Lymph Node Metastases Displaying Lower Ki-67 Immunostaining Activity than the Primary Breast Cancer

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Abstract. *The aim of the study was to verify by Ki-67 immunostaining if any difference exists in the cell proliferating fraction between primary breast tumors (PTs) and matching positive axillary lymph nodes (ALNs). Patients and Methods: Immunohistochemistry with the monoclonal antibody against Ki-67 was performed in 160 node-positive breast carcinomas and in their respective lymph node metastases. Results: An increase of Ki-67 immunoreactive cells in ALN compared with that of PTs was observed in 84% of cases (ALN: mean 17%, PTs: mean 8%; $p<0.001$), whereas 16% of the cases showed Ki-67 value two to six times lower in the ALNs than in the corresponding PTs (ALN: mean 3.2%, PTs mean 12.5%; $p<0.005$). The decrease of Ki-67 positive cells in the ALN was independent from the histotype and the histological grade of the tumor. Conclusion: A different cell proliferation fraction between PTs and matching positive ALNs was demonstrated and underlined that the existence of a group of patients with decreased number of Ki-67 immunoreactive cells in lymph node metastases compared with that of the primary tumors could be taken into account in the choice of therapeutic strategy.*

Breast cancer is the first cause of death in women. Its biological behavior is highly variable and many efforts have been made over the years to further characterize this neoplasia, its potential aggressiveness and the probability of response to treatment.

The most important prognostic factors in breast cancer are the expression of estrogen and progesterone receptors, C-Erb2, p53 and the proliferation index (PI). The PI is usually assessed through the immmunohistochemical evaluation of Ki-67, a monoclonal antibody which reacts with a nuclear antigen expressed in all the proliferating cells during the active phase of the cell cycle (G1, S, G2), and which is absent in G0 cells. The PI has a strong prognostic value even when considered alone (1) and plays a role in determining the efficacy of some chemotherapic agents (*i.e.,* 5-fluorouracil) that are generally cell-cycle dependent.

To date, immunohistochemical prognostic factors have been studied exclusively on primary tumors and their assessment has influenced the therapeutic strategies.

The purpose of this study was to verify, by Ki-67 immunostaining in primary breast tumors (PTs) and in matching positive axillary lymph nodes (ALNs), if any difference exists in the cell proliferating fraction of these two different breast cancer locations.

Patients and Methods

One hundred and sixty cases of PTs with diameters ranging from 2 to 5 cm (T2 according to the UICC-TNM staging system) and with positive ALNs, were consecutively selected between 2002 and 2005, from the Institute of Pathology of the University of Palermo (Italy). The cases consisted of 96 ductal infiltrating carcinomas (80 CDI, 9 mucinous carcinomas, 7 apocrine carcinomas), 51 lobular infiltrating carcinomas, 7 lobular and ductal infiltrating carcinomas and 6 tubulo-lobular carcinomas. Informed consent was obtained from all the patients included in the study.

The immunohistochemical evaluation was performed on tissue sections from formalin-fixed, paraffin-embedded samples from the PTs and corresponding ALNs. Tissue sections were treated using a microwave epitope retrieval technique with 10 mmol/L citrate buffer, pH 6.0, at high temperature for 20 min and were then incubated with antibody against Ki-67 by the avidin-biotin-peroxidase complex method. Appropriate positive controls were run concurrently for the antibody tested. In all cases, a subjective, qualitative evaluation of the areas showing the highest positivity for Ki-67 was carried out. In each case, approximately 10 non-overlapping high power fields (objective 40x) were selected from three section planes of the tissue block. High-power fields were accepted for evaluation when they did not contain areas of extensive necrosis, unspecific background staining or sectioning artefacts. The percentage of positive cells was counted in at least 1,000 tumoral cells, both in PTs and their matching ALNs, by two pathologists independently.

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Table I. *Mean percentage of Ki-67 immunoreactive cells in primary tumors (PTs) and in matching axillary lymph nodes metastases (ALNs).*

	PTs (%)	range	ALNs (%)	range	difference (%)	*p*-value
Category A (135 cases)	8	(3-13)	17	(10-23)	9	<0.001
Category B (25 cases)	12.5	(5-23)	3.2	(2-5)	9.3	<0.005

To assess the difference in Ki-67 expression between PTs and ALNs, statistical analysis was performed by the means of Student's *t*-test for paired independent samples.

