

Prognostic Factors for Triple-Negative Breast Cancer

Table 6 Univariate Analysis of Clinicopathological Factors for DFS and OS

	DFS		OS	
	Hazard Ratio (95% CI)	P	Hazard Ratio (95% CI)	P
Menopausal Status				
Pre	1	.157	1	.133
Post	0.62 (0.33–1.14)		0.63 (0.32–1.22)	
Familial History				
Negative	1	.988	1	.939
Positive	1.03 (0.35–2.40)		0.99 (0.29–2.50)	
BMI				
< 18.5	1	.931	1	.801
18.5–25	1.00 (0.36–4.17)		0.71 (0.25–2.98)	
> 25	0.76 (0.21–3.54)		0.70 (0.20–3.27)	
UICC Stage				
I	1	.318	1	.449
II	0.24 (0.05–4.33)		0.20 (0.04–3.60)	
III	0.42 (0.09–7.61)		0.32 (0.06–5.78)	
PST Regimen				
A+T	1	.891	1	.796
A	0.72 (0.04–3.33)		0.98 (0.05–4.59)	
T	0.82 (0.13–2.69)		NA	
Completion of PST				
Yes	1	.025	1	.0044
No	2.10 (1.07–3.94)		2.44 (1.19–4.78)	
Clinical Response				
CR	1	< .0001	1	< .0001
PR	3.61 (1.33–12.5)		4.25 (1.40–18.4)	
SD	6.56 (2.07–24.6)		4.66 (1.14–22.7)	
PD	19.6 (6.71–70.7)		28.0 (8.67–125.1)	
Surgical Procedure				
Mastectomy	1	.556	1	.546
WLR	0.86 (0.45–1.61)		0.88 (0.43–1.73)	
Radiation Therapy				
Yes	1	.296	1	.934
No	0.71 (0.33–1.40)		0.81 (0.36–1.66)	
pT (Except pCR)				
T1	1	.0009	1	.0024
T2	1.88 (0.81–4.46)		2.14 (0.86–5.52)	
T3	3.93 (1.84–8.88)		4.07 (1.79–10.1)	
pN				
N0	1	< .0001	1	< .0001
N1	4.58 (2.11–10.7)		4.05 (1.76–10.1)	
N2	4.88 (1.74–13.1)		4.65 (1.53–13.4)	
N3	15.4 (5.47–41.9)		10.7 (3.50–30.7)	
Lymphatic Invasion				
No	1	< .0001	1	< .0001
Yes	4.60 (2.46–8.91)		4.06 (2.07–8.28)	

Table 6 Continued

	DFS		OS	
	Hazard Ratio (95% CI)	P	Hazard Ratio (95% CI)	P
Vascular Invasion				
No	1		1	
Yes	7.56 (3.18–16.0)	< .0001	6.23 (2.48–13.7)	< .0001
Histologic Grade				
1	NA		NA	
2	1	.0047	1	.0063
3	2.84 (1.34–7.01)		3.45 (1.46–10.1)	
HER2 Status				
Score 0	1	.933	1	.613
Score 1	1.00 (0.51–1.88)		0.83 (0.39–1.66)	
Pathologic Response				
pCR	1	.001	NA	.0044
Non-pCR	13.5 (2.94–239.8)			

Abbreviations: A = anthracycline regimen; A+T = regimen containing both anthracycline and taxane; BMI = body mass index; CR = complete response; DFS = disease-free survival; OS = overall survival; pCR = pathologic complete response; PD = progressive disease; pN = pathologic nodal status; PR = partial response; PST = preoperative systemic chemotherapy; pT = pathologic invasive size; SD = stable disease; T = taxane regimen; UICC = International Union Against Cancer; WLR = wide local resection.

Table 7 Multivariate Analysis of Clinicopathologic Factors for DFS and OS

Factor	DFS (P)	OS (P)
Completion of PST (Yes or No)	.015	.039
Clinical Response (CR, PR, SD, PD)	.0007	.0002
pT (T1, T2, > T3)	.266	.099
pN (N0, N1, N2, N3)	.0003	.0022
Lymphatic Invasion (yes or no)	.562	.513
Vascular Invasion (yes or no)	.039	.061
Histologic Grade (1, 2, or 3)	.025	.016
Pathologic Response (CR or non-CR)	.428	.548

Abbreviations: CR = complete response; DFS = disease-free survival; OS = overall survival; PD = progressive disease; PR = partial response; pT = pathologic invasive size; SD = stable disease.

Chemotherapy showed that pCR was a favorable prognostic factor for patients who underwent preoperative chemotherapy.^{5,9,10} Kaplan–Meier analysis for DFS and OS demonstrated that both good clinical response with PST and pCR were correlated with a favorable prognosis. Our data also show that both DFS and OS of patients who achieve pCR is much better than that of patients who did not achieve pCR by Kaplan–Meier analysis. In addition, there were no breast cancer recurrences and no deaths among patients with pCR, although most recent metaanalysis has shown that prognosis of triple-negative breast cancer is worse than luminal types even if pCR is achieved.¹¹ However, pCR is not an independent prognostic indicator according to the multivariate analysis. This might be caused by relatively small sample size of our study, and of course, it cannot be concluded that pCR is not a surrogate marker for prognosis of TNBC patients. This implies that there is a strong relationship be-

tween pCR and clinical response and clinical response offsets the prognostic value of pCR. We emphasize that some other factors, such as clinical response and histologic grades are also important for TNBC as well as pCR.

In this study, statistical analysis was done with a median follow-up period of 49.2 months, which is too short to evaluate 10-year survival rates. Triple-negative breast cancer has biologically aggressive features, and the DFS curve plateaus 5 years after diagnosis and the OS curve plateaus 8 years after diagnosis.¹² Furthermore, in this study, because there were only 2 patients with UICC stage I disease, our study population included patients with more advanced disease that might result in earlier recurrence and breast cancer death. This is the reason why we analyzed the prognostic factors for TNBC with the current median follow-up time. In contrast, recurrence and breast cancer death in the current study were observed in about one-third of the TNBC patients in the previous study.¹⁰ More recurrences or breast cancer deaths might occur with more time in our study group; therefore, we need to continue observing these patients and reanalyze the prognostic factors in the future.

A recent report¹³ showed that women with T1–2 N0 TNBC treated with mastectomy without radiation therapy have a significantly increased risk of locoregional recurrence compared with those treated with partial mastectomy; however, distant metastasis-free survival or OS were not evaluated. Our data demonstrated that the type of surgical procedure, mastectomy or partial mastectomy, did not affect DFS or OS, perhaps because relatively few cases of locoregional recurrence were observed in our study (5.9%) compared with the previous report (10%).

We also found that completion of chemotherapy was a significant prognostic factor among TNBC patients. Multivariate analysis demonstrated that completion of chemotherapy was an independent prognostic factor despite the relationship with clinical response. Pre-

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operative systemic chemotherapy should be finished not only in clinical trials but also in routine practice unless unmanageable severe adverse events or obvious disease progression occurs. Furthermore, considering the poor prognosis of patients with clinical PD, another regimen should be considered for patients to avoid a PD clinical response.

There were 16 patients of PD (12%) and 16 of SD (12%) in our study. The rate of clinical nonresponders in our study was higher than that of a previous multiinstitutional randomized phase III trial, NSABP B-27.¹⁴ Our group included 112 of invasive ductal (83%), 9 of invasive lobular (7%), and 14 were special histologic types such as squamous cell carcinoma or spindle cell carcinoma (10%). Preoperative systemic chemotherapy for 7 out of 14 patients (50%) of special types resulted in PD. This might affect the higher PD rate and our results of statistical analysis.

We demonstrated the prognostic data of TNBC patients with PST, but there were 2 out of the 135 patients who received systemic chemotherapy after surgery as well. One patient received A regimen before surgery and T regimen after surgery. The other received T before and A after surgery. These 2 patients were included the 'A+T' group for analysis of prognosis. This might not affect the results because a randomized clinical trial showed that there was no difference in prognosis between preoperative AC-T and preoperative AC plus postoperative T.¹⁰

Family history is a not significant factor for prognosis. It has been reported that there is a strong correlation between the triple-negative subtype and *BRCA* mutations.¹⁵ Among Japanese women, hereditary breast cancer is strongly associated with the triple-negative phenotype¹⁶ and aggressive behavior. These reports suggest that TNBC patients with a family history of breast cancer have a poorer prognosis than patients with no family history. Our data suggest that the prognosis of TNBC patients with a family history of breast cancer is similar to those with sporadic TNBC. Of course, this might be because of the relatively low numbers of patients with a positive family history in our study, but our findings are supported by a previous report describing that the overall prognosis of breast cancer in *BRCA* carriers receiving PST is similar to patients with sporadic breast cancers receiving PST.¹⁷

Conclusion

Our study demonstrated that multivariate analysis demonstrates that pCR is not an independent significant prognostic marker for TNBC patients receiving PST. Clinical response is a stronger surrogate marker than pCR for a favorable prognosis. The importance of clinical response should be further investigated in multicenter clinical trials, and as well, novel treatment procedures need to be established for TNBC patients with unfavorable responses to PST.

