4. Discussion

The results of this study clearly showed that the grading system of LVTE is capable of being used to classify IDC patients with lymph vessel invasion into a low-, intermediate-, or high-risk group. The grading system also clearly demonstrated that IDCs with grade 1 LVTE and grade 0 LVTE are almost equally malignant. Because IDC patients with grade 1 LVTE accounted for almost half of the IDC patients with lymph vessel invasion, this grading system enabled us to accurately select many low-risk IDCs among IDCs with lymph vessel invasion. Indeed, the classification according to the number of lymph vessels invaded selected IDCs with 1 to 5 lymph vessels invaded almost equal the number to that of IDCs with grade 1 LVTE as low-risk IDCs, but it did not show any difference in the mortality rate between patients with IDC in which 6 to 20 lymph vessels had been invaded and patients with IDC in which more than 20 lymph vessels had been invaded. These findings strongly suggest that the malignancy of IDCs with lymph vessels invaded cannot be judged based only on the number of lymph vessels invaded by tumor cells. Furthermore, this study clearly demonstrated that one patient with IDC having only one grade 3 lymph vessel tumor embolus died of their disease, whereas 3 IDCs with more than 20 grade 1 LVTE were alive and well without tumor recurrence, strongly suggesting that the grading system for lymph vessel tumor embolus can be used to accurately assess the true degree of biological malignancy of IDCs with lymph vessel invasion independent of the number of lymph vessels invaded. It is therefore concluded that the grading system for lymph vessel tumor embolus in IDCs does not only enable pathologists to select IDCs with high risk but also selects IDCs with intermediate and low risks among IDCs having various numbers of lymph vessels invaded by tumor cells.

The results of the multivariate analyses also clearly demonstrated that the grading system for lymph vessel tumor embolus is very useful for accurately predicting the outcome of patients with IDC independent of adjuvant therapy status or nodal status. The prognostic power of grade 3 LVTE could not be analyzed for patients with IDCs whose invasive tumor size is 20 mm or less because there were no IDC patients with grade 3 LVTE. However, grade 2 LVTE sufficiently compensated for the lack of prognostic power of grade 3 LVTE in patients with IDC who received adjuvant therapy whose invasive tumor size is 20 mm or less. In addition, the prognostic power of this grading system is superior to the histologic grading system for the stroma-invasive tumor cells. It can therefore be concluded that the grading system for lymph vessel tumor embolus is an excellent histologic prognostic classification for IDCs that accurately predicts the outcome of patients with IDC.

This study also clearly demonstrated significant associations between increases in grade of LVTE and number of nodal metastases. Because the grading system is based on assessment of mitotic and apoptotic figures in tumor cells in lymph vessels, the tumor cells with a high turnover rate in the lymph vessels most likely have greater ability to spread tumor nests in lymph vessels than the tumor cells with a low turnover rate in the lymph vessels. It can therefore be concluded that factors that accelerate the turnover rate of tumor cells in the lymph vessels should be investigated.

Although no inflammatory breast cancer cases were included in this study, many LVTE were observed in IDCs with grade 2 and 3 LVTE, especially in the latter. Because IDCs patients with grade 3 LVTE had a very high tumor recurrence rate and a very high mortality rate, this finding strongly suggests that some inflammatory ductal carcinomas of the breast originate from IDCs with grade 3 LVTE. In addition, some IDCs with widespread large LVTE in nontumor breast tissue are very difficult to differentiate from intraductal carcinoma nests in IDCs with an abundant intraductal carcinoma component. Thus, pathologists should recognize the existence of IDCs with grade 2 and 3 LVTE that are difficult to differentiate from noninvasive ductal carcinomas or IDC with a predominantly intraductal carcinoma component in routine examinations.

In conclusion, the grading system of lymph vessel tumor embolus we devised is the best grading system for accurately predicting the outcome of patients with IDC of the breast. Although the method used in this grading system is somewhat cumbersome because of the need to count tumor cell mitotic and apoptotic figures in lymph vessels, pathologists can most accurately assess the true malignant potential of IDCs by using this grading system as a histologic prognostic classification of IDCs of the breast.

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REVIEW ARTICLE

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Clinical evidence of breast cancer micrometastasis in the era of sentinel node biopsy

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Abstract Sentinel node biopsy for early-stage breast cancer has been established as an excellent surgical and staging procedure developed to enhance the detection of minimal lymph node involvement such as micrometastases. Multisection and the proper use of immunohistochemical staining have led to the increased detection of micrometastases, and this has given rise to new questions about the treatment to be employed concerning micrometastasis. That is whether complete axillary lymph node dissection (ALND) and adjuvant systemic therapy are really required for patients with micrometastasis because of the low prevalence of nonsentinel lymph node metastasis. Some currently published case studies report that selected patients with micrometastases without further ALND would not suffer from a high incidence of regional recurrence. However, the long-term prognostic risk of systemic recurrence and local failure associated with residual axillary disease in the sentinel lymph node-positive patient electing for no further axillary surgery has not been defined. Numerous studies have investigated the impact of occult metastases, which may be regarded as micrometastases or a small tumor deposit. Although data from randomized controlled trials are lacking, these studies suggest that the prognosis of breast cancer patients with micrometastases should not be considered the same as that in truly node-negative patients. Patients with micrometastases should have some adjuvant systemic therapy. Ongoing randomized trials will provide prospective answers to the question of the optimal treatment for micrometastasis.

Key words Sentinel lymph node biopsy · Micrometastasis · Axillary lymph node dissection · Prognosis · Breast neoplasms

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Introduction

In patients with breast cancer, nodal metastasis in the axillary lymph nodes has historically been considered the most important prognostic factor for decision-making regarding adjuvant therapy and predicting the outcome for women afflicted with this disease. Until recently, to confirm the status of the axillary nodes, routine level I and level II axillary lymph node dissection (ALND) has been performed for breast cancer patients, except for those with noninvasive breast cancer. The majority of patients who have had ALND suffer from its attendant complications, including lymphedema, seroma, pain, and arm paresthesia.

The advent of sentinel node biopsy (SNB) has made a significant impact on the field of surgical oncology.[1,2] The SNB technique, as a staging procedure, is an extremely sensitive and specific method to predict whether breast cancer has metastasized to the regional lymph nodes, and SNB has decreased morbidity compared with ALND for patients with breast cancer.[3–6] Accordingly, in developed countries, SNB has become an acceptable alternative to ALND for women with clinically node-negative early-stage breast cancer. The American Society of Clinical Oncology (ASCO), in a special article, has recommended SNB for patients with early-stage breast cancer;[7] this has been welcomed by breast surgery teams who already use the technique.

However, although axillary node status is an excellent prognostic factor, about a quarter of all axillary node-negative patients followed-up using routine pathological examination with single sections and only hematoxylin and eosin staining (H&E) have a relapse within 10 years.[8] Breast cancer is conceptually accepted by many investigators as a systemic disease because early tumor-cell dissemination may occur even in patents with small tumors. At the same time, we have suspected that the methods used for the detection of metastatic involvement may be insufficient and, consequently, a large number of patients are "understaged" – due to the missing of minimal lymph node involvement – and thus undertreated.

Serial sectioning and the use of immunohistochemical staining (IHC) increase the yield,[9,10] but the methods cannot be applied to all dissected axillary nodes because they are too labor-intensive and expensive for routine use. However, the introduction of SNB allows the pathologist to focus on the small number of harvested sentinel lymph nodes (SLNs) most likely to contain tumor deposits. Detailed pathological examination of SLNs has led to a higher frequency of detection of minimal lymph node involvement (so-called "micrometastasis") than routine pathological examination.

