Axillary Lymph Node Nanometastases Are Prognostic Factors for Disease-Free Survival and Metastatic Relapse in Breast Cancer Patients

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Abstract

Purpose: Early breast cancer presents with a remarkable heterogeneity of outcomes. Undetected, microscopic lymph node tumor deposits may account for a significant fraction of this prognostic diversity. Thus, we systematically evaluated the presence of lymph node tumor cell deposits ≤0.2 mm in diameter [$pN_{0(i+)}$, nanometastases] and analyzed their prognostic effect.

Experimental Design: Single-institution, consecutive patients with 8 years of median follow-up ($n = 702$) were studied. To maximize chances of detecting micrometastases and nanometastases, whole-axilla dissections were analyzed. pN_0 cases ($n = 377$) were systematically reevaluated by lymph node ($n = 6676$) step-sectioning and anticytokeratin immunohistochemical analysis. The risk of first adverse events and of distant relapse of bona fide pN_0 patients was compared with that of $pN_{0(i+)}$, pN_{1mi}, and pN_1 cases.

Results: Minimal lymph node deposits were revealed in 13% of pN_0 patients. The hazard ratio for all adverse events of $pN_{0(i+)}$ versus $pN_{0(i-)}$ was 2.51 ($P = 0.00019$). Hazards of pN_{1mi} and $pN_{0(i+)}$ cases were not significantly different. A multivariate Cox model showed a hazard ratio of 2.16 for grouped $pN_{0(i+)}/pN_{1mi}$ versus $pN_{0(i-)}$ ($P = 0.0005$). Crude cumulative incidence curves for metastatic relapse were also significantly different (Gray's test $\chi^2 = 5.54$, $P = 0.019$).

Conclusion: Nanometastases are a strong risk factor for disease-free survival and for metastatic relapse. These findings support the inclusion of procedures for nanometastasis detection in tumor-node-metastasis staging.

The tumor-node-metastasis (TNM) staging system for breast cancer (1) has proven invaluable in categorizing the extent of neoplastic disease, and as a basis to estimate prognosis and to direct treatment (2). However, this has not lead to the definition of tightly homogeneous prognostic classes, as considerable heterogeneity of outcomes can be observed among disease cases currently categorized as similar. This is particularly evident in the case of small breast tumors (2). We argued that a diverse extent of lymph node dissemination at early stages of disease may account for diverse disease recurrence dynamics. The principle that the macroscopic burden of metastatic cells (e.g., number of invaded lymph nodes) dictates different risks of disease recurrence has been recognized (1, 3). This principle might be equally important at the low end of the spectrum, i.e., in the case of microscopic tumor cell deposits (1, 4).

Serial sectioning coupled to immunohistochemical analysis has considerably improved the detection of small tumor cell clusters in lymph nodes (5–10). Occult metastases can indeed be identified in up to 30% of cases previously classified as pN_0 (7–9), in 14% to 20% of the cases by single lymph node sections (9, 10). Studies based on these procedures have shown that axillary lymph node microinvasion is a prognostic factor for breast cancer patients, and is associated with poorer disease-free and overall survival (7, 8, 11–13). As a consequence, micrometastases (0.21-2 mm in diameter) have been identified as a relevant risk factor and their detection has been introduced in TNM staging procedures (2003 TNM edition; ref. 1).

Following these guidelines, isolated tumor cells or cell deposits smaller than 0.2 mm are currently required to be classified as $pN_{0(i+)}$ (1). This is because the associated risk is believed to be small or altogether nil. However, little clinical data are available to support this point of view, and the question of the lower size limit of lymph node metastases that may bear clinical significance remains open.

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Received 3/9/06; revised 7/21/06; accepted 8/28/06.

Grant support: Fondazione of the Cassa di Risparmio della Provincia di Chieti, Italian Association for Cancer Research, MIUR-FIRB Postgenomica grant RBNE0157EH, and EU NoE Biopattern (FP6-2002-IST-1 no. 508803).

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi:10.1158/1078-0432.CCR-06-0569

To tackle this issue, we systematically investigated the presence and prognostic effect of tumor cell deposits ≤0.2 mm in diameter in the axillary lymph nodes of breast cancer patients conventionally classified as pN_0. As these tumor deposits are up to six orders of magnitude smaller in volume than micrometastases, we propose to indicate them as "nanometastases."