Clinical Practice Points

- Previous clinical studies have revealed that pCR is a surrogate marker for prognosis after PST, and pCR is usually used as the primary end point of clinical trials involving PST instead of OS or DFS.
- However, there is no report focused on triple-negative breast cancer receiving PST.
- From the current study, Kaplan–Meier analysis demonstrated that patients achieving pCR have more favorable prognosis than the

others, but multivariate analysis of characteristics after adjustment for confounders showed that clinical response, nodal status, and vascular invasion instead of pCR were the significant for patients' prognoses.

- Metaanalysis demonstrates that triple-negative patients have a relatively poor prognosis compared with patients with luminal types even if pCR is achieved,¹¹ and to our knowledge, this is the first report that pCR is not an independent prognostic marker for triple-negative breast cancer patients.
- We believe these findings will be of great interest to oncologists, and particularly to researchers working on breast cancer clinical trials.

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Disclosures

The authors have stated that they have no conflicts of interest.

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Histological factors for accurately predicting first locoregional recurrence of invasive ductal carcinoma of the breast

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The accurate assessment of the risk of first locoregional recurrence is very important for improving the survival of patients with invasive ductal carcinoma of the breast. The present study investigated which histological factors (both well-known histological factors and factors that we have proposed) were the most capable of accurately predicting first locoregional recurrence among 1042 patients with invasive ductal carcinoma and various tumor statuses (overall, nodal status, Union Internationale Contre le Cancer pathological TNM stage, adjuvant therapy status, and adjuvant radiotherapy status) using multivariate analyses by the Cox proportional hazard regression model. The present study clearly demonstrated that the best factor for accurately predicting locoregional recurrence was grade 3 lymph vessel tumor embolus (>4 mitotic figures and >6 apoptotic figures in tumor embolus), followed by type 2 invasive ductal carcinoma (negative for fibrotic foci but positive for atypical tumor-stromal fibroblast), grade 2 lymph vessel tumor embolus (1–4 mitotic figures and >0 apoptotic figures in tumor embolus; >0 mitotic figures and 1–6 apoptotic figures in tumor embolus), primary invasive tumor cell-related factors (>19 mitotic figures, presence of tumor necrosis, presence of skin invasion) and >5 mitotic figures in metastatic carcinomas to the lymph node. Our proposed factors were superior to well-known histological factors of primary invasive tumors or clinicopathological factors for the accurate prediction of first locoregional recurrence in patients with invasive ductal carcinoma of the breast. (*Cancer Sci* 2013; 104: 1252–1261)

Locoregional recurrence is an important prognostic factor for patients with invasive ductal carcinoma of the breast,⁽¹⁾ and several studies have been performed to clarify factors that are significantly associated with locoregional recurrence.^(2,3) These studies demonstrated that lymph vessel invasion, histological grade, tumor size, hormone receptor status, and HER2 status are very important predictors of locoregional recurrence in patients with invasive ductal carcinoma. We have already reported histological factors that are significantly associated with distant-organ metastasis or the tumor-related death of patients with invasive ductal carcinoma of the breast.⁽⁴⁾ Since the publication of our previous study,⁽⁴⁾ we have performed additional studies that identified the following new histological factors as predictors of the outcome of patients with invasive ductal carcinoma of the breast⁽⁵⁾: (i) type of invasive ductal carcinoma,⁽⁶⁾ (ii) grading system for lymph vessel tumor emboli,⁽⁷⁾ (iii) number of apoptotic figures in blood vessel tumor emboli,⁽⁸⁾ (iv) number of mitotic figures in metastatic carcinomas to the lymph node,⁽⁹⁾ and (v) maximum dimension of metastatic carcinomas to the lymph node.⁽⁸⁾ Although our

studies clearly demonstrated that the factors we previously reported were very useful for accurately predicting tumor recurrence, distant-organ metastasis or tumor-related death,^(4–9) we have not yet investigated whether these factors are significantly associated with the locoregional recurrence of invasive ductal carcinoma of the breast. We are confident that clarification of the recurrent or metastatic patterns of invasive ductal carcinomas based on their histological features will provide clinicians, pathologists, and scientists with very important clues for accurately evaluating the true biological characteristics of invasive ductal carcinomas. Such a result would likely contribute to the establishment of targeted therapies for patients with invasive ductal carcinoma of the breast.

The purpose of the present study was to investigate which histological factors were most capable of accurately predicting first locoregional recurrence in patients with invasive ductal carcinoma of the breast.

Materials and Methods

Patients and histological examinations. The subjects of this study were 1042 consecutive patients with invasive ductal carcinoma of the breast who did not receive neoadjuvant therapy and were surgically treated at the National Cancer Center Hospital between January 2000 and December 2005 (the same case series as that used in our previous study).⁽⁵⁾ The invasive ductal carcinomas were diagnosed preoperatively using needle biopsy, aspiration cytology, mammography, or ultrasonography. All the patients were Japanese women, ranging in age from 23 to 72 years old (median, 55 years). All the tumors were classified according to the pathological UICC-TNM (pTNM) classification.⁽¹⁰⁾ The protocol (20–112) for this study was reviewed by the institutional review board of the National Cancer Center.

The clinicopathological factors, well-known histological factors and the eight factors that we previously proposed were evaluated and we arranged the above mentioned factors into five groups (Table 1). The eight factors that we previously proposed are as follows (Tables 1 and 2): (i) fibrotic focus;^(11,12) (ii) type of invasive ductal carcinoma (Fig. 1a–c);⁽⁶⁾ (iii) grading system for lymph vessel tumor emboli (Fig. 1d–f);⁽⁷⁾ (iv) number of apoptotic figures in blood vessel tumor emboli;⁽⁸⁾ (v) grade of stromal fibrosis in metastatic carcinomas to the lymph node;⁽⁸⁾ (vi) maximum dimension of metastatic carcinomas to the lymph node;⁽⁸⁾ (vii) number of extranodal blood vessel tumor emboli;⁽⁸⁾ and (viii) number of mitotic figures in

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Table 1. Groups and factors

<i>Clinicopathological group</i>				
1	Adjuvant therapy			
	None	Endocrine therapy	Chemoendocrine therapy	Chemotherapy
2	Adjuvant radiotherapy			
	Not received		Received	
3	Age (year)			
	≤39		>39	
4	Allred scores for estrogen receptors in tumor cells			
	0 or 2	3 to 6	7 or 8	
5	Allred scores for progesterone receptors in tumor cells			
	0 or 2	3 to 6	7 or 8	
6	HER2 category			
	0 or 1	2	3	
<i>Primary invasive tumor cell-related group</i>				
1	Histologic grade			
	Grade 1	Grade 2	Grade 3	
2	Invasive tumor size (mm)			
	≤20	>20 to ≤50	>50	
3	Nuclear feature of primary invasive tumors			
	Small	Moderate	Marked	
4	Number of mitotic figures in the primary invasive tumors			
	≤9	>9 to ≤19	>19	
5	Skin invasion			
	Absent		Present	
6	Tumor necrosis			
	Absent		Present	
<i>Primary tumor-stromal fibroblast-related group</i>				
1	Fibrotic focus, dimension (mm)			
	Absent	≤8	>8	
2	Types of invasive ductal carcinoma			
	Type 1	Type 2	Type 3	Type 4
<i>Tumor embolus-related group</i>				
1	Grading system for lymph vessel tumor embolus			
	Grade 0	Grade 1	Grade 2	Grade 3
2	Number of apoptotic figures in blood vessel tumor emboli			
	Absent	≤2	>2	

Table 1. (continued)

Metastatic carcinomas to the lymph node-related group					
1	UICC pN category				
	pN0	pN1mi	pN1	pN2	pN3
2	Grade of stromal fibrosis in metastatic carcinomas to the lymph node				
	No nodal metastasis	None, mild and moderate		Severe	
3	Maximum dimension of metastatic carcinomas to the lymph node (mm)				
	No nodal metastasis	≤20		>20	
4	Number of extranodal blood vessel tumor emboli				
	No nodal metastasis	≤2		>2	
5	Number of mitotic figures in metastatic carcinomas to the lymph node				
	No nodal metastasis	≤5		>5	

pN0, no nodal metastasis, but including lymph node with isolated tumor cell clusters (single tumor cells or small clusters of cells not more than 0.2 mm in greatest dimension); pN1mi, cases with micrometastasis (larger than 0.2 mm, but none larger than 2.0 mm in greatest dimension); pN1, 1–3 nodal metastases; pN2, 4–9 nodal metastases; pN3, 10 or more nodal metastases; no nodal metastasis, pN0 cases excluding the seven cases with lymph nodes containing isolated tumor cell clusters; Grade 0 of grading system for lymph vessel tumor embolus, no lymph vessel invasion.

metastatic carcinomas to the lymph node.⁽⁹⁾ In the present study, seven of the 598 pN0 cases had isolated tumor cell clusters (ITC)⁽¹⁰⁾ (Table 3). We excluded these seven cases from the pN0 cases and these cases showed no stromal fibrosis in metastatic carcinomas to the lymph node, showed a ≤20 mm maximum dimension in metastatic carcinomas to the lymph node, showed ≤2 extranodal blood vessel tumor emboli, or showed ≤5 mitotic figures in metastatic carcinomas to the lymph node (Table 3). Thus, we classified these seven cases as cases with no grade of stromal fibrosis, those with a ≤20 mm maximum dimension, those with ≤2 extranodal blood vessel tumor emboli, or those with ≤5 mitotic figures in metastatic carcinomas to the lymph node (Table 3).