Results

The immunoreactivity of the Ki-67 antibody was distributed in two categories (Table I).

Category A consisted of 135 cases (84%) of the following histological types: 68 infiltrating ductal carcinomas NOS (Elston and Ellis system scoring: grade 1=24 cases; grade 2=27 cases; grade 3=17 cases), 7 mucinous carcinomas, 5 apocrine carcinomas, 48 infiltrating lobular carcinomas, 3 lobular and ductal infiltrating carcinomas and 4 tubulo-lobular carcinomas. All cases showed an increased number of Ki-67-immunoreactive cells in ALNs compared to the PTs (ALN: mean 17%, range 10-23%; PTs: mean 8%, range 3-13%; mean difference 9%; $p<0.001$).

Category B consisted of 25 cases (16%) of the following histological types: 12 infiltrating ductal carcinomas NOS (Elston and Ellis system scoring: grade 1=4 cases; grade 2=5 cases; grade 3=3 cases), 2 mucinous carcinomas, 2 apocrine carcinomas, 7 infiltrating lobular carcinomas, 1 lobular and ductal infiltrating carcinoma and 1 tubulo-lobular carcinoma. In this category, the cases showed a decreased number of Ki-67 immunoreactive cells in the ALNs compared to the PTs (ALN: mean 3.2%, range 2-5%; PTs: mean 12.5%, range 5-23%; mean difference 9.3%; $p<0.005$).

In category B, the ALNs showed a percentage of Ki-67-positive cells two to six times lower than that observed in the corresponding PTs (PT/ALN ratio=3.7; range=2-6.5). The decrease of Ki-67-positive cells in the ALN proved to be independent from the histotype and the histological grade of the tumor.

Figure 1 shows the increase of Ki-67 immunostaining in ALN compared to the PT in a case from category A (Figure 1a-b) and the decrease of Ki-67 immunostaining in ALN compared to the PT in a case from category B (Figure 1c-d).

The distribution of the 160 cases according to the PI in PT and ALN, is shown in Figure 2. Those cases with a higher PI in ALN (classic) and those with a higher PI in PT (variant) show a distinct distribution.

When the 160 selected cases were analyzed as a whole, the mean percentage of Ki-67 immunoreactive cells was significantly higher in the ALNs than in the PTs (ALN: mean 15%, range 2-30; PTs: mean 9%, range 3-36%. The mean difference was 6%; $p<0.001$).

Discussion

Breast cancer has a very high frequency worldwide and is the first cause of death in women. The median survival of metastatic breast cancer ranges from 2 to 3 years, although there is a wide variability among the patients. The activity of some of the commonly used chemotherapic agents (*i.e.,* 5-fluorouracil, cyclophospamide, methotrexate, anthracyclines) is generally cell-cycle dependent and, thus, cell growth fraction, assessed by Ki-67, could play a role in the efficacy of these drugs (2).

In primary breast tumors, the PI (assessed by Ki-67) is significantly associated with the PT stage, the axillary lymph node status and the tumor grading, and is inversely related to progesterone and estrogen receptor status (1-4). To date, Ki-67 and the other immunohistochemical prognostic factors (estrogen and progesterone receptors, C-Erb2, p53) are exclusively assessed by the primary tumor. Recently, differential expression of these molecular markers has been shown between invasive mammary ductal carcinoma associated with ductal carcinoma *in situ versus* invasive breast cancer alone, confirming the important role of these markers in tumor progression (5).

Fehm *et al.* (6) showed that the bone marrow involvement was not influenced by tumor size and lymph node involvement, but it was significantly related to p53 expression, hormone receptor status, HER2 and Ki-67 in keeping the role of tumor biological factors of the primary tumor for tumor cell dissemination.

De la Haba-Rodriguez *et al.* (7) reported that in 60% of their cases, the immunohistochemical expression of some proteins, such as estrogen, progesterone, Ki-67, p53 and HER-2, was modified during tumor development and dissemination, and led to immunophenotypical differences between PTs and ALNs. They suggested that this could be related to the intrinsic hetereogeneity of the neoplastic cells. In addition, it was also suggested that as different clones are present in primary breast carcinomas, it is possible that only a small sub-population of the PT cells metastasize (3).