Patients and Methods

Seven hundred and two single-institution, consecutive cases with unilateral breast cancer, who had undergone surgery with axillary lymph node dissection between January 1989 and December 1993, were studied. The median follow-up time was 8 years; the last update was in December 2002.

Table 1. Characteristics of the patients enrolled in the study

	Reevaluated pN_0 cases (%)		pN_1 cases (%)
Total number	377		184
Age (y)			
29-40	22 (5.8)		18 (9.8)
41-50	75 (19.9)		43 (23.4)
51-70	203 (53.9)		88 (47.8)
>70	77 (20.4)		35 (19.0)
Histotype	Primary*	Nanometa†	
Ductal	261 (69.2)	33 (67.3)	153 (83.2)
Lobular	63 (16.7)	12 (24.5)	24 (13.0)
Tubular	19 (5.0)	2 (4.1)	3 (1.6)
Mucinous	15 (4.0)	1 (2.0)	3 (1.6)
Cribriform	6 (1.6)	0	0
Papillary	6 (1.6)	0	0
Medullary	5 (1.3)	1 (2.0)	0
Rare histotypes	2 (0.5)	0	1 (0.5)
pT			
pT_1	284 (75.3)		106 (57.9)
pT_2	90 (23.9)		75 (41.0)
pT_3	3 (0.8)		2 (1.1)
NA	0		1
Grading			
G_1	96 (25.5)		27 (14.8)
G_2	223 (59.1)		115 (62.8)
G_3	58 (15.4)		41 (22.4)
NA	0		1
Nodal status	$pN_{0(i-)}$‡ 328 (87.0)		$pN_{1(1+)}$§ 92 (50.0)
	$pN_{0(i+)}$ 24 (6.4)		$pN_{1(2+)}$ 56 (30.4)
	pN_{1mi} 25 (6.6)		$pN_{1(3+)}$ 36 (19.6)
ER-positive cells (%)‖			
0-9	52 (14)		34 (19)
10-49	83 (22)		45 (24)
50-100	200 (53)		89 (48)
NA	42 (11)		16 (9)
Neu-positive cells (%)‖			
0-9	207 (55)		93 (51)
10-24	57 (15)		23 (12)
25-49	47 (13)		29 (16)
50-74	20 (5)		12 (7)
75-100	31 (8)		19 (10)
NA	15 (4)		8 (4)

Abbreviations: NA, not available; ER, estrogen receptor.
*Histotype of the primary tumor.
†Histotype of the tumor cases with nanometastases or micrometastases.
‡2002 TNM staging system (1).
§Number of macroscopically invaded lymph nodes.
‖Percentage of positive cells in individual tumors.

Clinical and pathologic status and follow-up data (radiotherapy, chemotherapy, or hormone therapy; site of relapse; last follow-up time or date/cause of death) were recorded (Tables 1 and 2; Supplementary Table S1). Histologic grade was reevaluated on the original pathology slides. Patients (Table 1) were selected according to the following criteria: T_1 to T_3; availability of at least 10 resected axillary lymph nodes (14); absence of synchronous bilateral tumors and of other malignancies before breast cancer diagnosis and up to 6 months after surgery; absence of distant metastases at diagnosis and up to 6 months after surgery; no neoadjuvant therapy. Three hundred and seventy-seven patients were classified as node negative (pN_0) at diagnosis (median follow-up, 9 years), according to European guidelines for mammographic screening (15). The latter recommended the analysis of three macrosections for lymph nodes >5 mm and of two for those <5 mm. pN_0 patients were reevaluated for the occurrence of lymph node microinvasion by the procedures outlined below. Patients with one [$pN_{1(1+)}$] or two to three [$pN_{1(2+3+)}$] macrometastatic axillary lymph nodes (184 cases; median follow-up, 9 years) were used for comparison of risk estimates with micrometastatic and nanometastatic cases. The protocol of this study was approved by the board of the Ministry of the University and Research ("Identification and validation of new markers of metastasizing phenotype of breast cancer," prot. MM06095812_006, year 2000).