The following antibodies were used for immunohistochemistry: anti-estrogen receptor mouse monoclonal antibody ER88 (BioGenex, Fremont, CA, USA), anti-progesterone receptor mouse monoclonal antibody PR88 (BioGenex), and anti-HER2 mouse monoclonal antibody CB11 (BioGenex). Allred scores for estrogen receptor or progesterone receptor were assessed according to our previously study.⁽¹³⁾ We defined an Allred score of 0 or 2 for ER or PR as being negative for ER or PR and Allred scores of 3 or more for ER or PR as being positive for ER or PR. HER2 expression in tumor cells was categorized according to the definition of Wolf.⁽¹⁴⁾ All types 2 and 4 invasive ductal carcinomas were immunohistochemically studied using monoclonal antibodies to keratins (AE1/3) to confirm that the atypical tumor-stromal fibroblasts were not modified invasive tumor cells, and fibroblasts that were negative for keratins were considered as atypical tumor-stromal fibroblasts (Fig. 1b). We also performed immunohistochemical staining for alpha-smooth muscle actin for types 2 and 4 invasive ductal carcinomas to investigate whether atypical tumor-stromal fibroblasts are myofibroblasts (Fig. 1c). Some invasive ductal carcinomas contained large lymph vessel tumor emboli, especially in invasive ductal carcinomas containing a grade 2 or grade 3 lymph vessel tumor emboli, and it was difficult to determine whether they were true lymph vessel tumor emboli or a non-invasive ductal carcinoma component by hematoxylin and eosin staining alone. We therefore performed immunohistochemical staining with D2-40 antibody (monoclonal mouse antibody, Signet, Dedham, MA, USA, 1:200) to confirm that

the lymph vessel tumor emboli identified by hematoxylin and eosin staining were true tumor emboli in some invasive ductal carcinomas with grade 2 or grade 3 lymph vessel tumor emboli (Fig. 1e). Histologic grade, nuclear feature of primary invasive tumors, and mitotic activity index in primary invasive tumors were evaluated according to the criteria of Elston and Ellis.⁽¹⁵⁾ Tumor necrosis in primary invasive tumors was evaluated according to the definition of Gilchrist.⁽¹⁶⁾

Patient outcome and statistical analysis. Survival was evaluated using a median follow-up period of 98 months (range: 63–134 months) until March 2011. Of the 1042 invasive ductal carcinoma patients, first locoregional recurrence was observed in 47 out of 1042 patients with invasive ductal carcinoma. The first locoregional recurrence-free survival period was calculated using the time of surgery as the starting point. The factors that were significantly associated with first locoregional recurrence in the univariate analyses were then entered together into multivariate analyses using the Cox proportional hazard regression model. In addition, we conducted to compare the power of grading system for lymph vessel tumor emboli with that of the following three lymphatic parameters for accurately predicting the first locoregional recurrence in multivariate analysis using the Cox proportional hazard regression model: (i) the presence or absence of lymph vessel invasion; (ii) real numbers of lymph vessel invasion;⁽¹⁷⁾ and (iii) location of lymph vessel tumor emboli⁽¹⁸⁾ (inside area of the tumor, advanced area within the tumor and outside area of the tumor). In this study, we were unable to perform multivariate analyses for first locoregional recurrence because of a small sample size (fewer than 10 patients) in patients who did not receive adjuvant therapy. The case-wise and step-down method was applied until all the remaining factors were significant at a *P*-value of <0.05. First locoregional recurrence-free survival curves were drawn by the Kaplan–Meier method. All the analyses were performed using Statistica/Windows software (StatSoft, Tulsa, OK, USA).

Results

Patients. All of the patients had a solitary lesion; 498 patients were premenopausal, and 544 were postmenopausal. A partial mastectomy had been performed in 458 patients, and a

Table 2. Histological features, criteria or assessing methods of the five factors that we have proposed

1	<i>Histological features of atypical tumor-stromal fibroblasts and the type of invasive ductal carcinoma</i>			
(1)	The presence of atypical tumor-stromal fibroblasts was defined based on the presence of one or more atypical tumor-stromal fibroblasts in the tumor stroma inside and outside of the fibrotic foci in invasive ductal carcinoma. Although atypical tumor-stromal fibroblasts are occasionally distributed at random locations in the tumor stroma, they tend to exist within the cellular area of the tumor-stromal fibroblasts			
(2)	The number of nuclei in an atypical tumor-stromal fibroblast is one or more. The nuclear size of an atypical tumor-stromal fibroblast is two or more times larger than that of an ordinary tumor-stromal fibroblast. The nuclear features of an atypical tumor-stromal fibroblast include an irregular or convoluted shape, and also include various bizarre shapes			
(3)	An obvious small to large size nucleolus or nucleoli are seen in the nucleus or nucleoli of atypical tumor-stromal fibroblasts and some atypical tumor-stromal fibroblasts show a coarsely granulated nuclear chromatin pattern			
Type	Fibrotic focus	Atypical tumor-stromal fibroblast not forming a fibrotic focus	Atypical tumor-stromal fibroblast forming a fibrotic focus	
1	Absent	Absent	Not applicable	
2	Absent	Present	Not applicable	
3	Present	Not assessed	Absent	
4	Present	Not assessed	Present	
2	<i>Grading system for lymph vessel tumor embolus</i>			
Grade 0	Invasive ductal carcinomas with no lymph vessel tumor embolus			
Grades 1–3	Invasive ductal carcinomas with lymph vessel tumor embolus or emboli			
		<u>No. of mitotic figures</u>	<u>No. of apoptotic figures</u>	
Grade 1		0	0	
		0	Any	
		Any	0	
Grade 2		1–4	>0	
		>0	1–6	
Grade 3		>4	>6	
(1)	The numbers of tumor cell mitotic figures and apoptotic figures in lymph vessels are counted in 20 high-power fields. In carcinomas containing a small number of lymph vessel tumor emboli, the mitotic figures and apoptotic figures are counted in fewer than 20 high-power fields			
(2)	A large lymph vessel tumor emboli located far from the stroma-invasive tumor margin is selected and the mitotic figures and apoptotic figures in the lymph vessel tumor emboli or embolus are counted			
(3)	The numbers of mitotic figures and apoptotic figures in tumor cells composing the lymph vessel tumor embolus or emboli in the high-power field containing the largest number of mitotic figures, and/or the largest number of apoptotic figures are recorded as the number of mitotic figures and apoptotic figure in the lymph vessel tumor emboli or embolus. The cumulative numbers of tumor cell mitotic figures and apoptotic figures in the lymph vessel tumor emboli in all 20 high-power fields are not used			
3	<i>Grade of stromal fibrosis in metastatic carcinomas to the lymph node</i>			
None	Metastatic carcinoma with no tumor-stromal fibrosis			
Mild	Metastatic carcinoma occupied by ≤30% tumor-stromal fibrosis			
Moderate	Metastatic carcinoma occupied by >30 to ≤80% tumor-stromal fibrosis			
Severe	Metastatic carcinoma occupied by >80% tumor-stromal fibrosis			
4	<i>Extranodal blood vessel tumor embolus or emboli</i>			
Tumor embolus or emboli in blood vessel or vessels with a smooth muscle-supported endothelial lining in perinodal adipose tissues was/were assessed as extranodal blood vessel tumor embolus or emboli				
5	<i>Mitotic figures in metastatic carcinomas to the lymph node</i>			
(1)	A random search for mitotic figures in metastatic mammary carcinoma to the lymph nodes is performed using high-power magnification fields (×10 or ×20) of the tumor area			
(2)	Next, one high-power magnification field (×40) of the tumor area containing the highest number of mitotic figures is selected to determine the largest number of metastatic mammary carcinoma to the lymph nodes exhibiting mitotic figures			

modified radical mastectomy had been performed in 584. The surgical margins of all the partial mastectomy materials were histologically examined to confirm whether tumor cells were absent or present at the surgical margins of the materials; we confirmed that all the materials had been completely resected because the outermost edges of the tumors were 5 mm or further from the surgical margin of the materials. A Level I and II

axillary lymph node dissection had been performed in all the patients, and a Level III axillary lymph node dissection had been performed in some of the patients. Of the 1042 patients, 873 received adjuvant therapy, consisting of chemotherapy in 217 patients, endocrine therapy in 281 patients, and chemoendocrine therapy in 375 patients. The chemotherapy regimens used were anthracycline-based with or without taxane and