Before the era of SNB, there had been speculation that such micrometastasis in axillary lymph nodes was clinically insignificant. Because there have been hardly any prospective randomized studies of the prognosis of breast cancer patients with micrometastases, the clinical implication of the size of lymph node involvement continues to raise substantial uncertainty and controversy.[11–16] In the era of SNB, we have a dramatically increased chance to treat patients with micrometastasis, owing to the efforts of pathologists. At the same time, in about a quarter to half of patients with micrometastases, the SLN is the sole site of regional node metastasis.

Usually, SNB as a standard procedure in surgical management demands that complete ALND is necessary in all patients with a positive SLN. However, acceptance of SNB raises the question of whether any positive SLN mandates complete ALND and what the exact prognostic role is for nonSLN metastasis which might remain behind in the axilla in SLN-positive patients who are spared an ALND.

This review was undertaken to examine the definition, detection, and frequency of micrometastasis, and the prevalence of nonSLN metastases and their prognostic impact on decision-making for local control and adjuvant systemic therapy. This article does not deal with SNB after neoadjuvant chemotherapy.

Definitions of micrometastases and isolated tumor cells

There has long been a suspicion that very small metastases found in the regional lymph nodes of breast cancer patients may have little if any clinical implication. A major difficulty with the analysis and comparison of clinical research studies, however, is that there has never been a standard definition of the size of a tumor deposit small enough to be termed a "micrometastasis". In studies of breast cancer, various authors have been using the term "micrometastasis", which is described simply as small deposits of metastatic tumor in the regional lymph nodes. Various other definitions have also been reported: metastasis less than 1.0 mm[2,17] metastasis occupying less than 20% of the lymph node sectional area,[18] and even differentiating between metastases less than 1.3 mm and metastases 1.3 to 2.0 mm.[19] The size criteria are subjectively defined in the medical literature. Therefore, a widely accepted definition is urgently needed.

In 1997, the fifth edition of the American Joint Committee on Cancer (AJCC) Cancer Staging Manual[20] adopted the term "micrometastasis" as denoting a single focus of metastasis no larger than 2.0 mm in the greatest dimension of the lymph node.[10,21] The sixth edition of the AJCC Cancer Staging Manual[22–24] has been subsequently revised, and contains extensive and significant modifications. The principal changes, as compared with those presented in the fifth edition, are related to the size, number, location, and method of detection of regional metastases to the lymph nodes. Micrometastases are distinguished from isolated tumor cells (ITCs) on the basis of size. A micrometastasis is defined as a single nodal deposit of tumor larger than 0.2 mm in diameter but no larger than 2.0 mm and is classified as pN1mi. ITCs are defined as single cells or small clusters of cells not greater than 0.2 mm in their largest dimension, usually with no histological evidence of malignant activity (e.g., proliferation or stromal reaction) and are classified as pN0.

In the original sixth edition of the AJCC Cancer Staging Manual,[22] identifiers have been added to indicate the use of SLN dissection and immunohistochemical or molecular techniques (e.g., (i+), (mol+)), which are related to the growing use of new technology. However, the designation (i) had been unclear, confusing "immunohistochemical" with "isolated tumor cells". Afterward, the identifier (i) was amended to indicate "isolated tumor cells" rather than "immunohistochemical."

All metastatic lesions no larger than 0.2 mm, whether detected by H&E or IHC, will be designated as pN0(i+), while a designation of pN0(i–) will be used to indicate no detectable tumor cells by either H&E or IHC. If lymph node staging is based only on SLN biopsy, then (sn) is added to the pN classification (e. g., pN0(i+)(sn)). The designation pN1mi with no additional identifiers will be used for micrometastases greater than 0.2 mm but no greater than 2.0 mm in their greatest dimension.

On the other hand, the term "occult metastasis" has been also used in previous studies. Occult metastases have been defined as metastases missed by initial screening and identified on subsequent screening, and they have also been defined as metastases that are identified through the additional examination of paraffin-embedded lymph node blocks with deeper levels into the block and/or with IHC stains.[25] In many cases, an occult metastasis may be a micrometastasis. However, it may be larger and is not defined by size, because portions of the lymph nodes were discarded at the initial pathological examination.

Detection of micrometastases

Multi-section and using immunohistochemical analyses

SNB is an appropriate initial alternative to routine staging ALND for patients with early-stage breast cancer with clinically negative axillary nodes. Generally, at many institutions, the routine pathological examination for all dissected lymph nodes has been just a single section stained with H&E. This method is so rough for SLNs that it is easy to

overlook small-volume tumor deposits. Currently, many investigators accept that SLNs should be confirmed as malignant by a more detailed pathological examination rather than a rough routine examination. Pathological diagnostic approaches, including serial sectioning and IHC, have been developed for the optimal assessment of metastatic involvement of the SLN. However, the pathological examination of SLNs varies considerably and is not standardized. Cserni et al.[26] reported a high degree of variability among European pathologists in the methods they used.

The clinical significance of micrometastases is best addressed by a prospective study with a defined initial lymph node evaluation strategy that includes slicing the lymph nodes as close to 2.0 mm in the greatest thickness as possible prior to embedding them in paraffin. Serial sectioning and the use of IHC have led to the increased detection of minimal lymph node involvement, classified as micrometastases (pN1mi) and ITCs (pN0(i+)), as occurring in 4%–62% of patients in protocols not using IHC consecutively.[27–32] There is no doubt that IHC is more sensitive than conventional H&E.

Stage migration from pN0 to pN1mi will be encountered in some patients by the use of IHC. The use of serial sectioning of the SLN has been established, but it is still leaves the question open as to whether routine evaluation of SLNs by IHC is necessary[33] and whether the detection of metastasis using IHC can have an impact on the prognosis.[31] The routine use of IHC may increase the detection of false-positive tumor deposits and the detection of metastases of no biological relevance.

Molecular techniques

Pathologists can also use currently established highly sensitive molecular techniques, such as the polymerase chain reaction (PCR), that are capable of detecting trace amounts of keratin messenger RNA (mRNA) produced by epithelial cells. Published studies show that PCR-based assays will detect evidence of epithelial cells in SLNs that appear negative for metastatic carcinoma by routine H&E and IHC.[34] However, it is not clear what exactly is the biological and clinical significance of an SLN whose only evidence of metastatic involvement is a positive PCR, because there can be no morphologic assessment of the cells responsible for the positive reaction.

We must remember that the positive PCR reaction can result from any epithelial cells; metastatic carcinoma or benign epithelial cells carried into the lymph node by chance or by intraoperative contamination. Furthermore, many investigators using PCR for SLNs divide the SLNs in half, with half submitted for routine H&E and IHC, and the other half submitted for PCR.[34–39] When tumor foci exist in only one half of an SLN specimen, a discrepancy will occur between the morphologic assessment and the molecular assessment.[40] Therefore, the results are difficult to interpret regarding whether a positive PCR result means a "true" metastasis in the SLN. The problem is not the detection rate and sensitivity, but the specificity. The clinical value of histologically negative but PCR-positive SLNs can only be determined with long-term follow up.

Frequency of sentinel lymph node micrometastases and nonsentinel lymph node metastasis

Frequency of micrometastases

Table 1 shows the reported frequency in the literature of SLN micrometastases, and the prevalence of nonSLN metastasis with micrometastatic involvement of SLNs.[29,30,35,41–59] SLNs were positive in 21% to 47% of patients in various series. Of these positive patients, the frequency of SLN micrometastases was 18% to 59% (average, 38%). Of these micrometastatic patients, the prevalence of nonSLN metastasis showed a relatively wide range, of 0% to 57%. Several investigators (Table 1) showed a relatively high-rate of nonSLN macrometastases in spite of micrometastatic involvement of the SLN.