Histopathologic and immunohistochemical analysis. Guidelines for metastasis identification and data collection were adopted, which followed the 2003 TNM classification (1). Dissection procedures were chosen to maximize the efficiency of detection of micrometastases (14, 16–18). These included a threshold level of 10 available lymph nodes per patient (14), eight sectioning levels per node, and a spacing of 100 μmol/L between levels (Fig. 1; refs. 5, 16–18). More in detail, the original H&E slides of the two halves of each dissected lymph node were reevaluated. The corresponding blocks were remounted and paired, and a pair of sections was cut for H&E staining and immunohistochemical analysis for cytokeratin expression (AE1-AE3-PCK26 antibody, Ventana, Tucson, AZ). Two further pairs of consecutive sections were obtained at 100 μmol/L steps from both lymph node halves (Fig. 1).

Immunohistochemistry was done with an automated immunostainer (Ventana NEXES Medical System, Tucson, AZ) run with Ventana kits (Strasbourg, France). Two pathologists (R.R. and A.R.L.) independently examined all lymph node sections; each occult micrometastasis case was reviewed by a third, senior pathologist (P.Q.). The diameter of lymph node metastases was measured with a computerized image analyzer (EUREKA, Menarini Diagnostic, Florence, Italy). Lymph node deposits were classified as follows: nondetectable, $pN_{0(i-)}$; isolated tumor cells/nanometastases (largest diameter ≤0.2 mm), $pN_{0(i+)}$; micrometastases (largest diameter 0.21-2 mm), pN_{1mi}. Staining for estrogen receptor was done with the 6F11 mouse monoclonal antibody (Ventana). Staining for Her2/neu was done with the CB11 (Cell Marque, Hot Springs, AR).

Statistical analysis. The occurrence of any adverse event over the follow-up period was considered as hard end point and was used to estimate the event-free survival of the patients under study. Cumulative incidence curves for a three-level classification [$pN_{0(i-)}$, $pN_{0(i+)}$, pN_{1mi}] according to the reevaluated lymph node status were estimated by the Kaplan-Meier method. The difference between the hazard of $pN_{0(i+)}$ versus $pN_{0(i-)}$, and of pN_{1mi} versus $pN_{0(i+)}$ was analyzed using a Cox model. The effect of the reevaluated lymph node status was adjusted for established prognostic factors [pathologic T stage (T_2-T_3 versus T_1), grading (G_2-G_3 versus G_1), and age (35 versus 55 years)] by a multivariate Cox model. Age was modeled using a spline function (19). The assumption of proportional hazards was checked using Schoenfeld residuals (20).

Among all adverse events, the occurrence of distant metastases was the end point of main interest. In the analysis of the latter, methodologies that account for the presence of competing risks (i.e., local relapses, contralateral tumors, other neoplasias, or death without evidence of neoplastic disease) were used. The reevaluated lymph node

Table 2. Causes of first failure in $pN_{0(i-)}$, $pN_{0(i+)}$, and pN_{1mi} patients

	$pN_{0(i-)}$ cases	$pN_{0(i+)}$ cases	pN_{1mi} cases
Total number	328	24	25
Cause of first failure or death*			
Local relapse	17 (1.5; 5.4)	3 (8.3; 12.5)	4 (12; 16)
Contralateral tumors	6 (1.5; 1.8)	0 (0; 0)	1 (4; 4)
Other neoplasias	15 (1.5; 4.8)	3 (4.1; 12.5)	2 (8; 8)
Deaths	41 (4; 13.1)	4 (4.1; 16.7)	2 (4; 8.3)
Metastases	25 (4; 7.9)	6 (4.1; 25)	3 (4; 12)
Total	104 (12.5; 33)	16 (20.6; 66.7)	12 (32; 48,3)
Therapy†			
None	181 (55.2)	14 (58.3)	11 (44)
Antihormone	67 (20.4)	6 (25)	7 (28)
Chemotherapy	20 (6.1)	1 (4.2)	4 (16)
Antihormone + chemotherapy	3 (0.9)	1 (4.2)	0 (0)
Incomplete records	57 (17.4)	2 (8.3)	3 (12)