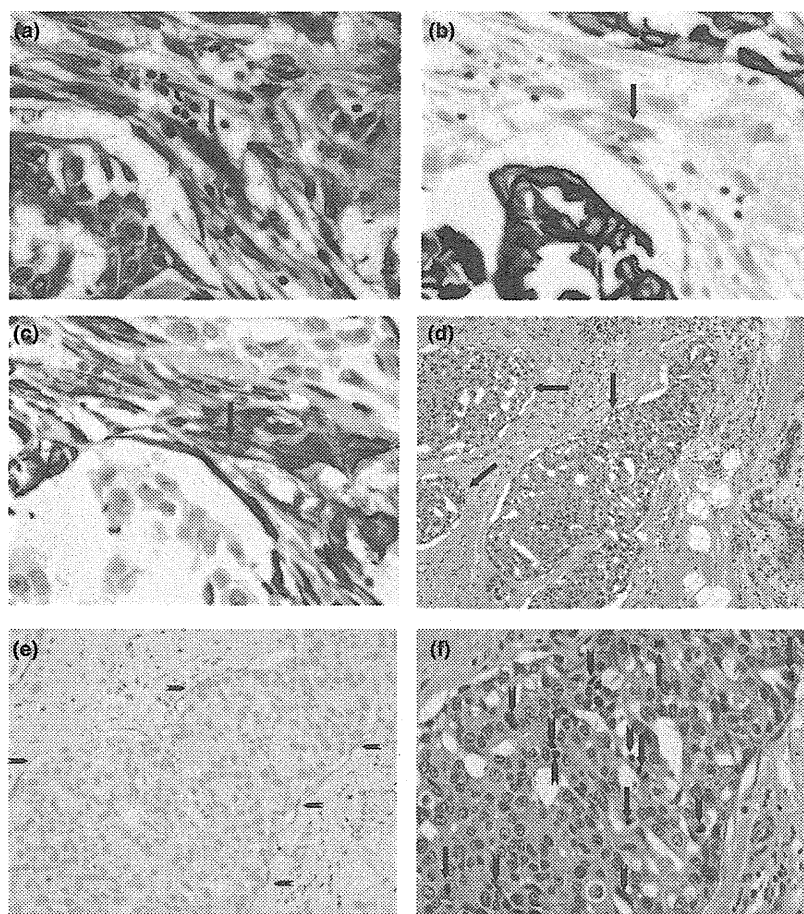


Fig. 1. (a–c) Type 2 invasive ductal carcinoma. One atypical tumor-stromal fibroblast with a large spindle nucleus is visible in the tumor stroma (arrow). The fibroblast was stained negative for AE1/3 (arrow, b) and positive for smooth muscle actin (arrow, c). The invasive tumor cells were stained positive for AE1/3 (b). (d–f) Grade 3 lymph vessel tumor emboli. Three large lymph vessel tumor emboli are present, and the wall of one of the tumor lymph vessels containing the embolus was positive for D2-40 (arrowheads, e). Five mitotic tumor cells (arrows) and eight apoptotic tumor cells (arrowheads) are visible within the tumor embolus (f).

non-anthracycline-based. The endocrine therapy regimens consisted of tamoxifen with or without a gonadotropin-releasing-hormone agonist, tamoxifen, with or without an aromatase inhibitor, an aromatase inhibitor alone, or a gonadotropin-releasing-hormone agonist alone. Of the 1042 patients, 466 patients received adjuvant radiotherapy.

Univariate analyses for first locoregional recurrence. Overall, age ($P = 0.026$), the Allred score for estrogen receptors in the tumor cells ($P = 0.017$), the histologic grade ($P = 0.009$), the invasive tumor size ($P < 0.001$), nuclear features of the primary-invasive tumor cells ($P < 0.001$), the number of mitotic figures in the primary-invasive tumor cells ($P = 0.002$), tumor necrosis ($P = 0.019$), the type of invasive ductal carcinoma, the grading system for lymph vessel tumor emboli, the UICC pN category, the grade of stromal fibrosis in metastatic carcinomas to the lymph node, the maximum dimension of metastatic carcinomas to the lymph node, the number of extranodal blood vessel tumor emboli, and the number of mitotic figures in metastatic carcinomas to the lymph node were significantly associated with first locoregional recurrence in the univariate analyses (Table 3). The fibrotic focus dimension (Table 3), the number of apoptotic figures in blood vessel tumor emboli (Table 3), adjuvant therapy, adjuvant radiotherapy, the Allred score for progesterone receptors in the tumor cells, the HER2 category, and the skin invasion were not significantly associated with first locoregional recurrence in the univariate analyses (data not shown). Atypical tumor-stromal fibroblast was observed in 69 (7%) cases (type 2 and 4 invasive ductal carcinoma cases) among 1042 cases (Table 3). The presence of atypical tumor-stromal fibroblasts stained positive for alpha-

smooth muscle actin was observed in 60 (87%) out of 69 types 2 and 4 invasive ductal carcinomas (type 2: 35/40 cases, 88%; type 4: 25/29 cases, 86%).⁽⁶⁾

Multivariate analysis for clarifying the best lymphatic factor for accurately predicting first locoregional recurrence. Number of lymph vessel invasion ranged from 0 to 494 (median number and standard error: 0 and 1.1) in the present study. Only the grading system for lymph vessel tumor emboli significantly increased the hazard ratio for first locoregional recurrence in the multivariate analysis ($P = 0.002$). The presence of lymph vessel invasion ($P = 0.158$), real number of lymph vessel tumor emboli ($P = 0.144$), or location of lymph vessel tumor emboli (inside area of the tumor: $P = 0.227$; advanced area within the tumor: $P = 0.512$; outside area of the tumor: $P = 0.425$) failed to significantly increase the hazard ratio for first locoregional recurrence in the multivariate analysis.

Multivariate analyses for first locoregional recurrence. Overall ($n = 1042$), lymph vessel tumor embolus grade 2 ($P < 0.001$, Fig. 2a) and 3 ($P < 0.001$, Fig. 2a), and type 2 invasive ductal carcinoma ($P < 0.001$, Fig. 2b) significantly increased the hazard ratios for first locoregional recurrence in the multivariate analyses (Table 8). Lymph vessel tumor embolus grade 3 was significantly associated with first locoregional recurrence in a manner that was independent of almost all the tumor statuses, except for adjuvant radiotherapy status (received adjuvant radiotherapy) (Tables 4–8). Type 2 invasive ductal carcinoma was significantly associated with first locoregional recurrence among the overall patients who had received adjuvant therapy ($P < 0.001$), the UICC pN0 patients (Tables 4 and 8), the UICC pN1-3 patients (Tables 4 and 8), the UICC

Table 3. Frequencies of first locoregional recurrence of the eight histological factors that we have proposed and UICC pN category

	Cases (%) 1042	No. patients (%)		
		First locoregional recurrence		
		Present 47	Absent 995	P-value
Primary tumor-stromal fibroblast-related group				
Fibrotic focus, dimension (mm)				
Absent	667	30 (5)	637 (95)	0.624
≤8	221	9 (4)	212 (96)	
>8	154	8 (5)	146 (95)	
Types of invasive ductal carcinoma				
Type 1	627	23 (4)	604 (96)	<0.001
Type 2	40	7 (18)	33 (82)	
Type 3	346	15 (4)	331 (96)	
Type 4	29	2 (7)	27 (93)	
Tumor embolus-related group				
Grading system for lymph vessel tumor embolus				
Grade 0	666	20 (3)	646 (97)	<0.001
Grade 1	250	6 (2)	244 (98)	
Grade 2	97	12 (12)	85 (88)	
Grade 3	29	9 (31)	20 (69)	
Number of apoptotic figures in blood vessel tumor emboli				
Absent	890	36 (4)	854 (96)	0.071
≤2	78	6 (8)	72 (92)	
>2	74	5 (7)	5 (93)	
Metastatic carcinomas to the lymph node-related group				
UICC pN category				
pN0	598	17 (3)	581 (97)	<0.001
pN1mi	20	0	20 (100)	
pN1	291	16 (6)	275 (94)	
pN2	85	6 (7)	79 (93)	
pN3	48	8 (17)	40 (83)	
Grade of stromal fibrosis in metastatic carcinomas to the lymph node				
No nodal metastasis	591	17 (3)	574 (97)	<0.001
None, mild and moderate	415	25 (6)	390 (94)	
Severe	36	5 (14)	31 (86)	
Maximum dimension of metastatic carcinomas to the lymph node (mm)				
No nodal metastasis	591	17 (3)	574 (97)	<0.001
≤20	396	26 (7)	370 (93)	
>20	55	4 (7)	51 (93)	
Number of extranodal blood vessel tumor emboli				
No nodal metastasis	591	17 (3)	574 (97)	<0.001
≤2	423	25 (6)	398 (94)	
>2	28	5 (18)	23 (82)	
Number of mitotic figures in metastatic carcinomas to the lymph node				
No nodal metastasis	591	17 (3)	574 (97)	<0.001
≤5	286	12 (4)	274 (96)	
>5	165	18 (11)	147 (89)	

