHO response criteria were used. The median duration of follow-up was 18 months (range 3–58).

Forty-seven patients (62%) had tumors with low, and 29 (38%) had tumors with a high rate of proliferative activity. The two groups were well balanced in terms of important variables such as disease-free survival, performance status, age, menopausal status, and the type of first-line chemotherapy (anthracycline-based regimens versus cyclophosphamide-methotrexate-5-fluorouracil). The estrogen receptor (ER) content, measured by means of immunohistochemical assay, was markedly different in the two groups, with 27/47 tumors with low proliferative activity (57%) and 6/29 with high-proliferative activity (21%) being ER positive (≥ 45% of stained cells) ($p = 0.003$). Moreover, a significant difference in the metastatic pattern was also evident, with a higher incidence of bone and a lower incidence of soft tissue metastases in the group of patients with tumors with low proliferative activity ($p = 0.004$). Overall, 10/47 responses (21%: PR = 7, and CR = 3) were observed in the group with a low rate of proliferative activity, versus 14/29 (48%: PR = 9, and CR = 5) in the group with highly proliferative tumors, the difference being statistically significant ($p = 0.03$). When a multivariate analysis was performed, the only factor that retained independent prognostic significance was the predominant site of disease, particularly soft tissues ($p = 0.003$). Despite the difference in response rate, when survival analysis was performed according to the Kaplan-Meier method, no significant difference was observed in the two groups, but when the analysis was limited to responsive patients, the median survival observed in those with a low and those with a high rate of proliferation was 35 and 19 months respectively ($p = 0.02$). The same results were obtained when multivariate survival analysis was carried out using Cox's regression model. These data suggest that there is a link between tumor proliferative activity and response to chemotherapy in advanced breast cancer, and may indicate the need to use more intensive treatments in selected patients with highly proliferative tumors.

Presented in part at the Annual Meeting of the American Society of Clinical Oncology, May 14–17, 1994, Dallas, TX, USA
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Introduction

Tumors contain both proliferating cells (i.e. cells actively progressing toward mitosis in each phase of the cell cycle) and non-proliferating or quiescent cells [1]. The ratio between proliferating cells and the total number of cells in a given tumor sample (growth fraction) [2], varies greatly from one tumor type to another, epithelial cancer usually having a lower growth fraction than embryonal tumors or non-Hodgkin's lymphomas [3].

The proliferative activity of tumors can be determined in a number of ways, including the counting of the number of mitoses on a histologic section, the incorporation of tritiated thymidine (thymidine labeling index, TLI) or 5-bromo-2-deoxyuridine, the cytometric flow evaluation of the proportion of cells in the S- or S-G2 phases, or the histologic staining of monoclonal antibodies which recognize the antigens expressed only by proliferating cells [4].

The Ki-67 monoclonal antibody reacts with a nuclear antigen expressed during all of the phases of the cell cycle except G0 [5, 6], and is regarded as a marker of cell proliferation [7]. A correlation between Ki-67 positivity and the cell proliferation data obtained using other techniques, such as TLI [8–10], S-phase fraction [11], and 5-bromo-2-deoxyuridine incorporation [12] has been reported.

Given that cytotoxic chemotherapy mainly acts by killing dividing cells, it might be expected to be more active against rapidly proliferating tumors, as suggested by the results obtained in high-grade non-Hodgkin's lymphoma, lymphoblastic leukemia, and germ-cell tumors.

In advanced breast carcinoma, there are no biological markers to indicate the likelihood of a response to systemic chemotherapy and so the patients to be treated in this way are usually selected by means of a process of exclusion [13]. The identification of a link between proliferative activity and the response to chemotherapy may help in selecting those patients who could benefit from this treatment modality. The present study was designed to examine the relationship between tumor proliferative activity (as assayed by Ki-67) and the response to first-line chemotherapy in advanced breast cancer.

Patients and methods

Patients

At our Institution, Ki-67 and estrogen receptor (ER) immunostainings are routinely performed on fresh breast cancer samples. All of the patients with histologically documented breast cancer who experienced a relapse between January 1989 and April 1994, and in whom the Ki-67 and ER content of the primary tumors (55 patients) or at the relapse site (21 patients) could be determined, were included in this study. The patients were considered eligible if they had measurable or evaluable disease; they were considered ineligible if the only manifestation of disease was a malignant effusion, a previously irradiated lesion, brain metastasis, or nuclide scan evidence of disease. Clinical staging was based upon a complete history, physical examination, a routine biochemical profile, a complete blood cell count and the results of imaging procedures for all patients before the beginning and after three cycles of chemotherapy. Response was evaluated according to standard WHO criteria [14].