*Distribution of the causes of first failure or death in $pN_{0(i-)}$, $pN_{0(i+)}$, pN_{1mi} patients. In parentheses are the CCI at 3 and 8 years of follow-up.
†Distribution of the therapy assigned to the three different classes of patients. Percentages are in parentheses.

status was considered both in a three-level classification or grouping $pN_{0(i+)}$ and pN_{1mi} cases. Nonparametric estimates of the probability of occurrence of metastases as the first observed event [crude cumulative incidence (CCI)] were obtained (20). CCI curves were compared by the Gray's test (21), which is the equivalent, in the presence of competing risks, to the log-rank test for the comparison of survival curves. A procedure based on the proportional subdistribution hazard regression model was used to assess the difference among CCI curves of $pN_{0(i+)}$ versus $pN_{0(i-)}$, and of pN_{1mi} versus $pN_{0(i+)}$ (22). This model is the natural extension of the Gray's test and can be thought of as the equivalent of the Cox regression model in the presence of competing risks. To quantify the effect of the reevaluated lymph node status adjusted for the established prognostic factors described above, a multiple regression subdistribution hazard model was used. Model estimates of CCI curves for $pN_{0(i+)}/pN_{1mi}$ versus $pN_{0(i-)}$ were plotted for high- and low-risk groups of patients, defined according to the above prognostic factors. Proportional subdistribution hazard assumptions were checked using Schoenfeld-type residuals (22). The risk attributable to "exposure" (23) to micrometastases and nanometastases was estimated for both end points.

CCIs for $pN_{1(1+)}$ and $pN_{1(2+3+)}$ patients were compared with those of $pN_{0(i+)}/pN_{1mi}$ and $pN_{0(i-)}$ cases by plotting nonparametric estimates. SAS system[5] and S-plus software were used throughout this study. The *Cmprsk* library for S-plus[6] was applied for competing risk analysis.

Results

Complete information at 8 years was available for 82% of pN_0 and 85% of pN_1 patients. During follow-up, 34 metastases (14 to the bones, 10 to the lungs, 5 to the liver, 4 to extraregional lymph nodes, and 1 to a serous membrane), 24 local relapses, 7 contralateral tumors, 20 other neoplasias, and 47 deaths were observed as first events in pN_0 patients. Thirty-two metastases, 18 local relapses, 3 contralateral tumors, 6 other neoplasias, and 22 deaths were observed as first events in pN_1 patients.

The reinclusion and restaging of 6,676 axillary lymph nodes of the pN_0 cases (median, 16.5 per patient) were systematically carried out. Including the H&E analysis at the time of surgery, a total of eight levels per lymph node was analyzed, six of which by both H&E staining and immunohistochemical analysis. On average, 250 lymph node sections were analyzed per patient (by H&E or immunohistochemistry), for a gross total of 90,000 data points. Noteworthy, all micrometastases detected by H&E were also identified by immunohistochemistry, but not vice versa, which is consistent with previous work (7–9). Thus, only immunohistochemical data underwent statistical analysis. After reevaluation, 328 patients (87%) were confirmed to be $pN_{0(i-)}$. Cancer cells were revealed in the axillary lymph nodes of 49 cases (13%). Twenty-four cases (6.4%) were classified as $pN_{0(i+)}$ (nanometastases); 11 of these presented with isolated tumor cells; 13 contained clusters of tumor cells that ranged between 0.036 and 0.2 mm in diameter (median value, 0.14 mm; Supplementary Table S2). Twenty-five cases (6.6%) were classified as pN_{1mi} (micrometastases; diameter range. 0.21-2 mm; Fig. 1).

Cumulative incidence curves for the time to any adverse event are presented in Fig. 2 (*left*). Of interest, the estimated cumulative incidence of pN_{1mi} patients is greater than that of $pN_{0(i+)}$ patients until about the first 40 months, whereas, at later times, this effect is lost (see also below). The unadjusted hazard ratio of $pN_{0(i+)}$ versus $pN_{0(i-)}$ for the three-level classification was 2.51 (P = 0.00019; 95% confidence interval, 1.55-4.07). On the other hand, there was no significant difference between the hazard of pN_{1mi} and $pN_{0(i+)}$ (hazard ratio, 0.728; confidence interval, 0.34-1.56; P = 0.41). No significant time-dependent effects were detected. Thus, $pN_{0(i+)}$ and pN_{1mi} cases were grouped for further analyses.