Lymph node metastasis is a multifactorial event. Several variables have been described as predictors of lymph node metastasis in breast cancer. The size of lymph node metastasis was significantly correlated with other prognostic features, such as the presence of vascular invasion, high grade, and large tumor size. Other studies have already reported the correlation of the degree of lymph node metastasis with other unfavorable prognostic factors. In particular, a significant correlation between micrometastases and large tumor size or the presence of vascular invasion has been reported.[7,60]

Prediction of nonSLN metastasis

In 32%–66% of patients with positive SLNs, the SLN is the sole site of regional node metastasis.[41–43,61–63] The risk of nonSLN metastasis is related to the size of disease in the SLN, being greatest for macrometastases, intermediate for micrometastases, and least for ITCs, which are usually detected by IHC.[54] Needless to say, the risk depends on the method of detection of the SLN metastasis. This should be taken into account when assessing the risk of omission of ALND after a positive SNB yielding micrometastatic or IHC-positive SLNs.[64]

Thus, it has become clear that the size of the metastasis in the SLN is one of the most powerful predictors of the nonSLN metastasis. Some investigators have reported that other risk factors (such as primary tumor size, lymphovascular invasion, and the number of positive and negative SLNs) were also potentially associated with nonSLN metastases on multivariate analyses.[41–43,61–63] Van Zee et al.,[65] at Memorial Sloan-Kettering Cancer Center, developed a nomogram to determine the probability of additional positive nonSLNs. Some validation studies have suggested that the nomogram may help predict an individual's risk and assist in patient decision-making regarding the benefit of ALND.[66–68]

Table 1. Frequency of SLN micrometastasis, and prevalence of nonSLN metastasis with micrometastatic involvement of SLNs

No.	Author	Year	Detected SLN	Positive SLN	Positive SLN/ detected SLN (%)	SLN with micro	Micro/positive SLN (%)	Performed ALND with micro	Positive nonSLN	Positive nonSLN/ALND with micro (%)	NonSLN macro/ SLN micro (%)
1	Reynolds[41]	1999	220	60	27%	27	45%	27	6	22%	NA
2	Chu[42]	1999	422	158	37%	69	44%	69	5	7%	NA
3	Turner[43]	2000	514	214	42%	111	52%	89	20	22%	8%
4	Liang[44]	2001	226	82	36%	15	18%	11	0	0%	NA
5	Viale[45]	2001	684	250	37%	86	34%	110	24	22%	NA
6	Mignotte[46]	2002	277	129	47%	76	59%	68	15	22%	NA
7	den Bakker[47]	2002	NA	NA	NA	32	NA	32	11	34%	6%
8	Fant[48]	2003	360	102	28%	27	26%	0	NA	NA	NA
9	Nos[49]	2003	800	263	33%	123	47%	123	8	7%	NA
10	Hwang[50]	2003	627	131	21%	30	23%	30	17	57%	NA
11	Fournier[51]	2004	194	48	25%	21	44%	16	1	6%	NA
12	Giard[52]	2004	525	142	27%	55	39%	40	6	15%	0%
13	Fan[29]	2005	390	114	29%	45	39%	18	3	17%	NA
14	Langer[53]	2005	224	101	45%	30	30%	0	NA	NA	NA
15	Viale[54]	2005	4207	1228	29%	434	35%	434	85	20%	13%
16	Leidenius[30]	2005	NA	NA	NA	84	NA	84	22	26%	10%
17	Schrenk[55]	2005	966	379	39%	138	36%	122	22	18%	18%
18	Rutledge[56]	2005	358	89	25%	29	33%	29	1	3%	3.4%
19	Nagashima[57]	2006	314	73	23%	19	26%	NA	NA	NA	NA
20	Gipponi[58]	2006	1284	NA	NA	117	NA	116	16	14%	NA
21	Houvenaeghel[59]	2006	NA	NA	NA	700	NA	700	94	13%	NA
22	van Rijk[33]	2006	2150	649	30%	253	39%	106	20	19%	NA

SLN, Sentinel lymph node; micro, micrometastasis; macro, macrometastasis; ALND, axillary lymph node dissection; NA, not available
Isolated tumor cells were included in the microcategory
SLNs in all studies were examined by multisections and by using immunohistochemistry

Sentinel lymph node micrometastases and locoregional control

It is standard practice that positive SLNs mandate complete ALND. In patients with SLN macrometastases (>2.0 mm), which can be easily detected even by routine pathological examination, a high frequency (39%–79%) of nonSLN metastases has been observed.[29,44,46,49,50,55,56] This justifies the idea that patients with SLN macrometastasis are the best candidates for ALND.

What should we do about minimal SLN involvement? Certainly, in approximately 80% of patients with SLN micrometastases, the SLN is the only involved axillary lymph node. These patients are unlikely to benefit from further axillary surgery. The guidelines of the ASCO panel recommend routine ALND for patients with SLN micrometastases found on SNB, regardless of the method of detection, but they make no recommendations regarding the significance of ITCs.[7] It remains unclear whether ITCs or micrometastases detected with H&E or IHC represent an adverse prognostic indicator and whether ALND should be carried out in all of such patients.

Omission of ALND for patients with SLN micrometastases

Recent retrospective studies of selected patients with micrometastases without further ALND suggest that this subset of patients will not suffer from a higher incidence of regional recurrence. Some authors have reported on the prognosis of patients with micrometastases without ALND. Fan et al.[29] demonstrated that 1 patient (with a single micrometastasis) of 27 patients without ALND developed regional and systemic failure 17 months after mastectomy. Nagashima et al.[57] reported that axillary relapse occurred in only 1 patient with micrometastasis 24 months after the surgery.

However, some limitations of these studies should be pointed out. Usually, ALND was not performed because of patients' preference after consultation with their physicians. Several investigators reported no local failure without ALND in a short follow-up period.[44,48,51,53,69,70] Systemic recurrence and local failure associated with residual axillary disease in SLN-positive patients electing to have no further axillary surgery has not been observed over a long-term period.

Radiation instead of ALND for patients with positive SLN

For several decades, breast-conserving surgery plus radiotherapy has been recognized as an acceptable alternative to mastectomy in patients with early-stage breast cancer. The management of regional lymph nodes in these patients, however, remains a highly complicated and controversial issue. A major subject of concern is the relative benefit of ALND versus axillary radiotherapy for SLN-positive patients. For axillary radiotherapy a portion of the axilla is covered in the radiation field for some patients. The majority of level I and level II axilla can be included in a breast-conserved radiation field when a high-tangent technique is utilized.[71] Pejavar et al.[72] reported that none of 16 SLN-positive patients treated with radiotherapy without ALND had nodal failure.

In the Axillary Lymphatic Mapping Against Nodal Axillary Clearance (ALMANAC) trial, which is a multicenter, randomized clinical trial to compare quality-of-life outcomes between patients with SNB and patients who received ALND, patients were randomized to undergo either SNB or ALND (or four-node axillary sampling). Patients with a positive SLN (based on paraffin section histology) were offered a choice of either delayed ALND or axillary radiotherapy, after discussion with the patient and the multidisciplinary team. Of the 121 patients with a positive SLN for whom axillary treatment information was available, 33 patients received axillary radiotherapy. At 12 months after surgery, there was no axillary local recurrence in this group.[6,73] Nodal radiotherapy may also be an effective treatment for SLN-positive patients.

Prognostic impact of micrometastasis

In general, current information on the prognostic value of lymph node metastasis is largely dependent on the results of a series of older retrospective studies collected over several years. Numerous studies have investigated the impact of occult metastasis. It is thought that almost all occult metastases detected by re-examination of serial sections and/or IHC were micrometastases or smaller. Historical and retrospective studies of occult metastases do not have controlled initial node sampling and therefore cannot be directly compared with modern SLN evaluation, in which nodes have been thinly sliced and totally embedded. Nevertheless, we may be able to assess the significance of micrometastasis in SLN from such studies.