The chemotherapy administered was the first for metastatic disease. Standard protocols were used: cyclophosphamide 600 mg/m^2, methotrexate 40 mg/m^2, 5-fluorouracil 600 mg/m^2 on day 1 (CMF = 43 patients); cyclophosphamide 500 mg/m^2, doxorubicin 50 mg/m^2 (or epirubicin 70 mg/m^2), 5-fluorouracil 750 mg/m^2 on day 1 (CAF or CEF = 27 patients); cisplatin 100 mg/m^2 on day 1, etoposide 80 mg/m^2 on day 1 through 3 (3 patients); vinorelbine 25 mg/m^2 (2 patients); carboplatin 50 mg/m^2 on day 1 through 3, 5-fluorouracil 375 mg/m^2 on day 1 through 5, folinic acid 250 mg/m^2 on day 1 through 5 (1 patient). All of the drugs were given intravenously in cycles with a 3 or 4 week interval, except for vinorelbine which was given weekly.

Immunohistochemical Ki-67 and ER assay

Immunohistochemical staining for replicative fraction cells was performed using Ki-67 monoclonal antibody (DAKO-PC) [15]. Air-dried thin frozen sections mounted on glass slides were immersed in

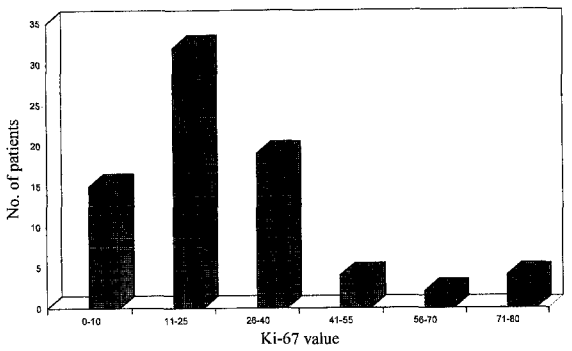

Fig. 1. Distribution of patients according to Ki-67 values.

acetone (– 20° C) for 10 min and then, after rinsing in PBS, incubated for 1 hour, at room temperature, with anti- proliferation-associated antigen Ki-67 antibody (dilution 1:10) and immunostained using the APAAP technique. Counterstaining was performed using 3% methyl green. Field selection sought areas of highest Ki-67 expression evident by lower power scanning. Typically, the total cell count exceeded 1000 tumor cells. Specific staining was observed as red stained nuclei in Ki-67 positive cells. The results were expressed as the percentage of positive cells among the total number of cells.

The immunohistochemical ER staining procedure was performed using ERICA monoclonal kits (Abbott, Chicago, IL) [16]. At least 1000 tumor cells were examined, and the ER-positive cells were recognized by their brown stained nuclei. In a previous study [17], an ERICA threshold value of 45% gave the best level of sensitivity (0.810) and specificity (0.804) in comparison with the classical DCC (dextran coated charcoal) ER assay, and so the tumors were considered ER-negative if there was less than 45% of positive cells and ER-positive if there was 45% or more.

Statistical analysis

Crude and stratified analysis of the differences between groups were performed using the χ^2 statistics or Fisher's exact test [18]. Multivariate analysis, using unconditional logistic regression [19], was conducted in order to investigate the prognostic role of Ki-67 value and other explanatory covariates with respect to the response rate, measured as a dichotomous variable. Overall survival (OS) estimates were obtained according to the Kaplan-Meier method [20] and the significance of the differences in survival time between the two groups was measured by the log-rank test [21]. In addition, a multivariate survival analysis was performed using the Cox's regression model [22]. Logistic regression was performed with SAS [23] and survival analysis was carried out using KMSURV [24] and COXSURV packages [22].

Results

The study involved 76 breast cancer patients who had had a relapse between January 1989 and April 1994, and for whom measurements of tumor proliferative activity by Ki-67 immunostaining and ER content were available. The stains were obtained either on the primary tumors (55 patients; 72%) or at the relapse site (21 patients; 28%). The distribution of Ki-67 at the two sites was similar, with 32/55 (58%) primary tumors and 15/21 (71%) metastases presenting a low proliferative rate (p = n.s.).

There was a substantial range of Ki-67 expression in our patients (0% to 80%), with a median value of 25% (mean ± SD = 25% ± 17%). The distribution of the patients according to Ki-67 status is shown in Fig. 1.