NA, not available; pN0, no nodal metastasis, but including lymph node with isolated tumor cell clusters (single tumor cells or small clusters of cells not more than 0.2 mm in greatest dimension); pN1mi, cases with micrometastasis (larger than 0.2 mm, but none larger than 2.0 mm in greatest dimension); pN1, 1–3 nodal metastases; pN2, 4–9 nodal metastases; pN3, 10 or more nodal metastases; no nodal metastasis, pN0 cases excluding the seven cases with lymph nodes containing isolated tumor cell clusters; Grade 0 of grading system for lymph vessel tumor embolus, no lymph vessel invasion.

pTNM stages I and II patients (Tables 5 and 8), the patients who had received endocrine therapy (Tables 6 and 8), the patients who had received chemotherapy (Tables 6 and 8), the patients who had not received adjuvant radiotherapy

Table 4. Multivariate analyses for first locoregional recurrence in invasive ductal carcinoma patients who received adjuvant therapy according to UICC pN category

	First locoregional recurrence			
	pN0 (n = 453)		pN1-3 (n = 420)	
	Hazard ratio (95% CI)	P-value	Hazard ratio (95% CI)	P-value
Grading system for lymph vessel tumor embolus				
Grade 0	1.0		1.0	
Grade 1	1.0		1.5 (0.5–4.5)	0.503
Grade 2	11.9 (3.0–46.6)	<0.001	2.2 (0.7–6.7)	0.163
Grade 3	11.9 (3.0–46.6)	<0.001	11.7 (3.4–39.9)	<0.001
Types of invasive ductal carcinoma				
Type 1	1.0		1.0	
Type 2	6.1 (1.2–29.9)	0.025	6.3 (2.0–20.0)	0.002
Type 3	2.0 (0.5–8.6)	0.362	0.9 (0.4–2.2)	0.810
Type 4	9.8 (0.9–105.8)	0.059	NA	
Number of mitotic figures in the primary invasive tumors				
≤9	1.0		1.0	
>9 to ≤19	3.4 (0.3–40.1)	0.323	–	
>19	4.7 (1.2–18.4)	0.023	–	

–, not significant; NA, not available; no nodal metastasis, pN0 cases excluding the seven cases with lymph nodes containing isolated tumor cell clusters.

Table 5. Multivariate analyses for first locoregional recurrence in invasive ductal carcinoma patients who received adjuvant therapy according to UICC pTNM stage

	First locoregional recurrence			
	Stages I and II (n = 692)		Stage III (n = 181)	
	Hazard ratio (95% CI)	P-value	Hazard ratio (95% CI)	P-value
Grading system for lymph vessel tumor embolus				
Grade 0	1.0		1.0	
Grade 1	1.8 (0.6–5.4)	0.319	0.3 (0.03–2.10)	0.210
Grade 2	7.9 (2.9–20.9)	<0.001	0.7 (0.1–3.2)	0.596
Grade 3	15.8 (3.2–77.3)	<0.001	8.1 (2.4–28.1)	<0.001
Types of invasive ductal carcinoma				
Type 1	1.0		1.0	
Type 2	6.4 (2.3–18.2)	<0.001	–	
Type 3	1.2 (0.5–3.3)	0.685	–	
Type 4	NA		–	
Tumor necrosis				
Absent	1.0		1.0	
Present	2.4 (1.0–5.8)	0.045	–	

–, not significant; NA, not available.

(Tables 7 and 8) and the patients who had received adjuvant radiotherapy (Tables 7 and 8). Lymph vessel tumor embolus grade 2 was significantly associated with first locoregional recurrence among the overall patients who had received adjuvant therapy ($P < 0.001$), the UICC pN0 patients (Tables 4 and 8), the UICC pTNM stages I and II patients (Tables 5 and 8) and the patients who had received chemoendocrine therapy (Tables 6 and 8). Twenty or more mitotic figures in primary invasive tumors, the presence of tumor necrosis, and the presence of skin invasion were significantly associated with first locoregional recurrence among the UICC pN0 patients (Tables 4 and 8), among the UICC pTNM stages I and II patients (Tables 5 and 8) and among the patients who had

Table 6. Multivariate analyses for first locoregional recurrence in invasive ductal carcinoma patients who received adjuvant therapy according to adjuvant therapy status

	First locoregional recurrence					
	Endocrine (n = 281)		Chemoendocrine (n = 375)		Chemotherapy (n = 217)	
Grading system for lymph vessel tumor embolus						
Grade 0	1.0		1.0		1.0	
Grade 1	0.6 (0.1–3.4)	0.602	1.7 (0.3–9.1)	0.545	0.8 (0.09–6.40)	0.795
Grade 2	1.6 (0.2–12.0)	0.667	6.8 (1.3–36.8)	0.026	0.8 (0.09–7.30)	0.866
Grade 3	25.8 (1.2–560.0)	0.038	9.8 (1.4–70.8)	0.024	27.5 (6.3–119.1)	<0.001
Types of invasive ductal carcinoma						
Type 1	1.0		1.0		1.0	
Type 2	37.2 (3.6–369.7)	0.002	–		18.6 (3.6–90.7)	<0.001
Type 3	7.4 (0.9–59.0)	0.058	–		1.5 (0.4–6.1)	0.579
Type 4	NA		–		5.4 (0.6–52.6)	0.145
Number of mitotic figures in metastatic carcinomas to the lymph node						
No nodal metastasis	1.0		1.0		1.0	
≤5	3.1 (0.7–12.9)	0.120	–		–	
>5	20.1 (1.3–312.3)	0.032	–		–	
Skin invasion						
Absent	1.0		1.0		1.0	
Present	–		–		5.4 (1.4–21.6)	0.014

–, not significant; NA, not available.

Table 7. Multivariate analyses for first locoregional recurrence in invasive ductal carcinoma patients who received adjuvant therapy according to adjuvant radiotherapy status

	First locoregional recurrence			
	No adjuvant radiotherapy (n = 576)		Adjuvant radiotherapy (n = 466)	
	Hazard ratio (95% CI)	P-value	Hazard ratio (95% CI)	P-value
Types of invasive ductal carcinoma				
Type 1	1.0		1.0	
Type 2	3.2 (1.1–9.5)	0.041	6.0 (1.2–29.3)	0.026
Type 3	1.3 (0.5–3.4)	0.563	0.9 (0.2–3.3)	0.843
Type 4	3.1 (0.3–29.8)	0.334	NA	
Grading system for lymph vessel tumor embolus				
Grade 0	1.0		1.0	
Grade 1	1.7 (0.5–5.1)	0.366	–	
Grade 2	3.5 (0.9–16.7)	0.051	–	
Grade 3	129.8 (29.1–578.0)	<0.001	–	
Number of mitotic figures in metastatic carcinomas to the lymph node				
No nodal metastasis	1.0		1.0	
≤5	–		1.6 (0.3–8.4)	0.603
>5	–		5.6 (1.8–17.4)	0.003

–, not significant; NA, not available.

received chemotherapy (Tables 6 and 8), respectively. Six or more mitotic figures in metastatic carcinomas to the lymph node were significantly associated with the first locoregional recurrence among the patients who had received endocrine therapy (Tables 6 and 8), and the patients who had received adjuvant radiotherapy (Tables 7 and 8).