Buxant *et al.* found a significantly higher PI in ALNs than in PTs, suggesting an increased aggressiveness of metastatic neoplastic cells compared to their PT counterpart. They

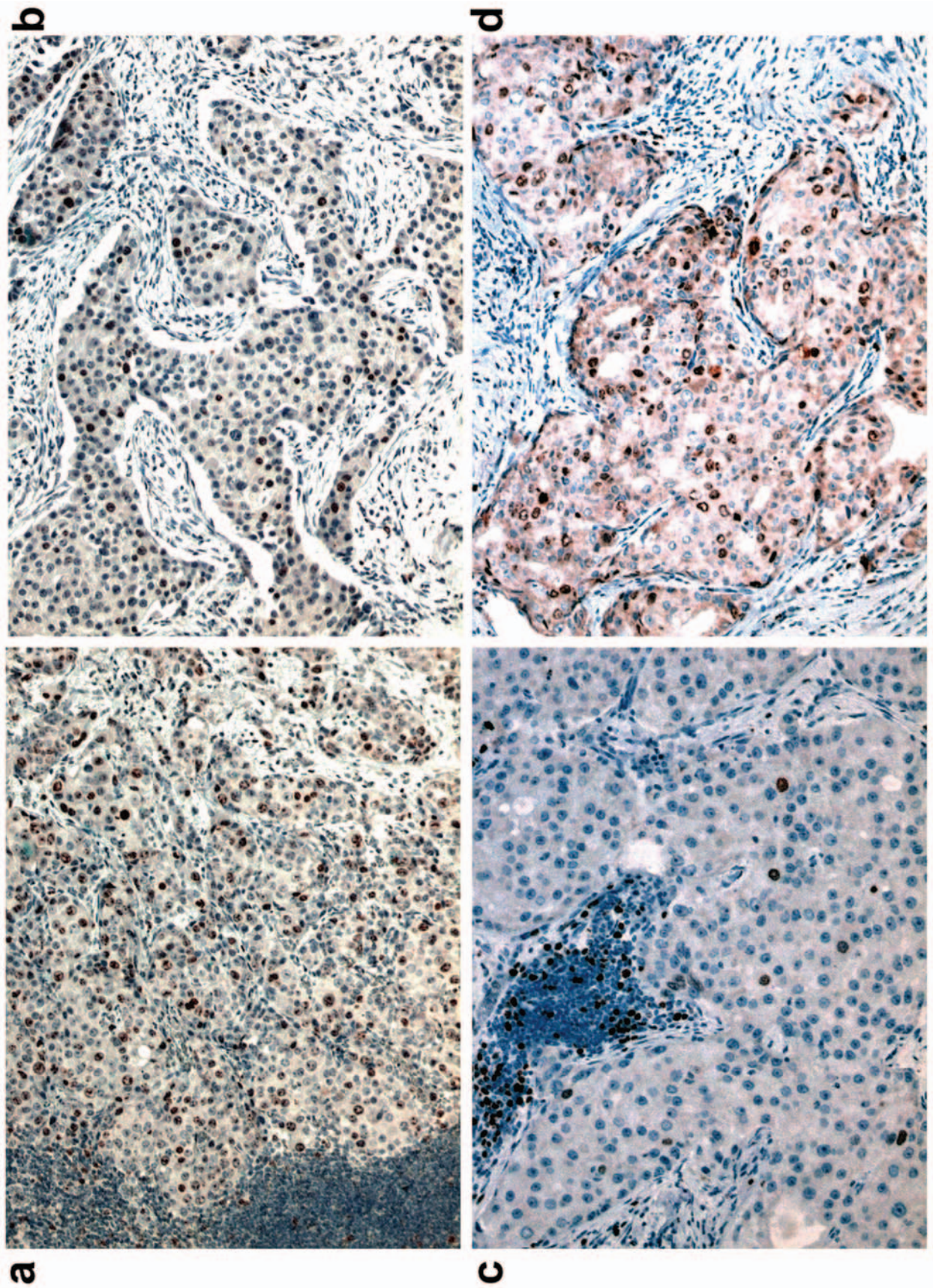

Figure 1. *a, b, c, d: Category A: increase of Ki-67 immunostaining in ALN (1a) when compared with PT (1b). Category B: decrease of Ki-67 immunostaining in ALN (1c) when compared with PT (1d).*