Dowlatshahi et al.[74] produced a review (1948–1996) which attempted to evaluate the role of occult lymph node micrometastases and their relevance to disease recurrence. Recent published studies (1999–2007) of occult (micro-) metastases and their prognostic significance are summarized in Table 2.[27,75–79] The recent studies that showed survival differences had larger patient populations, a relatively long follow-up period (4–25 years), or the detection of occult lymph node metastases by multi-section and/or IHC. These various studies suggest that the prognosis of breast cancer patients with micrometastases should not be considered the same as that of truly node-negative patients.

The International Breast Cancer Study Group reassessed the axillary nodal status (negative for metastases by routine histology) of 921 patients (International Breast Cancer Study Group Trial V) after re-evaluation by serial sectioning and staining with H&E; they showed that the presence of micrometastases in patients was associated with an increased chance of relapse when compared with patients

Table 2. Recent published studies of occult metastases and their prognostic significance

No.	Author	Year	No. of occult metastases	No. of pN0	Detection methods	Size of occult metastasis	Follow-up period (years)	DFS	OS	Comments
1	Grabau[75]	2007	427	4767	H&E, IHC, not serial	=<2.0 mm	10	NA	$P = 0.04$ (premenopausal)	IHC was used when H & E was not clear
2	Kahn[76]	2006	27	186	H&E, IHC	>0.2 mm to 2.0 mm	8	$P = 0.32$	$P = 0.67$	95% of patients did not receive adjuvant systemic therapy
3	Colleoni[27]	2005	232	1400	H&E, IHC	=<2.0 mm	4	$P = 0.047$	$P = 0.037$ for DM	By multivariate analysis
4	Kuijt[77]	2005	87	4263	H&E, single section	=<2.0 mm	25 (Maximum)	NA	$P = 0.09$	Only patients without adjuvant systemic therapy
5	Susnik[78]	2004	21	75	H&E, IHC, serial section	=<2.0 mm	15	NA	$P = 0.004$ for DM	21 Patients, only with occult micrometastases, by multiple logistic regression model
6	Cote[79]	1999	148	588	H&E, IHC, serial section	Various sizes	12	$P = 0.09$	$P = 0.10$	All patients
			53	290				$P = 0.01$	$P = 0.003$	Only postmenopausal women

DFS, Disease-free survival; OS, overall survival; H&E, hematoxylin and eosin; IHC, immunohistochemical staining; NA, not available; DM, distant metastasis

found to have no micrometastases on reassessment.[80] Furthermore, Cote et al.[79] showed that similar results, in terms of worse disease-free survival (DFS) and overall survival (OS), were reported when the analysis was conducted with immunohistochemistry, although the results were statistically significant only in postmenopausal patients.

Recently, Colleoni et al.[27] published a large prospective study testifying to the significance of micrometastasis in breast cancer patients as assessed by multivariate analysis. This study demonstrated that a statistically significant difference in DFS and risk of distant metastases was also observed for patients with minimal lymph node involvement versus patients with node-negative disease (hazard ratio [HR], 1.58; 95% confidence interval [CI], 1.01 to 2.47; $P = 0.047$ for DFS; HR, 1.94; 95% CI, 1.04 to 3.64; $P = 0.037$ for distant metastases). Minimal lymph node involvement was associated with poorer DFS independently of whether it was detected in SLN or after ALND.

Another large retrospective study showing similar results was also recently reported. Kuijt et al.[77] analyzed 10 111 patients with invasive breast cancer over a nearly 25-year period ending in 2002. This study showed the effect of adjuvant systemic treatment in patients with a single axillary lymph node micrometastasis. Among the patients without adjuvant systemic treatment, patients with micrometastases had a significantly higher risk of dying as compared to patients with node-negative breast cancer (HR, 1.51; 95% CI, 1.11–2.06; $P = 0.009$). Lymph node micrometastases remained a significant independent predictor of mortality on multivariate analysis.

The study by Grabau et al.[75] is the largest study to date showing population-based national figures for incidence rates and the prognostic value of micrometastases, on multivariate analysis. In this study, of patients with three or fewer metastatic axillary lymph nodes, patients with micrometastases ($n = 427$) experienced a significantly worse OS compared with node-negative patients ($n = 4767$), irrespective of menopausal status. (relative risk,1.20; 95% CI, 1.01–1.43; $P = 0.04$ in premenopausal women and $P = 0.03$ in postmenopausal women).

On the other hand, Kahn et al.[76] suggest that breast cancer patients with occult micrometastases (not included in macrometastasis) in axillary lymph nodes have a prognosis similar to those with no micrometastases in a median 8-year follow-up.

Although data from randomized controlled trials are lacking, it is surprising that most studies have documented a poorer prognosis for patients with micrometastasis compared with that for node-negative patients, suggesting that such minimal lymph node involvement cannot be safely overlooked. Patients with micrometastases should receive some adjuvant systemic therapy.

Prospective randomized controlled trials

Ongoing or completed/closed randomized trials will help resolve the above-mentioned issue of whether further axil-

lary treatment is mandatory when the SLN is positive, with risk stratification based on the metastatic load.

The International Breast Cancer Study Group (IBCSG) 23-01 study[81] is designed to determine the prognostic significance of minimal (2.0 mm or less) metastatic involvement of SLNs in breast cancer. The nodes are examined extensively by multisection staining with H&E; IHC is used only when the H&E findings are not clear. Patients with SLN micrometastases were randomized to either SNB alone or ALND.

The National Surgical Adjuvant Breast and Bowel Project (NSABP) Protocol B-32[82,83] is a randomized phase III clinical trial that compares SNB alone to ALND in clinically node-negative breast cancer patients. This large multicenter study is designed to examine the long-term survival effect of SNB alone and its morbidity compared with these parameters for axillary dissection. This trial will also determine whether the survival of patients who have occult tumor cells is worse than that of patients who are negative by both H&E and IHC.

By contrast, the American College of Surgeons Oncology Group (ACOSOG) Z0010 trial[84,85] is a prospective multicenter trial designed to evaluate the prognostic significance of micrometastases in the SLNs and bone marrow aspirates of patients with early-stage breast cancer. Patients with negative SLN by H&E should not have an ALND. Patients with positive SLN may be eligible for registration and randomization to the Z0011 trial. The objective of the Z0010 trial is to evaluate the hazard rate for regional recurrence in women whose SLNs are negative by H&E and to estimate the prevalence and to evaluate the prognostic significance of SLN micrometastases detected by IHC. In the ACOSOG Z0011 trial,[86] SLN-positive patients, irrespective of tumor size in the SLN, were randomized into either an ALND or a no-further-axillary-therapy group. The objective of the Z0011 trial was to evaluate the differences in axillary recurrence and overall survival between the two groups. Unfortunately, this trial has now been stopped as a result of poor accrual.

To investigate the effects of nodal radiotherapy for patients with positive SLN, the following randomized trial is ongoing. The European Organization for Research and Treatment of Cancer (EORTC) 10981–22023 AMAROS (After Mapping of the Axilla: Radiotherapy or Surgery) trial[87] is a phase III study comparing a complete ALND with radiotherapy of the axilla in SLN-positive patients, whereas SLN-negative patients are followed for the endpoints of the study as well. The main objective of the trial is to prove equivalent local regional control for patients with proven axillary lymph node metastasis by SNB if treated with axillary radiotherapy instead of ALND, with reduced morbidity.

ALND will provide maximum staging information and minimize the risk of leaving residual nodal disease in the axilla. This is important for good local regional control of disease, systemic treatment decisions, and possibly patients' overall survival. However, it should be noted that ALND may represent overtreatment for some patients, if the nodal micrometastases prove to have limited biological and clinical significance. These trials will answer the question of the appropriate treatment for minimal involvement in SLN in the near future.