The correlation between the proliferative activity revealed by Ki-67 and the response to chemo-

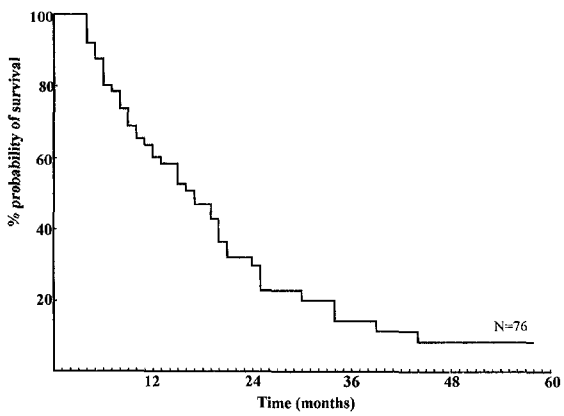

Fig. 2. Overall survival of patients.

therapy made it possible to identify the median value of 25% as a cut-off level separating two groups with a significant difference in response rate. The overall response rate was 32% (24 out of 76: partial responses [PR] = 16, and complete responses [CR] = 8; 95% confidence interval [CI], 22%–42%). The median duration of PR was 8 months (range 4–24), as was that of CR (range 2–17). Figure 2 shows the overall survival of all of the patients; the median duration of survival was 17 months.

When the response to chemotherapy was related to the predominant site of disease, regardless of the Ki-67 content, CR and PR were observed in 12/22 soft tissue (55%), 8/34 visceral (24%), and 4/20 bone metastases (20%) (p = 0.02).

In the 47 patients with slowly proliferating tumors (≤ 25% positive cells), 10 responses were observed (PR = 7, CR = 3) (21%; 95% CI, 9%–33%); in the 29 patients with highly proliferative tumors (> 25% positive cells) there were 14 responses (PR = 9, CR = 5) (48%; 95% CI, 30%–66%). This difference was significant (p = 0.03).The two groups were well balanced in terms of a number of important variables that may have influenced outcome, such as disease-free survival, performance status, age, menopausal status, and the type of first-line chemotherapy. The ER content differed markedly between the two groups, with 27/47 slowly proliferating tumors (57%) and 6/29 highly proliferative tumors (21%) being ER-positive (p = 0.003). Moreover, there was also a significant difference in the metastatic pattern, with a higher incidence of bone and a lower incidence of soft tissue metastases in the group with slowly proliferating tumors (p = 0.004) (Table 1).

The highest response rate (83%) was observed in the patients with a Ki-67 value of 50% or more. However, this cut-off point created a disproportion

Table 1. Patient characteristics according to Ki-67 status

Characteristic	Low Ki-67 (n = 47)		High Ki-67 (n = 29)		p value
	No.	%	No.	%	
Median age (range) (years)	57 (25–73)		54 (30–71)		n.s.
Performance status					
0	28	60	16	55	
1-2	19	40	13	45	n.s.
Menopausal status					
pre- or peri-	11	23	7	24	
post	36	77	22	76	n.s.
Estrogen receptor					
negative	20	43	23	79	
positive	27	57	6	21	0.003
Dominant disease site					
soft tissues	9	19	13	45	
viscera	20	43	14	48	
bone	18	38	2	7	0.004
Median DFS (range) (months)	20 (0–120)		16 (0–180)		n.s.
Adjuvant chemotherapy					
CMF	10	21	9	31	
FAC	4	9	4	14	n.s.
Prior hormonotherapy					
Adjuvant	13	28	3	10	
Metastatic	25	53	8	28	
Type of first-line chemotherapy					
CMF	29	62	13	45	
Anthracycline-based	13	28	14	48	
Other	5	10	2	7	n.s.
Median follow-up (range) (months)	17 (3–58)		17 (4–28)		

Table 2. Number of responses (CR-PR) according to the predominant disease site and Ki-67 status

Predominant disease site	(No. of patients)	Low Ki-67	High Ki-67
		CR-PR/No. of patients (%)	CR-PR/No. of patients (%)
Soft tissues	(22)	3/ 9 (33%)	9/13 (69%)
Viscera	(34)	3/20 (15%)	5/14 (36%)
Bone	(20)	4/18 (22%)	0/ 2

in the sample, with only six patients in the highly proliferative group.