The multivariate Cox model supported the evidence of a heavy prognostic effect of the reevaluated lymph node status (hazard ratio, 2.16; confidence interval, 1.42-3.28; P = 0.0005; Supplementary Table S3). The estimated risk attributable to exposure over time showed a trend of slight decrease from 0.54 to 0.44 in the high-risk group and from 0.54 to 0.47 in the low-risk group.

The distributions of the different causes of failure and of deaths (CCI at 36 and 96 months) and of the therapies administered to the three different patient classes are reported in Table 2. Notably, the estimated CCI of all events for $pN_{0(i-)}$ cases are consistently lower than the estimated CCI for the other two classes. The estimated CCI for all failure causes different from metastases is greater in pN_{1mi} cases with respect

[5] http://www.sas.com.
[6] http://biowww.dfci.harvard.edu/~gray/.

to $pN_{0(i+)}$ at early follow-up times. CCI curves of metastatic relapse in $pN_{0(i-)}$, $pN_{0(i+)}$, and pN_{1mi} cases are shown in Supplementary Fig. S1 (*left*). The difference among CCIs was statistically significant (Gray's test: $\chi^2 = 7.90$, $P = 0.019$, degrees of freedom = 2). The percentages of treated $pN_{0(i+)}$ and $pN_{0(i-)}$ patients were fairly close (33% and 27%, respectively). The percentage of treated pN_{1mi} patients (44%) was only slightly greater. Thus, differences in the CCI for $pN_{0(i-)}$ cases with respect to the other two classes are unlikely to have been caused by treatment imbalance.

The unadjusted subdistribution hazard ratio for distant metastases of $pN_{0(i+)}$ versus $pN_{0(i-)}$ for the three-level classification was 3.43 ($P = 0.0054$; 95% confidence interval, 1.44-8.19). On the other hand, there was no significant difference between the CCIs of pN_{1mi} and $pN_{0(i+)}$ (hazard ratio, 0.40; confidence interval, 0.12-1.82; $P = 0.27$). The CCI curves for distant metastases in the two-level classification are shown in Supplementary Fig. S1 (*right*; Gray's test, $\chi^2 = 5.54$, $P = 0.019$; degrees of freedom = 1). The presence of micrometastases and nanometastases had a heavy effect on the CCIs for distant metastases (unadjusted hazard ratio, 2.47; confidence interval, 1.16-5.26, $P = 0.019$; adjusted hazard ratio, 2.10; confidence interval, 0.96-4.58; $P = 0.063$; Supplementary Table S4). The model estimates of distant metastasis CCIs for patients at high risk (tumor grading G_2 or G_3, pathologic T_2 or T_3 stage, 35 years of age) or low risk (tumor grading G_1, pathologic T_1 stage, 55 years of age) are shown in Fig. 3. A significant difference in the respective CCI curves was observed in both cases. The estimated risk attributable to exposure over time showed an approximately constant value of 0.52, i.e., 50% of the risk of distant relapse was accounted for by the presence of nanometastases and micrometastases in axillary lymph nodes.

The CCIs of distant metastases of the $pN_{0(i+)}/pN_{1mi}$ cases were compared with those of pN_1 patients. $pN_{1(1+)}$ and $pN_{1(2+3+)}$ cases were separately considered to permit a better assessment of risk versus diameter of lymph node metastases (Fig. 2, *right*). Of interest, the risk of relapse in $pN_{0(i+)}/pN_{1mi}$ cases at long follow-up times seems close to that of $pN_{1(1+)}$ patients, although the small number of events in $pN_{0(i+)}/pN_{1mi}$ cases limits the accuracy of this comparison.

Discussion

Most studies conducted to date on the prognostic effect of minimal lymph node invasion in breast cancer patients suffer from limited sample size (5–8, 11–13, 24, 25). A larger study by Cote et al. (9) was based on immunohistochemical analysis on single lymph node sections, and this can be affected by serious sampling errors (13). Nevertheless, taken together, these analyses provided a coherent indication on an adverse prognostic effect of micrometastases (1, 7–11, 24). Only occasional, small studies reported a lack of prognostic value of micrometastases (5, 6) or borderline clinical significance (12). On the other hand, heterogeneity in micrometastasis diameter cutoff values (7–9, 11, 13) prevented an equally reliable assessment of the clinical effect of the smallest lymph node deposits. A 0.2-mm cutoff was introduced with the 2003 edition of the TNM guidelines (1) and was adopted as a standard in the current study.