Discussion

The results of the present study clearly exhibited an excellent power for the tumor embolus-related group for the accurate prediction of first locoregional recurrence in patients with invasive ductal carcinoma since this group was significantly associated with the first locoregional recurrence independent of the tumor-status categories except among patients who had received adjuvant radiotherapy (Table 8). Especially, the results

of the present study clearly exhibited an excellent power for lymph vessel tumor embolus grade 3 for the accurate prediction of first locoregional recurrence in patients with invasive ductal carcinoma independent of the tumor statuses (Table 8). In contrast, a grade 1 lymph vessel tumor embolus was not a significant predictor for first locoregional recurrence and had a similar predictive power to grade 0 lymph vessel tumor embolus (Fig. 2a); more than half of the 376 patients with lymph vessel invasion were classified as having lymph vessel tumor embolus grade 1 (Table 3). These results suggest that the lymph vessel tumor embolus grade was capable of selecting not only patients with the worst prognosis, but also patients with a good prognosis among patients with lymph vessel invasion. Although many studies have already reported that the presence or absence of lymph vessel invasion or the number of invaded lymph vessels is an important factor for accu-

Table 8. Groups and factors significantly associated with first locoregional recurrence in patients with invasive ductal carcinoma

First locoregional recurrence											
A			B: Patients who received adjuvant therapy (n = 873)								
Total	All	All	UICC pN category		UICC pTNM stage		Adjuvant therapy status			Adjuvant radiotherapy status	
11			pN0	pN1-3	I and II	III	Endocrine therapy	Chemoendocrine therapy	Chemotherapy	None	Yes
Tumor embolus-related group											
10	G3	G3	G3	G3	G3	G3	G3		G3	G3	•
	G2	G2	G2	G2	G2			G2			
Primary tumor-stromal fibroblast-related group											
9	T2	T2	T2	T2	T2	•	T2	•	T2	T2	T2
Primary invasive tumor cell-related group											
3	•	•	MF19	•	Tumor necrosis	•	•	•	Skin invasion	•	•
Metastatic carcinomas to the lymph node-related group											
2	•	•	•	•	•	•	MF5	•	•	•	MF5
Clinicopathological group											
0	•	•	•	•	•	•	•	•	•	•	•

•, not significant; A, overall patients; G3, grade 3; G2, grade 2; T2, type 2 invasive ductal carcinoma; MF19, number of mitotic figure, >19; MF5, number of mitotic figures, >5.

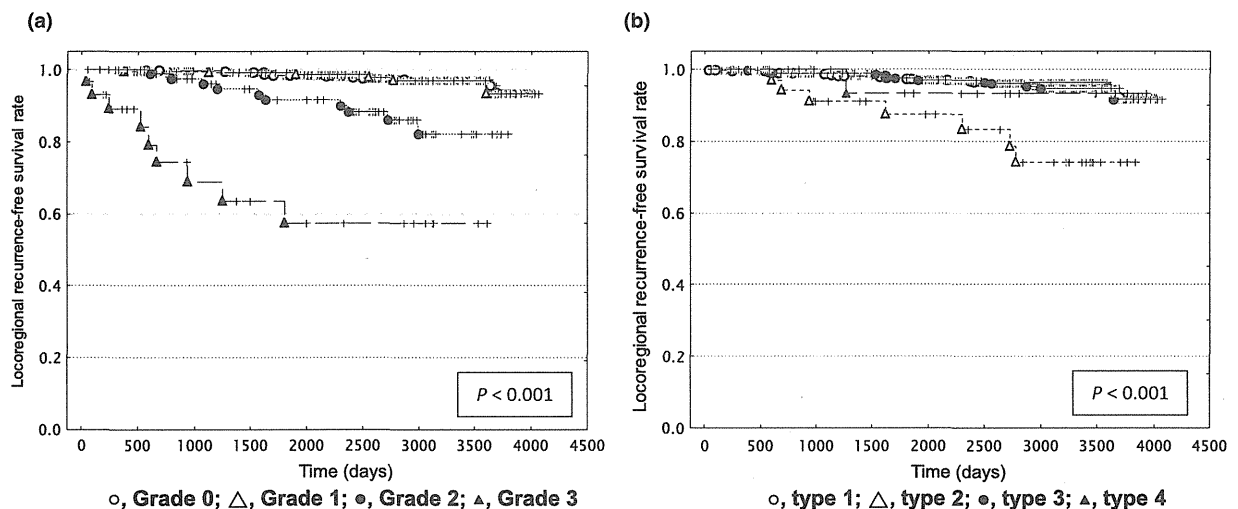


Fig. 2. First locoregional recurrence-free survival curves for overall patients with invasive ductal carcinoma (a and b). (a) Patients with grade 3 lymph vessel tumor emboli had the shortest locoregional recurrence-free survival curve. Patients with grade 2 lymph vessel tumor emboli also had a significantly shorter locoregional recurrence-free survival curve than patients with grade 1 lymph vessel tumor emboli or patients with grade 0 lymph vessel tumor emboli. (b) Patients with type 2 invasive ductal carcinoma had a significantly shorter first locoregional recurrence-free survival curve than patients with type 1 invasive ductal carcinoma, patients with type 3 invasive ductal carcinoma and patients with type 4 invasive ductal carcinoma.

rately predicting the locoregional recurrence of invasive ductal carcinoma,^(19,20) we confirmed that the grading system for lymph vessel tumor emboli is superior to the presence or absence of lymph vessel invasion, the number of invaded lymph vessels or the location of lymph vessels invaded for accurately predicting first locoregional recurrence in this study. Thus, we can conclude that the lymph vessel tumor embolus grade is the only lymph vessel assessment parameter that can accurately divide patients with lymph vessel invasion into a good prognosis group and a poor prognosis group. However, the locoregional predictive power of the lymph vessel tumor embolus grade was inferior to type 2 invasive ductal carcinoma or >5 mitotic figures in metastatic carcinomas to the lymph node in patients who had received adjuvant radiother-

apy; this finding strongly suggests that adjuvant radiotherapy prevents locoregional recurrence in patients with lymph vessel tumor embolus grades 3 or 2.⁽²¹⁾ Since the lymph vessel tumor embolus grade is assessed based on the numbers of mitotic figures and apoptotic figures in tumor cells in the lymph vessel,⁽⁷⁾ adjuvant radiotherapy probably inhibits the acceleration of the cell cycle in tumor cells in the lymph vessel. Thus, adjuvant radiotherapy may contribute to improving the outcome of patients with lymph vessel tumor embolus grade 3 or those with lymph vessel tumor embolus grade 2. From these, we can conclude that the lymph vessel tumor embolus grade in the tumor embolus-related group was the best grade for accurately predicting first locoregional recurrence among patients with invasive ductal carcinoma of a low-risk, intermediate-risk or high-risk

class. In addition, the results of the study also exhibited no predictive power for number of apoptotic figures in blood vessel tumor emboli for the accurate prediction of first locoregional recurrence in patients with invasive ductal carcinoma.

The next most-important group was the primary tumor-stromal fibroblast-related group, because this group accurately predicted first locoregional recurrence in nine of the 13 tumor statuses (Table 8). Especially, the results of the present study clearly exhibited a useful power for type 2 invasive ductal carcinoma for the accurate prediction of first locoregional recurrence in patients with invasive ductal carcinoma independent of the tumor statuses (Table 8). Type 2 invasive ductal carcinoma and type 4 invasive ductal carcinoma have atypical tumor-stromal fibroblasts, and the former does not have a fibrotic focus within them but the latter has a fibrotic focus with atypical tumor-stromal fibroblasts.⁽⁷⁾ Thus, the presence of atypical tumor-stromal fibroblasts alone probably plays an important role in the establishment of first locoregional recurrence under the condition of the absence of fibrotic foci in invasive ductal carcinomas. We have previously reported that atypical tumor-stromal fibroblasts exhibit a significantly higher frequency of p53 protein expression than ordinary tumor-stromal fibroblasts;^(6,22) this finding clearly indicates that the presence of atypical nuclear features is closely associated with p53 expression in tumor-stromal fibroblasts. p53 mutations in tumor-stromal fibroblasts are relatively common among primary breast cancers and have been reported to exert a positive effect on cancer growth.^(23,24) p53 gene abnormalities or specific reactive changes in p53 immunoreactivity in tumor-stromal fibroblasts produced by tumor cell-stromal cell interactions inside and outside of the fibrotic foci probably lead to the expression of p53 in tumor-stromal fibroblasts. Consequently, some tumor-stromal fibroblasts expressing p53 inside and outside of fibrotic foci probably transform into atypical tumor-stromal fibroblasts. Furthermore, since many atypical tumor-stromal fibroblasts were also stained for smooth muscle actin,⁽⁶⁾ one can conclude that many of the atypical tumor-stromal fibroblasts have the biological characteristics of myofibroblasts.^(25,26) Thus, these atypical tumor-stromal fibroblasts likely play important roles in the first locoregional recurrence of invasive ductal carcinomas of the breast.