The response rate of each predominant site of disease is shown in Table 2, according to the proliferative rate of the tumors. The highly proliferative tumors showed a higher response rate among soft tissues and visceral metastases, whereas the tumors with a low proliferative rate showed a higher response rate in bone metastases (only two tumors with a high level of Ki-67 staining had metastasized predominantly to the bone). However, according to the multivariate analysis, the only factor that retained independent prognostic significance across the different fitted models was the predominant site of disease, particularly soft tissues ($p = 0.003$), whereas borderline significance was observed for Ki-67 value ($p = 0.05$).

A better response rate in high proliferative tumors was observed either in patients receiving CMF ($n = 13$; CR-PR = 5, 38%) or an anthracycline-based regimen ($n = 14$; CR-PR = 8, 57%); however, this difference was not statistically significant.

Table 3 shows the characteristics of the patients who responded to chemotherapy, according to their Ki-67 status.

Since an inverse relationship between the prolif-

Table 3. Characteristics of patients achieving an objective response according to Ki-67 status

Characteristic	Low Ki-67 (n = 47)	High Ki-67 (n = 29)
	No.	No.
Partial response	7	9
Complete response	3	5
Objective response	10 (21%)	14 (48%)
Performance status		
0	6	10
1-2	4	4
Menopausal status		
pre- or peri-	3	5
post	7	9
Estrogen receptor		
negative	4	13
positive	6	1
Dominant disease site		
soft tissues	3	9
viscera	3	5
bone	4	0
Median DFS (range) (months)	19 (0–52)	10 (0–32)
Type of first-line chemotherapy		
CMF	6	5
Anthracycline-based	4	8
Other	0	1
Median follow-up (range) (months)	18 (4–46)	9 (4–24)

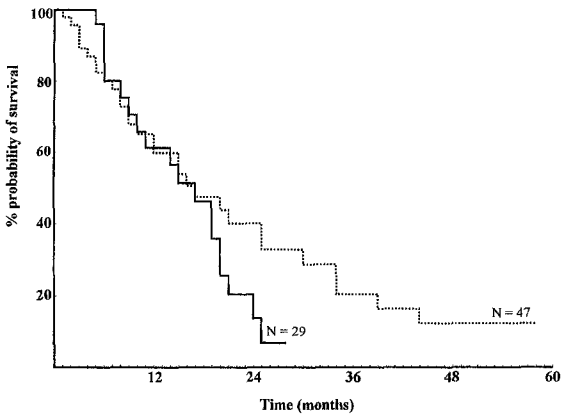

Fig. 3. Overall survival of patients according to Ki-67 values. (---) low Ki-67 values; (——) high Ki-67 values (p = ns).

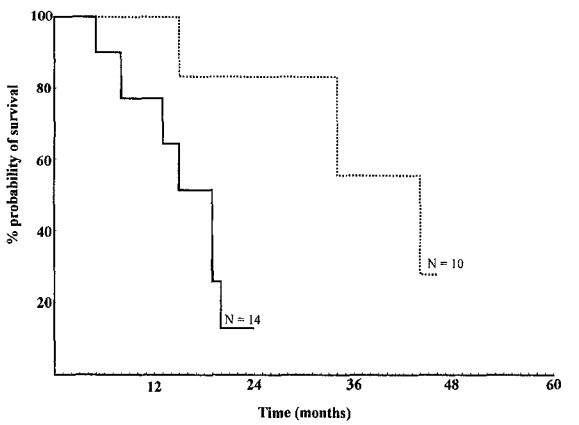

Fig. 4. Overall survival of responsive patients according to Ki-67 values. (---) low Ki-67 values; (——) high Ki-67 values (p = 0.02).

erative activity revealed by Ki-67 and ER content was observed, the correlation between response to chemotherapy and ER was evaluated. In the 43 ER-negative tumors, 17 responses were observed (39%) (95% CI, 24%–54%); in the 33 ER-positive tumors there were 7 responses (21%) (95% CI, 7%–35%) (p = ns).

However, despite the better response rate observed in the tumors with a high level of Ki-67 staining, the survival curves of the two groups were not significantly different when the Kaplan-Meier method was applied (Fig. 3).

In the subset of responsive patients, a median survival of 35 and of 20 months was observed in those with slowly and rapidly proliferating tumors, respectively (p = 0.02) (Fig. 4). The longer survival in low Ki-67 tumor patients achieving an objective response to first-line chemotherapy is mainly due to a more effective control of the disease played by the endocrine treatment administered later in the evolution of the disease. In fact, a hormonal treatment prescribed to 8/10 patients with low-proliferative tumors resulted, in 5 patients, in a stabilization of the tumor ranging from 6 to 12 months; in high proliferative tumors, mainly because of the aggressive course of the cancer, a hormonal treatment was given only to 2 patients, without observing any response or stabilization.