Whole-axilla dissection was privileged in the current study for two major reasons. The first one was the requirement for a long median follow-up time. This was satisfied by the enrollment of patients who underwent surgery between 1989 and 1993, a time where sentinel lymph node analysis had not been introduced yet in clinical practice (26). The second reason was that an analysis of at least 10 lymph nodes per patient was shown to possess the highest sensitivity in the detection of micrometastases (14, 25). The detection of nanometastases was further optimized by using frequent, narrow-sectioning intervals, which were previously shown to provide a 90% to 95% sensitivity (9, 16–18). Several factors are likely to contribute to this accuracy level: Among them are the larger information volume of sections near the equatorial region of a lymph node,

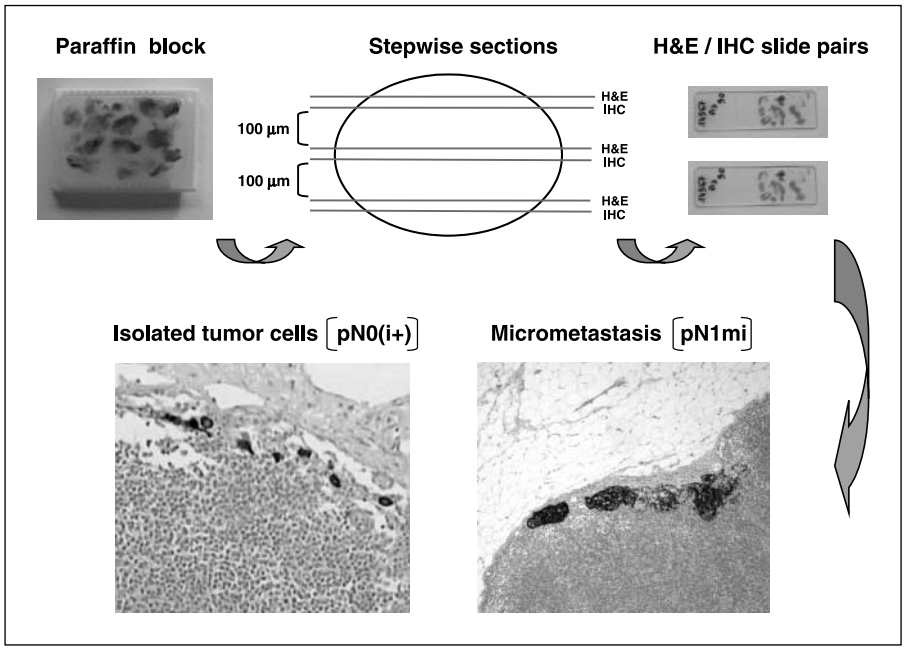

Fig. 1. Scheme of the restaging procedure of pN_0 cases. Examples of nanometastasis (isolated tumor cells) and micrometastasis (0.38 mm in diameter).

a possible preferential regional colonization (27), and the occurrence of multiple micrometastases in a given lymph node.

Our analysis shows a heavy effect of occult metastases on the event-free survival of the bearing patients (unadjusted hazard ratio of 2.51 for $pN_{0(i+)}$ versus $pN_{0(i-)}$, $P = 0.00019$; multivariate Cox model hazard ratio of 2.16, $P = 0.0005$). A corresponding effect was shown on metastatic relapse. This unfavorable prognostic value was maintained in multivariate analyses accounting for competing risks and adjusted for grading, pathologic T stage, and age, although the smaller number of events limits the accuracy of the estimate. Notably, occult lymph node metastases were shown to account for 50% of metastatic recurrences in bearing patients. Hence, extremely small lymph node tumor cell clusters, for which we propose the term nanometastases, possess a strong negative prognostic effect on the event-free and relapse-free survival of bearing patients. Thus, we suggest these cases to be upstaged from the $pN_{0(i+)}$ class to a novel pathologic category, for which we propose the name pN_{1na}.