In conclusion, the present study clearly demonstrated that the following factors that we have proposed play very important roles in the establishment of first locoregional recurrence:

(i) lymph vessel tumor embolus grade; and (ii) atypical tumor-stromal fibroblast outside a fibrotic focus, and also clearly demonstrated that the primary invasive tumor cell-related group, the metastatic carcinomas to the lymph node-related group, and the clinicopathological group were strikingly inferior to the above two factors for the prediction of first locoregional recurrence (Table 8). Thus, we can conclude that the above two factors are very useful surrogate markers for accurately predicting first locoregional recurrence of patients with invasive ductal carcinoma of the breast. Clinicians usually plan the follow-up care of patients after the initial operation has been completed, deciding whether patients should be treated with adjuvant therapy and which type of adjuvant therapy should be performed based on pathological reports of the clinicopathological findings for the invasive ductal carcinomas. Thus, pathology reports of invasive ductal carcinomas that are based on the assessment of our proposed factors would probably provide clinicians with more important clues for the selection of patients with a high likelihood of locoregional recurrence among patients with invasive ductal carcinoma, compared with ordinary pathology reports of invasive ductal carcinomas, throughout the follow-up period after the initial operation. Since it has recently been reported that the gene expression profile and protein expression profile of the tumor stroma play a very important role in tumor progression in carcinoma,^(27,28) key proteins that are expressed in tumor cells with highly-accelerating cell cycle in the lymph vessels, but also by atypical tumor-stromal fibroblasts should be carefully investigated to develop targeted therapies that eradicate tumor cells with highly-accelerating cell cycle or atypical tumor-stromal fibroblast expressing key proteins, resulting in the improved outcome of patients with invasive ductal carcinoma of the breast.

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Disclosure Statement

The authors have no conflict of interest.

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Original article

Sentinel and nonsentinel lymph node assessment using a combination of one-step nucleic acid amplification and conventional histological examination



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ABSTRACT

Background: Clinical significance of intraoperative sentinel lymph node (SLN) metastases detection using one-step nucleic acid amplification (OSNA) has not been thoroughly investigated. The aim of this study was to assess the usefulness of using a combination of OSNA and conventional histological examinations. **Materials and methods:** We included 772 consecutive patients with clinical node-negative cTis–cT3 primary breast cancer who underwent SLN biopsy with intraoperative OSNA and multi-section histological examination at our institution. We estimated the concordance rate and compared SLN metastases detection rates between the two methods. We also compared non-SLN metastasis detection rate between patients who tested positive in OSNA and those who tested positive in histology.

Results: Among 772 patients, SLN metastases were intraoperatively detected in 211 (26.4%) by either OSNA or histology, in 168 (21.8%) by OSNA, and in 150 (19.4%) by histology. The concordance rate between OSNA and histological examination was 89.2%, but only 123 (58.8%) patients tested positive in both OSNA and histology; 45 were positive in OSNA only and 43 were positive in histology only.

SLN status as per both OSNA and histology was significantly correlated with the presence of non-SLN metastases and multivariate analysis-identified independent predictive factors of non-SLN metastases. **Conclusions:** Intraoperative SLN metastases detection may be more accurate with a combination of OSNA and histological examination than with OSNA or histological examination alone. By using both methods, we can reduce the risk of false negative rate in SLN biopsy, and may prevent physicians from overlooking patients with non-SLN metastases.

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Introduction

Conventional intraoperative histological examinations in sentinel lymph node (SLN) biopsy are well known to show high (10–30%) false-negative results for metastatic foci because only a few thin sections from a lymph node are examined in this technique. The suboptimal quality of frozen section slides and oversights by pathologists increase the false-negative detection rate. Moreover, use of more intensive methods, such as serial-section examination of each SLN, is impractical because it requires a heavy workload for pathologists [1].

Molecular assays have been developed to overcome these shortcomings. The one-step nucleic acid amplification (OSNA) assay (Sysmex, Kobe, Japan), which involves amplification and

quantitative measurement of cytokeratin 19 (CK19) mRNA levels, can detect lymph node metastases as accurately as can conventional histological examination, is faster [2], and detects more low-volume tumor nodal involvement than do conventional histological methods. However, whether these techniques can verify the need for further axillary treatment is unclear [3].

This study compared detection rates between OSNA and histological examination, both for intraoperative SLN metastases and for non-SLN metastases. We also discuss the possibility of omitting axillary lymph node dissection (ALND) for some patients with positive SLN metastases (SLN⁺)—specifically, those histological micro-metastases or isolated tumor cells (ITC), and OSNA 1⁺ patients.

Materials and methods

Subjects comprised 772 consecutive patients with clinically node-negative Tis–T3 primary breast cancer who underwent SLN

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biopsy with combined intraoperative OSNA and histological examination between February 2010 and June 2012 at the National Cancer Center Hospital, Tokyo, Japan. Patients who received neoadjuvant therapy, and male patients were excluded.

Clinical and pathological *T* and *N* factors were based on the Cancer Staging Manual of the American Joint Committee on Cancer (AJCC), 7th edition [4]. Patient characteristics are listed in Table 3. The cut-off value for ER and PR positivity was 10% positive cells for both, irrespective of intensity. HER2 positivity was defined as an HER2 score of >3 (>30% strong membrane immunoreaction-positive cells) or an HER2 gene/centromere 17 ratio of ≥ 2.0 as assessed by fluorescence in situ hybridization.

SLN biopsy procedure

First, 0.1 ml of ^{99m}Tc -phytate was prepared. Half of this solution was injected into the dermis of the areola while the remainder was injected into the dermis over the tumor on the day before surgery. In all patients, lymphoscintigraphy was performed 3 h after injection. In addition, 3–4 ml blue dye or 1 ml indocyanine green was injected into the peritumoral space or areola at the time of surgery. SLNs were identified using a hand-held gamma probe guided by nodal staining. Nodes that responded to near-infrared light, were stained with blue dye, or had high radioactive count were considered as SLNs. No more than four resected SLNs per patient were intraoperatively evaluated by both OSNA and histological examination. We omitted axillary dissection in patients with no SLN metastases and performed axillary dissection in patients with histological macrometastases, micrometastases, or ITCs in SLNs or positivity in OSNA. These patients were considered SLN⁺ in this study.

Preparation of SLNs

Excised SLNs were cut into 2-mm slices along the short axis and were alternately prepared for OSNA and histological examination.

Histological examination of SLNs

The sliced tissue specimens for histological examination were first subjected to intraoperative frozen-section diagnosis. These sliced tissues were then fixed in 10% formalin overnight, embedded in paraffin, cut into 4- μm -thick sections, stained with hematoxylin and eosin (HE), and subjected to permanent-section diagnosis.

Macrometastases were defined as SLN⁺ that measured >2 mm in greatest diameter, micrometastases as SLN⁺ that measured 0.2–2 mm in greatest diameter, and ITC as small clusters of cells ≤ 0.2 mm across their greatest diameter, as detected by HE staining or immunohistochemistry. Although ITCs are recommended to be classified as pNO(i⁺), they were considered as metastases in the present study.

OSNA assay for SLN examination

The details of the OSNA assay based on the RT-LAMP method were previously described by Tsujimoto et al. [5] Briefly, resected SLNs were homogenized with 4 ml lysis buffer solution and centrifuged at 10000 \times g at room temperature. The RD-100i system (Sysmex, Kobe, Japan) was used to analyze 2 μl of the lysed SLN supernatant.

Using OSNA, SLNs were considered to be SLN⁻ when the CK19 mRNA copy number was $< 2.5 \times 10^2/\mu\text{l}$, SLN⁺ 1⁺ when the copy number was $2.5 \times 10^2/\mu\text{l}$ – $5.0 \times 10^3/\mu\text{l}$, and metastases-positive 2⁺ when the copy number was $> 5.0 \times 10^3/\mu\text{l}$. A 1:10 dilution of homogenized lymph node solution was always prepared for each patient and analyzed simultaneously because excess protein may

interfere with the RT-LAMP reaction [5]. Lymph node lysates showing >250 copies/ μl of CK19 mRNA only in the 1:10 diluted solution were classified as positive and designated as +1 (inhibition positive). Permanent histological tissue sections were immunostained for CK19 when samples that were SLN⁻ by OSNA were histologically SLN⁺.

Permanent histological examination of non-SLNs

ALND was performed when specimens were SLN⁺ by either OSNA or histology. All non-SLNs were bisected along the long axis after formalin fixation. For each non-SLN, only one permanent HE tissue section for the representative cut surface was histologically examined.

Statistical analysis

We used the Mann–Whitney test to compare age and BMI between SLN⁺ and SLN⁻ patients, the χ^2 test to compare other variables, and performed logistic regression analysis to investigate odds ratios of individual parameters for non-SLN metastases. $P < 0.05$ was considered statistically significant. Confidence intervals (CIs) were set at the 95% level. SPSS statistical software (version 19, IBM SPSS Statistics, Chicago, IL, USA) was used for all statistical analyses.

Results

Concordance rate between histology and OSNA

SLN metastases, including ITC, were detected in 211 (27.3%) of the 772 patients: 145 (18.8%) by intraoperative examination of frozen HE-stained sections, 168 (21.8%) by OSNA, and 166 (21.5%) by the examination of permanent HE-stained sections (Table 1). Because we regarded ITC as histological metastases, ALND was performed for patients with ITC in SLNs.

The concordance rate between OSNA and intraoperative histological diagnosis was 88.2%, and that between OSNA and permanent histological diagnosis was 88.6%. The kappa value between OSNA diagnosis and permanent histological diagnosis was 0.66, indicating substantial concordance.