Conclusions

This article provides an overview of SLN micrometastases for breast cancer. SNB has been established as an excellent surgical and staging procedure developed to enhance the detection of minimal lymph node involvement. For pathological examination, it is recommended to obtain serial SLN slices as close to 2.0 mm as possible prior to embedding the specimen in paraffin, but the prognostic value of the routine use of IHC is not clear. The substantial increase in the number of patients with micrometastases discovered using multi-section has resulted in new problems, especially the question of whether complete ALND and adjuvant systemic therapy are really required for these patients.

On consideration, ALND may be omitted in patients with micrometastases because of the low prevalence of nonSLN metastasis. Undissected axillary lymph nodes, which may contain undetected axillary metastases, could potentially give rise to future local relapse or systemic metastases. Some argue that surgical removal of subclinical nodal disease is associated with a small but non-zero survival benefit, while others argue that current adjuvant systemic and/or radiotherapy would likely treat the majority of patients adequately. The clinical relevance of resecting additional nodal disease remains unknown.

Micrometastases will most likely have some role in influencing the prognosis and management of breast cancer. Ongoing randomized clinical trials will help to resolve questions about the treatment of micrometastasis over the next few years.

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Sentinel node biopsy in primary breast cancer: Radioactive detection and metastatic disease

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Abstract

Aim: To examine the relationship between the intensity of the radioactive counts and the presence of tumor metastasis in sentinel lymph nodes (SLNs) in order to correctly identify the number of SLNs to be removed.
Patients and methods: Five hundred three breast cancer patients with successful radioisotope localization of SLNs using the combined blue dye and radioisotope method were analyzed. SLN biopsy was continued until all the blue-stained and radioactive nodes were removed.
Results: The mean number of harvested SLNs was 1.7 ± 0.9, and the number of radioactive SLNs among the harvested nodes was 1.6 ± 0.8. SLN metastasis was found in 123 of the 503 cases. The metastasis was detected in the SLN with the highest radioactive count (the hottest SLN) in 94 of the 123 cases with positive SLNs. The positive rate in the hottest SLN was 89% in 61 cases with a single radioactive SLN, and 65% in 62 cases with multiple radioactive SLNs. Of the 29 cases with positivity in other than the hottest SLNs, the metastasis was detected in the second hottest SLN in 16 cases, in the third hottest SLN in one case, in a mixture of negative radioactive SLNs and blue-dye-stained in four cases, and in the negative SLNs and positive non-SLNs (false-negative) in eight cases. Of 123 node-positive cases, 111 cases had metastasis that was detected within the first three hottest SLNs.
Conclusions: These data suggest that lymph node metastasis may not always be detected in the hottest SLN. Thus, in practice, all radioactive and/or blue-dye-stained nodes should be removed for further examination.
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Keywords: Breast neoplasms; Sentinel lymph node biopsy; Lymph node metastasis; Radioisotope; Gamma-probe

Introduction

Sentinel lymph node biopsy (SNB) is fast emerging as the preferred alternative to standard axillary lymph node dissection (ALND) in breast cancer patients, because it offers information on the axillary lymph node status with little morbidity as compared to the significant morbidity associated with ALND. Numerous validation studies[1,2,3,4] have demonstrated that SNB is a highly sensitive and minimally invasive technique for the detection of lymph node metastasis. However, the diverse approaches used for sentinel lymph node (SLN) mapping have raised controversies regarding the most appropriate definition of a SLN.[5,6,7] At present, it is considered that the combined use of vital blue dye and radioisotope (RI) may be superior to the use of either tracer alone for accurate nodal staging.[8]

The development of SLN detection techniques has contributed significantly to the accuracy of detection of metastases, however, some problems have also emerged that require further investigation. These issues are mainly related to the intensity of the radioactive counts in the lymph nodes. When multiple SLNs are detected using a gamma probe, the metastatic disease is often encountered not in the most radioactive, or the "hottest" node, but in the less radioactive nodes. It is not clear how many radioactive

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nodes should be harvested and examined to accurately predict the nodal status. Therefore, we conducted a study to verify the relationship between the intensity of radioactivity and the presence of tumor metastasis in SLNs.

Patients and methods

Patients

The outcomes of 551 breast cancer patients with a clinical tumor size of <5.0 cm and a clinically node-negative axilla (T1–2, N0, M0, clinical stage I or IIA), who underwent SNB between January 1999 and December 2003 were reviewed. This study was approved by National Cancer Center Hospital East's Institutional Review Board. Informed consent for the SNB had been obtained prior to the surgery in all the patients.

Lymphatic mapping and surgical technique

The technique employed for the SNB has been described in detail elsewhere.[9,10] In brief, 30–60 MBq of a radioactive tracer was injected subcutaneously one day prior to the surgical procedure at one or two sites over the primary tumor, but not into the tumor or the biopsy cavity. We used 99m technetium (Tc)-human serum albumin and 99mTc-tin colloid (Nihon Medi-Physics, Tokyo, Japan) or 99mTc-phytate (Daiichi Radioisotope Laboratories, Tokyo, Japan) as the tracer, and the tracers were not filtered. A preoperative lymphoscintigraphy was performed using a large-field scintillation camera. Then, at the beginning of the surgery, 4–5 ml of the dye indigocarmine (4 mg/ml) (Daiichi Pharmaceutical, Tokyo, Japan) was injected subcutaneously at the same site as that of injection of the radioactive tracer. SNB was then performed based on a combination of the presence of blue-dye-staining and radioactivity as detected by a hand-held gamma ray detection probe (Navigator; USSC, Norwalk, CT USA). Successful blue dye localization was defined as a lymph node with visible blue staining, directly contiguous blue-stained afferent lymphatics, or both. Any lymph node that showed radioactivity was removed. No specific SLN-to-background ratio was applied for defining the SLNs in this study, because the background counts can be quite variable depending on the tracer particle size, location of the primary tumor, and site of placement of the probe. All the SLNs were individually and separately checked for the maximum radioactivity count ex vivo with a gamma probe for a period of five seconds. A harvested SLN that showed any radioactivity ex vivo was considered to be a RI success.

Histological analysis

Intraoperatively, all of the SLNs were evaluated by frozen section analysis. Patients with positive frozen sections immediately underwent Level I–II, or higher-level ALND. The SLNs and other dissected non-SLNs were later examined in permanent single sections by routine hematoxylin-eosin staining, without immunohistochemical analysis.

Statistical analysis

All values were expressed as mean ± standard deviation (SD). The amount of radioactivity was expressed as count per second (CPS). Statistical analysis was performed using the Kruskal–Wallis ANOVA or the chi-squared test. Differences between groups were considered to be significant when the p values were <0.05.

Results

Results of sentinel node biopsy

In total, 551 patients underwent SNB. The numbers of cases with harvested SLNs as showing both blue-dye-staining and radioactivity, radioactivity alone, and blue-dye-staining alone were 453, 55, and 36, respectively. No SLNs were identified in seven cases. Our concern in this study was cases with radioactive SLNs. Therefore, cases in which the SLNs were identified by the presence of blue-dye -staining alone and those in which the SLNs were not identified at all were excluded from this study. Five cases in which the radioactivity count could not be recorded were also excluded. The eight cases showing involvement of non-SLNs in which the metastasis could not be identified in the SLNs (false-negative cases) were included in this study. Among these false-negative cases, the SLNs were identified by the presence of both blue-dye-staining and radioactivity in five cases and by the presence of radioactivity alone in three cases. Finally, a total of 503 cases with radioactive SLNs were eligible for the analysis in this study.