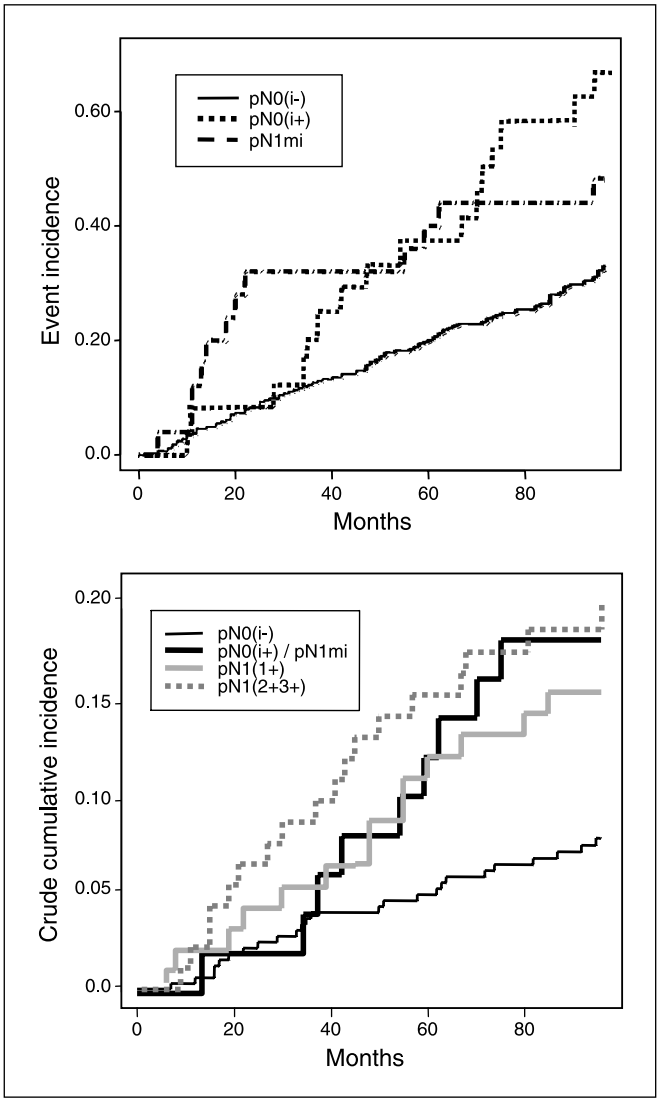

Fig. 2. *Left,* cumulative incidence curves for the time to any adverse event for $pN_{0(i-)}$, $pN_{0(i+)}$, and pN_{1mi} cases. *Right,* nonparametric estimates of CCI for metastatic relapse of $pN_{0(i-)}$, $pN_{0(i+)}/pN_{1mi}$, $pN_{1\,(1+)}$, and $pN_{1\,(2+3+)}$ patients as a function of time.