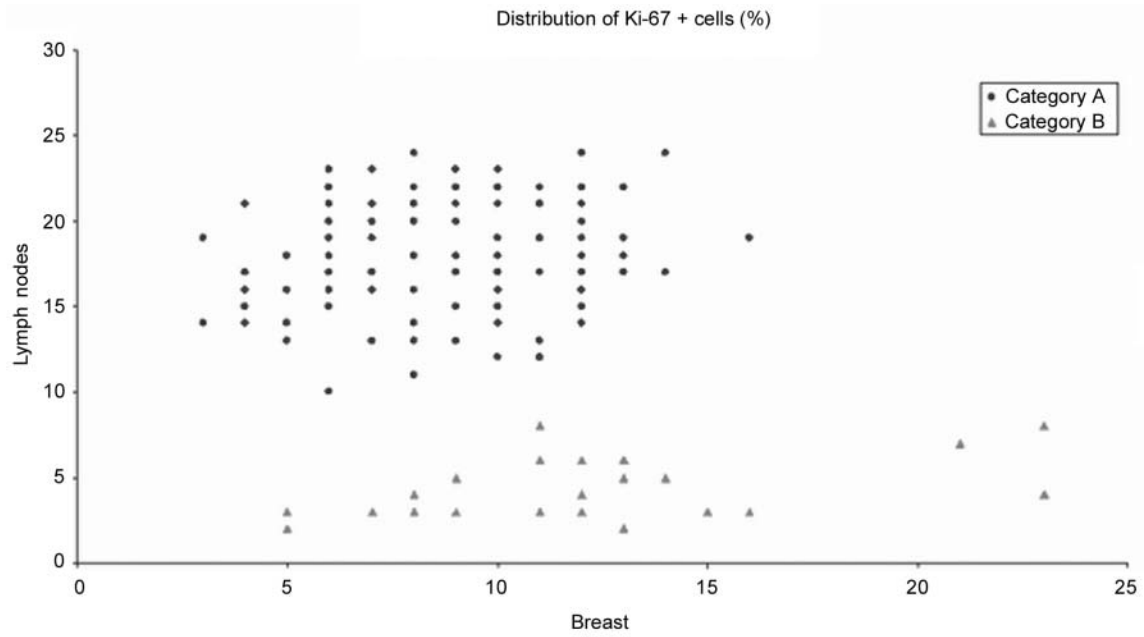

Figure 2. *Distribution of cases according to the proliferation index in primary tumors and in axillary lymph nodes.*

concluded that those cells with "the most aggressive potential (*i.e.,* high PI) were the most likely to escape from the primary tumor" or, that "cells that escape from primary tumor, lose down-regulator factors or suppressor genes and become more aggressive" (2).

In our study, the mean Ki-67 value was found to be higher in ALNs than in PTs when considering all the cases as a whole (ALN: mean 15%, range 2-30; PTs: mean 9%, range 3-36%; mean difference: 6%; $p<0.001$). Our results, obtained from the statistical analysis of Ki-67 mean values of PTs and ALNs, fit well with those of the Buxant *et al.* study (2), showing that the mean Ki-67 value was higher in PTs than in ALNs. Nevertheless, it is important to note a minority of cases included in the B category, in which the cell growing fraction (assessed by Ki-67 immunostaining) was significantly decreased in ALNs as compared to the PTs. In these cases, the Ki-67 value in the ALNs was two to six times lower than that in the corresponding PTs (PT/ALN ratio=3.7; range=2-6.5).

It is still unclear why some metastatic cells, which should have an elevated cell growth fraction because of their high aggressiveness, show a PI lower than that of PT cells. Perhaps their aggressiveness is related to other biological factors, which should be assessed in further specific studies. However, since the efficacy of chemotherapy is generally cell-cycle dependent, lower values of Ki-67 immunoreactivity in ALNs (revealing a majority of cells in G0-phase) could influence the clinician's therapeutic choice. Further studies are necessary to understand the importance of heterogeneity between PTs and ALNs, as well as the clinical relevance of assessing the cell growing fraction in ALNs.

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