Patient characteristics

The characteristics of the patients are listed in Table 1. A total of 498 patients underwent SNB for 503 axillary nodal basins. Five patients had bilateral breast cancer. Nine cases had both axillary and internal mammary SLNs. Besides that in the patients with positive SLNs, ALND was also performed in 53 other cases as part of a feasibility study and in keeping with the patients' wishes. As the relationship between the number of harvested SLNs and the number of radioactive SLNs, the number of which all harvested SLNs had radioactivity was 443. The remaining cases had a mixture of radioactive SLNs plus blue-dye-stained SLNs alone.

Features of SLNs

Table 2 shows the features of the SLNs according to the number of radioactive SLNs. Only a single radioactive SLN

Table 1
Patient characteristics

Case	503
Age median years (range)	54 (24–85)
Dominant primary site	
UOQ	257
UIQ	115
Central	42
LOQ	57
LIQ	32
Prior excisional biopsy	
Yes	52
No	443
Mean tumor size cm (range)	2.1 (0.4–5.0)
Clinical T stage	
T1a	5
T1b	44
T1c	221
T2	233
Clinical stage	
I	270
IIA	233
Surgery for breast	
Total mastectomy	138
Partial mastectomy	365
Surgery for axilla	
SNB alone	361
SNB + ALND	142
Mean number of harvested SLNs	1.7 ± 0.9
Mean number of radioactive SLNs in harvested SLNs	1.6 ± 0.8
Histopathological type	
IDC	412
ILC	26
Other	65
Lymphaticinvasion	
Present	139
Absent	364
Vascular invasion	
Present	242
Absent	261
Estrogen/Progesterone Receptor	
+/+	210
−/+	50
+/−	87
−/−	113
Unknown	43

U, upper; L, lower; O, outer; I, inner; Q, quadrant; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma.

was identified in 282 of all cases, whereas two or more (multiple) radioactive SLNs were identified in the remaining 221 cases. The greater the number of radioactive SLNs harvested, the greater the radioactive count of the hottest SLN was. Metastatic lymph nodes were found in 123 cases. As the number of radioactive SLNs increased, the rate of positive nodes also increased (22–44%). Of the 123 cases with positive SLNs, 94 cases corresponded to the node with the highest radioactivity count ("hottest SLN"). The positive rate of the hottest SLN was 89% in the 61 cases with a single radioactive SLN, but only 65% in the 62 cases with multiple radioactive SLNs; the difference between the two was significant ($p = 0.022$). Of the 29 cases with positivity in other than the hottest SLNs, metastasis was found in the second hottest SLN in 16 cases, and in the third hottest SLN in only one case. The radioactive counts in the hottest-SLN-negative and less than the-hottest-SLN-positive cases were more than 10% of that in the hottest SLN (so-called 10% rule). Moreover, four cases had a mixture of negative radioactive SLNs and at least one positive blue-dye-stained SLN. The remaining eight cases were false-negative (negative SLNs and positive non-SLNs). Finally, Of 123 node-positive cases, 111 cases had metastasis that was detected within the first three hottest SLNs.

Discussion

In general, most surgeons have the experience of detecting more than one SLN, irrespective of the technique used.[11] However, using the blue dye method alone, it might be difficult to find additional nodes after the first SLN is dissected, and there may be a tendency to be satisfied with the detection of just one SLN. When the RI injection method is used in combination with the blue dye injection method, it is possible to easily localize the SLNs based on high radioactive counts even after the detection of a SLN by the blue dye method. The use of an intraoperative gamma probe with the RI injection method facilitates the identification of SLNs.[12,13,14] Utilization of the RI injection method greatly increases the chances of detection multiple SLNs. In about half of the cases in this study, multiple SLNs were harvested. However, we cannot distinguish between the true first draining lymph node and the positive node among the SLNs. This could be attributable to the agents passing though the true SLN into other nodes, or an anatomic variation with more than one SLN draining a tumor. When multiple radioactive nodes are identified, which ones and how many should be removed? There is no clear consensus on which radioactive nodes should be removed or which may be true sentinel nodes.

In this study, we attempted to clarify the relationship between the radioactive counts and the presence of metastasis among the SLNs identified. We examined as many lymph nodes showing radioactivity as possible in this analysis, and calculated the ratio of the radioactivity counts in the SLNs to that in the hottest SLN, and not the axillary background, because the counts in some of the SLNs were nearly as low as that of the background. In our method, the injection of a RI a day before the surgery might lead to migration of the RI from the true SLNs into additional axillary nodes and reduction of the radioactivity because of the long time interval from the injection to the detection. Among the cases with multiple radioactive SLNs, 35% showed metastases in nodes other than the hottest SLN. If only the RI injection method without the blue dye method were used, with examination of only the hottest SLNs, 29 of the 123 patients with positive axillary lymph nodes

Table 2
Features of SLNs

	Total	Number of radioactive SLNs in harvested SLNs				p
		1	2	3	≧4	
Number of cases	503	282	163	49	9	
Mean CPS of the hottest SLN	36 ± 68	31 ± 64	39 ± 71	43 ± 70	107 ± 102	<0.001
All removed lymph nodes negative	380	221	119	35	5	
Metastatatic rate (%)	123/503 (24)	61/282 (22)	44/163 (27)	14/49 (29)	4/9 (44)	0.242
The hottest SLN positive	94	54	28	9	3	
The hottest SLN negative, the second hottest SLN positive	16	–	12	4	–	
The first two hottest SLNs negative, the third hottest SLN positive	1	–	–	–	1	
Radioactive SLNs negative, dye alone SLN positive	4	3	1	–	–	
Radioactive SLNs negative, non-SLNs positive*	8	4	3	1	–	
Positive rate of the hottest SLN in cases with lymph node metastasis (%)	94/123 (76)	54/61 (89)	28/44 (64)	9/14 (64)	3/4 (75)	0.022

* False-negative cases. However, all cases do not performed back-up axially lymph node dissection; SLN, sentinel lymph node; CPS, count per second.

would have been falsely negative. These data suggest that the metastasis is not always detected in the hottest SLN. Moreover, if back-up ALND was performed in all the cases, the false-negative rate might increase.

Camp et al. reported similar results;[15] they found that in 24% (8/33) of the cases, the metastasis was detected in other than the hottest node. Martin et al. conducted a detailed investigation on the significance of the hottest node by determining the count ratio of the SLN to the axillary background[16,17], and showed that in 369 of the 463 cases with positive SLNs, the metastasis was contained in the SLN with the highest count, whereas in the remaining 94 cases, the SLN with the highest count was benign, and SLN biopsy required the removal of all nodes containing the RI, the blue dye, and also of all clinically suspicious non-SLNs for maximum accuracy.

MacMasters et al. advocated the so-called "10% rule", in which SLNs are defined as lymph nodes showing an ex vivo count of 10% or more relative to that of the hottest SLN.[8] Reduction of the false-negative rate to 5.8% was accomplished in a multi-institutional study using a combination of this rule and the additional use of the blue dye method. In relation to our study results also, the "10% rule" seemed to be reliable. However, four cases with blue-dye-stained nodes showing no radioactivity were falsely excluded. In addition, we also demonstrated that the blue dye tracer could also efficiently detect SLNs with metastases. Thus, the use of the blue dye method combined with the RI method may probably minimize the rate of missing of SLNs.[18]

In conclusion, among the cases with multiple radioactive SLNs, the metastasis was detected in other than the hottest SLN in 35% of the cases. Furthermore, in 90% of the cases, the metastasis was detected in the first three hottest SLNs. However, the results were obtained using the RI injection method combined with blue dye injection, and measurement of the radioactive count was performed "ex vivo". In practice, we recommend that all nodes containing RI and/or showing positive staining with the blue dye should be removed.