Finally, when multivariate survival analysis was performed using the Cox's regression model, the results were comparable to those obtained with univariate analysis. More specifically, the independent prognostic role of Ki-67 value was confirmed in the subset of responsive patients (p = 0.03).

Discussion

In breast cancer, tumor proliferative activity, as revealed by Ki-67 immunostaining [25–28], TLI [29, 30], and S-phase fraction by DNA flow-cytometry [31–33], is a well defined prognostic factor, with highly proliferative tumors being associated with shorter disease-free and overall survival. However, while these data underline the more aggressive biological behavior of rapidly proliferating tumors, they are only marginally informative about the possible role of tumor proliferative activity as a predictor of response to chemotherapy. In early breast cancer, the majority of available data suggest a significant correlation between the pre-treatment tumor proliferative state measured by means of DNA flow-cytometry [34, 35] and TLI [36], and the response to preoperative chemotherapy. In advanced breast cancer, only one article dealing with the possible role of tumor proliferation (by TLI) in the response to chemotherapy has been published [37]. In this study of 25 patients the response to chemotherapy was significantly higher in tumors expressing a higher TLI.

In tumors characterized by a higher response rate to chemotherapy, such as non-Hodgkin's lympho-

mas, the relationship between tumor proliferation and response to chemotherapy is more complex and the results are conflicting. In general, there is a correlation between the rate of proliferation and the grade of lymphoma, with low-grade lymphomas expressing a low growth fraction and vice-versa [38–40]. In this disease, the impact of proliferation on survival is generally reported as being negative [41–44], and only rarely it is positively associated with response [45].

In the present study, a Ki-67 value of 25% allowed the identification of two groups of patients with a different probability of responding to chemotherapy. The only imbalance between the two groups was represented by the ER content and the dominant site of disease, with an excess of soft tissue involvement in the group with highly proliferative tumors and an excess of bone involvement in those with slowly proliferating tumors. This particular pattern of diffusion is probably due to a selectivity in the metastatic process related to differences in phenotypes. The importance of the predominant site of disease in determining the response to chemotherapy is well known, and it seems that the response rate of soft tissue and visceral metastases (the sites that most often respond to chemotherapy) is related to proliferative activity. The lack of statistical significance in our study could be due to the limited size of the sample.

The significant inverse relationship between Ki-67 expression and ER content is in agreement with a number of already published data relating tumor proliferation to ER content [46–50].

The response rate among highly proliferative tumors was significantly higher than in the group with slowly proliferating tumors. However, only 48% of the rapidly proliferating tumors responded to chemotherapy, thus making this index insuitable as a predictor of response; furthermore, although the response rate in the group with slowly proliferating tumors was lower (21%), it was certainly not negligible. Both a stable [51], but more often an increasing TLI-revealed proliferation rate from primary to methachronous lesions have been reported [52, 53]. In the present study, there was a temporal (and biological) gap between the time at which proliferative activity was determined (at cancer diagnosis, in 72% of the patients) and the beginning of chemotherapy for advanced disease, and this may have weakened the association between the two variables.

The survival curves of patients with rapidly or slowly proliferating tumors did not differ significantly. In the whole group, median survival was 17 months, which compares well with data from studies reporting a higher response rate [54]. Of interest is the analysis of the survival curves of responsive patients, which shows better survival in the group with slowly proliferating tumors. This fact is probably related to the differential effect of hormonotherapy in the two groups of patients.

The cell cycle is controlled by a number of factors: oncogenes substituting growth factors (e.g. *jun, fos, mos*) or promoting cell survival (*bcl-2*), and tumor-suppressor genes monitoring progression through the G_1 phase (*Rb, P53*) etc. [55], and these may play a role in the response to chemotherapy [56]. Furthermore, resistance to chemotherapy has been linked to the expression of membrane proteins, small cytoplasmic peptides (glutathione), enzymes, and many other factors [57], although their relationship to the proliferative state of the tumors has not yet been characterized. It is likely that only the simultaneous study of some of these factors would increase the possibility of predicting responsiveness to chemotherapy.

In conclusion, our data suggest the importance of studying the relationship between tumor proliferation and both the response rate to chemotherapy and patient survival. The higher response rate in rapidly proliferating tumors does not lead to better survival, thus confirming the view that malignancies are not curable by chemotherapy because they proliferate rapidly [58] and perhaps indicating the need for an intensification of treatment on the basis of the rate of tumor proliferation.

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