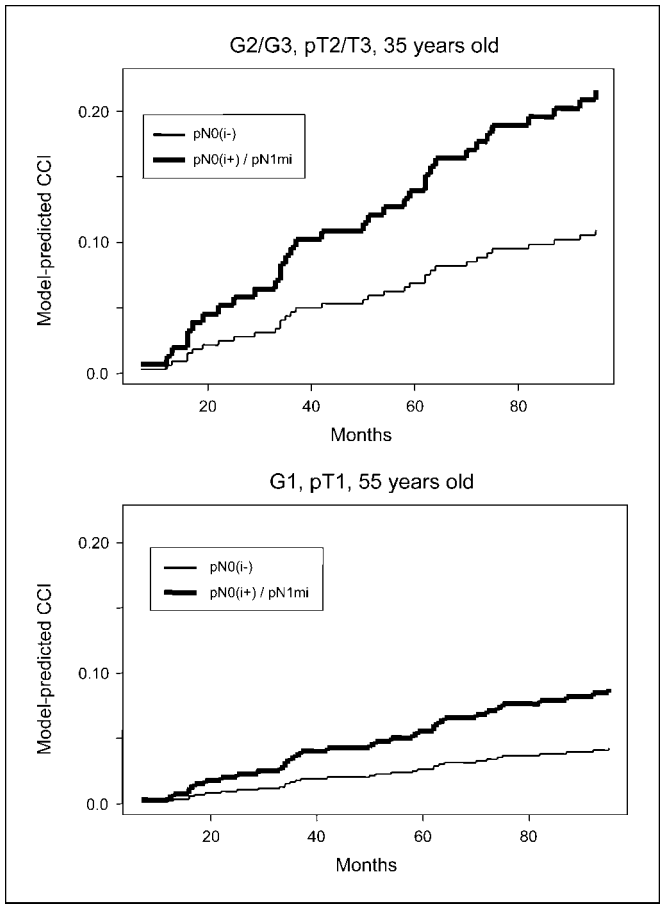

Fig. 3. Model-predicted CCI estimates for metastatic relapse of $pN_{0(i+)}/pN_{1mi}$ and $pN_{0(i-)}$ cases in different risk classes. High-risk (*left*) and low-risk (*right*) classes for metastatic relapse were identified according to tumor diameter, grading, and age.

Potential reasons for bias in our findings were investigated. Consistent with previous studies (28), histologic tumor types were found to correlate with the likelihood of macroscopic lymph node metastases. Ductal carcinomas carried a higher risk. On the other hand, lobular carcinomas and better prognosis tumor types (cribriform, medullary, mucinous, and tubular) showed a lower incidence of metastatic lymph nodes (28). Small occult metastases were detected in 13% of the cases, consistently with earlier reports (5, 6, 8–11, 24), and were more frequent in lobular than in ductal carcinomas (Table 1), consistent with previous reports (10, 13). An unbiased case distribution is also supported by corresponding estrogen receptor and Her-2/neu expression profiles in pN_0 and pN_1 cases, and by the younger age and worse average grading and pathologic T stage of pN_1 cases.

Planned therapy essentially coincided with actual therapeutic procedures, and the percentages of treated $pN_{0(i+)}$ and $pN_{0(i-)}$ patients were very similar. Thus, bias due to treatment imbalance seems unlikely. It should also be noted that tamoxifen is expected to reduce the risk of relapse by 35% at 8 years of follow-up (29). Polychemotherapy is correspondingly expected to reduce the risk by 29% to 32% (29). Only a minority of pN_0 patients was treated (8% with chemotherapy, 22% with

tamoxifen). Hence, a strong effect of therapy on the number of events recorded in this category seems unlikely. Treatment of pN_1 patients was much more frequent (80% of the cases) and a correspondingly larger effect is expected. This might have contributed to the flattening of the CCI curves of pN_1 patients at long follow-up times, which is consistent with previous observations (7).

In this context, it is interesting to note that the CCI curves for "true" pN_0 and $pN_{0(i+)}$ cases clearly diverge at ~36 months after surgery. Although a precise measurement of this effect was beyond the scope of this work, this observation raises several issues. Among them is the intuitive suggestion that distant metastases require time to grow to a detectable size (8). As these would be missed by studies with inadequate follow-up, the need for long observation times in breast cancer studies is emphasized, and the strategy chosen in this study is supported.

The results of this study seem to be important for a better understanding of metastasis development in breast cancer (30, 31). Indeed, the lack of a size threshold for lymph node metastases to bear an increased risk of distant relapse supports a model where microscopic lymph node tumor cell deposits are bona fide metastases at extremely early stages of disease. The constant risk of relapse over time also supports an unvaried metastatic ability over several years of tumor life. These results are consistent with recent comparative genomic hybridization (32) and transcriptomic (33) data. On the other hand, they raise questions on models where the development of metastases depends on the accumulation of successive, stochastic events (31).

In summary, our findings show that nanometastases are a risk factor for breast cancer patients. This supports the conduction of prospective clinical studies on the prognostic effect of occult metastases. These will provide an independent test of our conclusions and may lend decisive support to the inclusion of procedures for nanometastasis detection in TNM pathologic staging. In this context, an efficient compromise between the accurate but labor-intensive systematic detection of micrometastases and nanometastases by immunohistochemistry and reductionist approaches, e.g., sentinel lymph node analysis (25, 34, 35), should be investigated.

Acknowledgments

We thank Drs. M. Trerotola and R. Artusi for help during the course of this work.

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