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Original contribution

Accurate assessment of lymph vessel tumor emboli in invasive ductal carcinoma of the breast according to tumor areas, and their prognostic significance

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Summary Lymph vessel tumor emboli (LVTEs) within tumors are difficult to distinguish from stroma-invasive tumor foci. The purpose of this study was to evaluate staining of LVTEs with hematoxylin-eosin (HE) and with D2-40 to determine whether LVTEs identified by HE staining alone are D2-40–positive LVTE and whether the presence of LVTE identified by HE or D2-40 staining is an accurate predictor of outcome in 151 patients with invasive ductal carcinoma (IDC) of the breast. We first attempted to identify LVTE in the stroma-invasive tumor area (intratumor area), the advance area, and the nontumor area by HE staining alone, and then LVTE identified by HE staining was confirmed by D2-40 staining. The number of LVTE identified by HE staining and D2-40 staining successively increased from the intratumor area to the nontumor area. Although D2-40 staining detected larger numbers of LVTE than HE staining in all tumor areas, the highest positive predictive value of LVTE was observed in the intratumor area, and the next was in the advance area, and then the nontumor area, and significant correlations were found between the numbers of LVTE stained by HE and D2-40 in the same tumor areas. LVTE identified by HE staining or D2-40 staining in the intratumor area or nontumor area significantly increased the risk for tumor recurrence or death of patients with IDC, independent of hormone receptor status or nodal status. The results of this study demonstrate that the existence of intratumoral LVTE and that the presence of intratumoral LVTE identified by HE staining or D2-40 staining are accurate predictors of the outcome of patients with IDC of the breast.
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1. Introduction

Artifactual spaces often form around the nests of stroma-invasive tumor cells within an invasive carcinoma as a result of tissue shrinkage during processing, making it very

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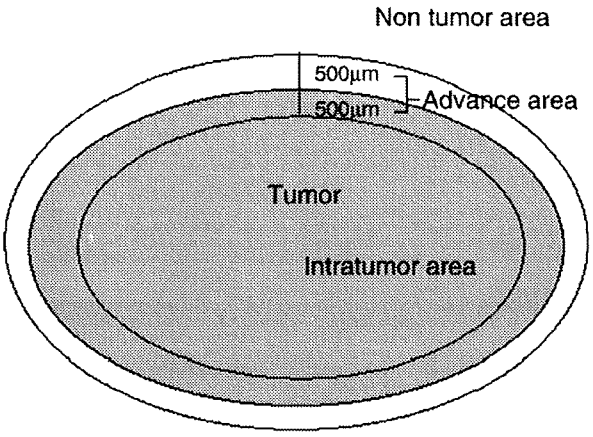

Fig. 1 The tumor areas examined for LVTEs and lymph vessel density. The advance area is defined as extending from 500 μm inside the tumor to 500 μm outside the tumor. The intratumor area consists of the area inside the advance area, and the nontumor area consists of normal mammary tissue surrounding the advance area. Tumor, the area shown in gray; nontumor, the area shown in white.

difficult to distinguish the artifacts from true intratumoral lymph vessel spaces. As a result, pathologists generally examine tumors for the presence of lymph vessel tumor emboli (LVTEs) at or beyond the border of the stroma-invasive tumor area. However, because lymph vessels within the invasive tumor area are surrounded by many stroma-invasive tumor cells that may give rise to nodal or lymphogenous distant organ metastasis, the detection of LVTEs in the invasive tumor area has a very important prognostic significance in patients with breast carcinoma.

Several putative lymphatic endothelial markers have recently been proposed [1-5], and D2-40 is a novel monoclonal antibody to an M_r 40000 O-linked sialoglycoprotein that reacts with a fixation-resistant epitope on the lymphatic endothelium. It does not stain the endothelium of blood vessels, including arteries, veins, and capillaries, and its usefulness for detecting intratumoral lymph vessels has been reported in several tumors [6-11].

The purpose of this study was to evaluate (1) whether LVTEs exist within the stroma-invasive tumors of invasive ductal carcinomas (IDCs) of the breast, (2) whether LVTEs identified by hematoxylin-eosin (HE) staining alone in stroma-invasive tumor area and LVTEs at or beyond the border of the stroma-invasive tumor area are true LVTEs, and (3) whether the presence of LVTEs identified by HE or D2-40 staining or D2-40 lymph vessel density is an accurate predictor of nodal metastasis or the outcome of patients with IDC. The results showed that intratumoral LVTEs do exist, and that the presence of intratumoral LVTEs identified by HE or D2-40 staining is an important accurate predictor of the outcome of patients with IDC of the breast.

2. Materials and methods

2.1. Cases

The subject of this study was 151 consecutive cases of IDC of the breast surgically treated at the National Cancer Center Hospital East, Chiba, Japan, between 1993 and 1995. Clinical information was obtained from the patients' medical records after complete histologic examination of all IDCs. All patients were Japanese women, and they ranged from 28 to 84 years old (mean age, 53 years). All patients had a solitary lesion. There were no cases of inflammatory breast cancer in this series.

For pathologic examination, the surgically resected tumor specimen was fixed in 10% formalin overnight at room temperature, and the entire tumor was cut into slices at intervals of 0.5 to 0.7 cm. The size and gross appearance of the tumors were recorded, and tumor size was confirmed by comparison with tumor size on the histologic slides. Multiple histologic sections were taken from each tumor to measure maximal tumor diameter and area. The sections were processed routinely and embedded in paraffin. Serial sections having the largest tumor surface area in each case were cut from the paraffin blocks, and 1 section was stained with HE and examined histologically to confirm the diagnosis. All tumors were classified according to the guidelines of the World Health Organization [12]. Histologic grade was assigned according to the modification of the Bloom and Richardson classification [13].

Fig. 2 Lymph vessels, LVTEs, and lymph vessel density in IDCs. A and B, Lymph vessel stained for D2-40. C, Seven lymph vessels positive for D2-40 are observed within the intratumor area at mid power magnification (×20), and the lymph vessel density of the tumor is 7. D and E, Three tumor emboli in the nontumor area are observed in lymph vessels positive for D2-40 (arrows), and the angiovessel has not stained for D2-40 (arrowhead). F, LVTEs in the intratumor area. G and H, The space around the tumor embolus is lined by endothelial cells (arrows), and the lymph vessels containing the tumor emboli assessed by HE staining exhibit linear staining for D2-40. I, LVTE in the advanced area. J and K, The space around the tumor embolus is lined by endothelial cells (arrows), and the lymph vessels containing the tumor embolus identified by HE staining show linear staining for D2-40 (arrow). L, LVTEs in the nontumor area in the vicinity of adipose tissue. M and N, The space around the tumor embolus is lined by endothelial cells (arrows), and the large lymph vessels containing the tumor emboli identified by HE staining show linear staining for D2-40, whereas the adipose tissue has not stained for D2-40. O and P, LVTEs in the intratumor area in the vicinity of adipose tissue. Although the 6 tumor cell nests were concluded to be intratumoral fat-invasive foci when stained with HE (O, arrows), 3 of them were located in the lymph vessel positive for D2-40 (P, black arrows). Although 1 of the 6 is located in a vessel-like lumen and faintly stained for D2-40, it was concluded not to be an LVTE in this study (P, blue arrow). The angiovessel adjacent to the LVTEs is negative for D2-40 (P, arrowhead), and the 2 tumor nests in adipose tissue are also negative for D2-40 (P).

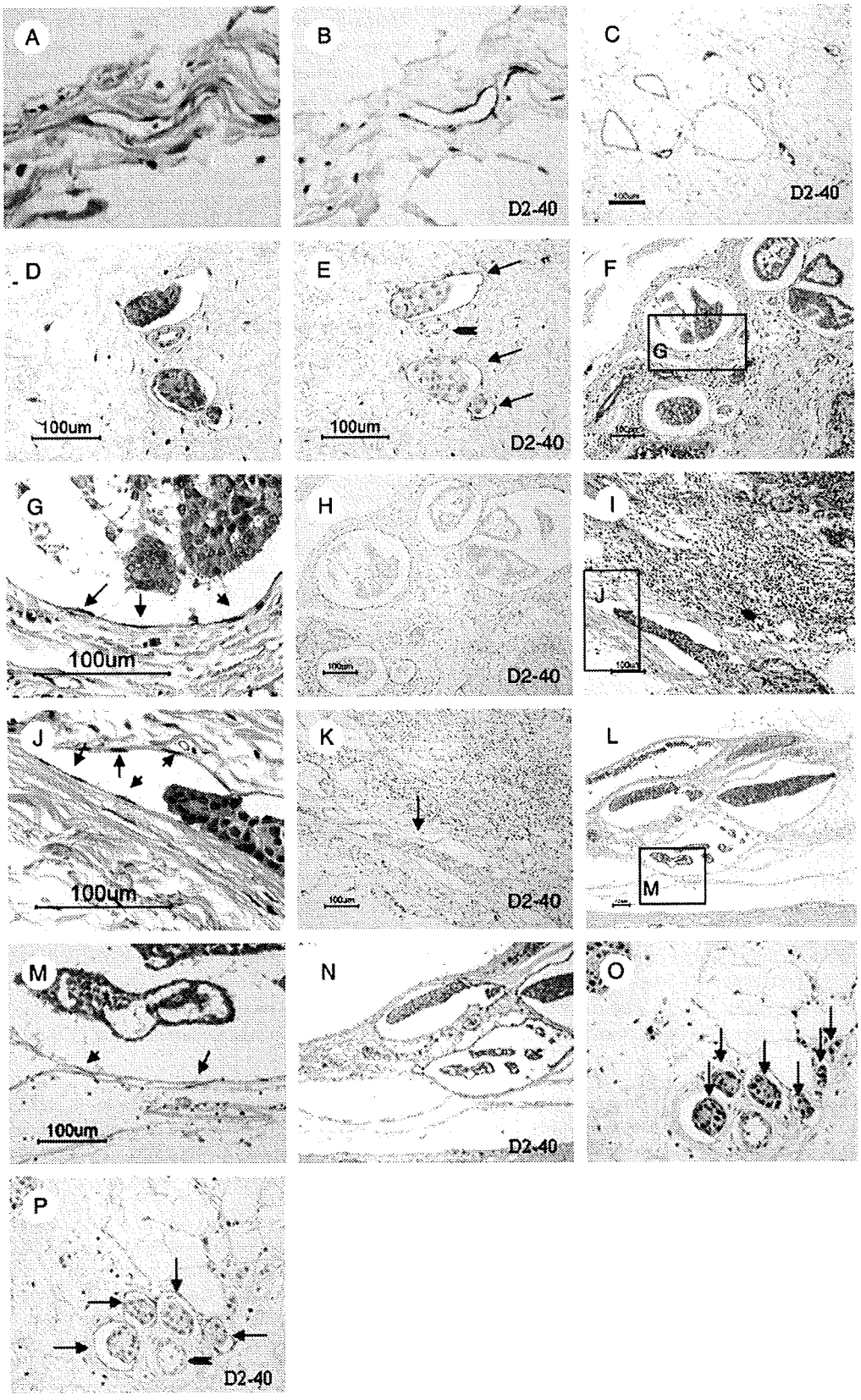
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Table 1 Maximum, minimum, median, and mean number of LVTEs detected by HE staining and by D2-40 staining, and D2-40 lymph vessel density according to tumor area

Areas	Cases	Positive cases (%)	Maximum	Minimum	Median	Mean ± SD
No. of LVTEs identified by HE staining						
Intratumor	151	19 (13)	84	0	0	1.1 ± 7.6
Advance	151	52 (34)	158	0	0	4.0 ± 18.8
Nontumor	151	35 (23)	243	0	0	7.3 ± 30.6
No. of LVTEs identified by D2-40 staining						
Intratumor	151	30 (20)	120	0	0	2.8 ± 14.2
Advance	151	69 (46)	247	0	0	6.1 ± 26.4
Nontumor	151	36 (24)	396	0	0	9.6 ± 42.4
Lymph vessel density determined by D2-40 staining						
Intratumor	151	48 (32)	27	0	0	3.3 ± 5.2
Advance	151	138 (91)	30	0	6.0	7.6 ± 5.6
Nontumor	151	148 (98)	27	0	9.0	9.4 ± 4.8

2.2. Immunohistochemical examination

Paraffin sections 5 μm thick were dewaxed and hydrated, and after inactivation of endogenous peroxidase with 3% hydrogen peroxidase for 20 minutes, antigen retrieval was performed by microwave oven heating for 20 minutes in tris buffer. Slides were incubated for 3 hours at room temperature with the D2-40 monoclonal antibody, a selective marker of lymphatic endothelium that does not react with blood endothelium [6-11] (1:200 dilution; Signet Laboratories, Dedham, MA). Immunohistochemical staining was performed using the EnVision + HRP DAB system (DAKO Cytomation, Carpinteria, CA).

2.3. Histologic assessment of LVTEs and lymph vessel density according to tumor area

In this study, we used HE staining to determine whether LVTEs were present and to count the actual number of LVTEs in 3 tumor areas: (1) the intratumor area, (2) the advance area, and (3) the nontumor area (Fig. 1). The advance area, the area at the border of stroma-invasive tumors, was defined as an area extending 500 μm inside and 500 μm outside the tumor area under high-power magnification (×400). The intratumor area was defined as the area of the stroma-invasive tumor consisting of the inner portion of the advance area, and the nontumor area as the area beyond the border of the stroma-invasive tumor, consisting of normal mammary tissue surrounding the advance area.

In this study, we defined "lymph vessel invasion by tumor cells" as tumor cell nests in spaces (Fig. 2D, F, I, and L) and around the clump of tumor cell nests that were lined by endothelium with no supporting smooth muscle or elastica, and/or that were filled with lymphatic fluid (Fig. 2G, J, and M). Tumor cell nests in spaces that were either not lined by endothelial cells or were lined by endothelial-like cells, probably tumor-stromal fibroblasts, were classified as stroma-invasive tumor cell nests. We first identified LVTEs in the 3 tumor areas by HE staining alone and then counted the numbers of LVTEs in each tumor area as the total number of LVTEs identified by HE staining.

Table 2 Cumulative actual numbers of LVTE identified by HE staining according to tumor area confirmed as true LVTE by D2-40 staining in all cases

	Tumor area			
	Total	Intratumor	Advance	Nontumor
No. of H-LVTE	1868	167	598	1103
No. of D-LVTE	2801	428	925	1448
Positive predictive value		95% (159/167)	88% (532/598)	88% (976/1103)
Sensitivity		37% (159/428)	58% (532/925)	67% (976/1448)
False-negative rate		63% (269/428)	42% (393/925)	33% (472/1448)
No. of H-LVTE undetected by HE staining according to tumor area (%)	1134	269	393	472
Reason for not being detected				
1. Angiovessel (%)	567	8 (3)	180 (45)	379 (80)
2. Stromal invasion (%)	453	261 (97)	173 (44)	19 (4)
3. Adipose invasion (%)	94	0	30 (8)	64 (14)
4. Error (%)	20	0	10 (3)	10 (2)

Abbreviations: H-LVTE, LVTEs identified by HE staining; D-LVTE, LVTEs identified by D2-40 staining.
NOTE. The actual numbers of LVTE are the numbers of H-LVTE confirmed as true LVTE by D2-40 staining.