Table 1
Concordance of sentinel lymph node metastasis between OSNA diagnosis and histological diagnosis.

A. Comparison with frozen section diagnosis			
	Number of patients (%)		
	Total	Intraoperative frozen section	
		Histology (+)	Histology (–)
OSNA (+)	168	111	57
OSNA (–)	604	34	570
Total	772	145	627
B. Comparison with frozen section + permanent section diagnosis			
	Number of patients (%)		
	Total	Intraoperative + permanent sections	
		Histology (+)	Histology (–)
OSNA (+)	168	123	45
OSNA (–)	604	43	561
Total $\kappa = 0.66$	772	166	606

Histology (+) includes macrometastasis, micrometastasis, and isolated tumor cells (ITC), whereas Histology (–) includes others. OSNA (–) includes OSNA 2+, 1+, and +1, whereas OSNA (+) includes OSNA–.

Table 2

Detailed comparison of OSNA results with histological results for sentinel lymph node metastasis.

OSNA	Number of cases (%)				
	Total	Histological diagnosis (permanent section)			
		Macrometastasis	Micrometastasis	ITC	Negative
2+	90	78 (87)	8 (9)	0 (0)	4 (4)
1+	72	16 (22)	15 (21)	5 (7)	36 (50)
+I	6	1 (17)	0 (0)	0 (0)	5 (83)
–	604	9 (1)	23 (4)	11 (2)	561 (93)
Total	772	104	46	16	606

Among the 168 OSNA⁺ patients, SLN metastases were histologically detected in 123 (73%), including five with ITC. The remaining 45 (27%) patients were histologically SLN[–] (Tables 1 and 2). When OSNA results were stratified, SLN⁺ rate per

permanent histological examination was 96% (86 of 90) for OSNA 2 + patients, 50% (36 of 72) for OSNA 1 + patients, and 17% (one of six) for OSNA + I patients. Among the 604 OSNA[–] patients, 43 (7%) were histologically SLN⁺.

In contrast, 123 (74%) of the 166 histologically SLN⁺ patients, including 16 with ITC in SLNs, were SLN⁺ using OSNA.

Clinicopathological correlation with SLN status by histology and OSNA

Clinicopathological characteristics of the 772 patients are listed in Table 3. For SLN statuses detected by both permanent histology and OSNA, SLN metastases significantly correlated with cT-factor, pT-factor, histological type, LVI (lymphovascular invasion) and histological grade.

There were no significant differences in characteristics between patients with SLN metastases detected by OSNA and histological

Table 3

Correlations of clinicopathological parameters with sentinel lymph node status, detected by histopathological examination and by OSNA method.

Parameter	Number of cases (%)							
	Total N = 772	SLN status (%)			p	SLN status (%)		p
		Histology (+) N = 166	Histology (–) N = 606	OSNA (+) N = 168		OSNA (–) N = 604		
Age								
Average (range)	56.3 (27–92)	54.9 (27–84)	56.8 (28–92)	NS	54.1 (27–92)	56.9 (28–92)	NS	
<50	256	59 (23)	197 (77)		64 (25)	191 (75)		
≥50	516	107 (21)	409 (79)		104 (20)	413 (80)		
Menopause								
Premenopausal	311	72 (23)	239 (77)	NS	78 (25)	233 (75)	NS	
Postmenopausal	457	94 (21)	363 (79)		90 (20)	367 (80)		
Unknown	4	0	4 (100)		0	4 (100)		
BMI								
Average (range)	22.1 (13.2–40)	22.6 (17–40)	22.0 (13.2–35.8)	NS	22.1 (17–40)	22.1 (13.2–35.8)	NS	
<25	645	138 (21)	507 (79)		147 (23)	498 (77)		
≥25	126	28 (22)	98 (78)		21 (17)	105 (83)		
Unknown	1	0 (0)	1 (100)		0 (0)	1 (100)		
CT-factor								
Tis	159	6 (4)	153 (96)	<0.0001	8 (5)	151 (95)	<0.0001	
T1	355	66 (19)	289 (81)		76 (21)	279 (79)		
T2	252	90 (36)	162 (64)		80 (32)	172 (68)		
T3	6	4 (67)	2 (33)		4 (67)	2 (33)		
PT-factor								
Tis	119	0 (0)	119 (100)	<0.0001	8 (7)	111 (93)	<0.0001	
T1	413	72 (17)	341 (83)		73 (18)	340 (82)		
T2	209	73 (35)	136 (65)		68 (33)	141 (67)		
T3	29	21 (72)	8 (28)		19 (66)	10 (34)		
Unknown	2	0 (0)	2 (100)		0 (0)	2 (100)		
Histological type								
Carcinoma in situ	119	0 (0)	119 (100)	<0.0001	8 (7)	111 (93)	<0.0001	
Invasive ductal	566	150 (27)	416 (73)		143 (25)	423 (75)		
Special	86	16 (19)	70 (21)		17 (20)	69 (80)		
Others	1	0	1 (100)		0 (0)	1 (100)		
Lymphovascular invasion								
Negative	533	65 (12)	468 (88)	<0.0001	74 (14)	459 (86)	<0.0001	
Positive	230	101 (44)	129 (56)		92 (40)	138 (60)		
Unknown	9	0 (0)	9 (100)		2 (22)	7 (78)		
Histological grade								
1	217	17 (8)	200 (92)	<0.0001	26 (12)	191 (88)	<0.0001	
2	351	95 (27)	256 (73)		84 (24)	267 (76)		
3	202	54 (27)	148 (73)		58 (29)	144 (71)		
Unknown	2	0 (0)	2 (100)		0	2 (100)		
Hormone receptor								
Negative	113	19 (17)	94 (83)	NS	21 (19)	92 (81)	NS	
Positive	658	146 (22)	512 (78)		146 (22)	512 (78)		
Unknown	1	1 (100)	0 (0)		1 (100)	0 (0)		
HER2								
Negative	671	148 (22)	523 (78)	NS	147 (22)	524 (88)	NS	
Positive	86	17 (20)	69 (80)		18 (21)	68 (89)		
Unknown	15	1 (7)	14 (93)		3 (20)	12 (80)		

Histology (+) includes macrometastasis, micrometastases, and isolated tumor cells (ITC), whereas Histology (–) includes others. OSNA (+) includes OSNA 2+, 1+, and +I, whereas OSNA (–) includes OSNA–.

method. However, despite no statistical significance, percentages of early and low-grade tumors tended to be larger in the former group: no patient with carcinoma in situ showed histological SLN metastases, whereas eight (7%) of 119 patients with carcinoma in situ showed positivity in OSNA. Similarly, LVI⁻ (14% vs 12%), and histological grade 1 (12% vs 8%) tumors tended to test positive more frequently in OSNA (Table 3).

Correlation of SLN status with non-SLN status in patients who underwent ALND

Among the 211 SLN⁺ patients, 206 underwent ALND after SLN biopsy. The correlation of SLN status with non-SLN status based on OSNA and permanent histological examination is summarized in Table 4.

Among the 206 SLN⁺ by OSNA or histology, 53 (26%) had non-SLN metastases. The overall incidence of non-SLN metastases among patients with histological SLN metastases was 32% (52 of 162): 40% (42 of 104), 20% (nine of 44), and 8% (one of 13) for patients with macrometastases, micrometastases, and ITC in SLNs, respectively. In contrast, only one (2%) of the 44 patients with OSNA⁺ but histology⁻ SLN metastases exhibited non-SLN metastases (Table 4).

Clinicopathological characteristics were analyzed in a multivariate logistic regression model. Histological SLN status (macrometastases/nonmalignant cells; odds ratio, 12.17; 95% confidence interval (CI), 1.45–102.34; $P = 0.020$) and OSNA-determined SLN status (OSNA2⁺ to OSNA⁻; odds ratio, 4.75; 95% CI, 1.23–17.35; $P = 0.018$) were identified as independent predictive factors for non-SLN metastases (Table 5). These data indicate that OSNA 1⁺ status was not an independent predictor for non-SLN metastases.

Discussion

The concordance rate of SLN metastasis detection between OSNA and histological diagnoses is reportedly high, ranging from 86.3% to 96.3% [12,7–14]. The SLN metastasis detection rate by OSNA was higher than that by histological examination because OSNA can detect tumor cells in whole tissues [14–16].

Table 4
Comparison between sentinel lymph node (SLN) status and non-SLN status.

SLN status			Number of cases (%)			
			Total	Non-SLN metastases		Subtotal
pN stage	Histology	OSNA	Positive	Negative		
PN1	Macrometastasis	2+	78	37 (47)	41	42/104 (40)
		1+	16	4 (25)	12	
		+1	1	0 (0)	1	
		–	9	1 (11)	8	
PN1mi	Micrometastasis	2+	8	1 (13)	7	9/45 (20)
		1+	15	5 (33)	10	
		–	22	3 (14)	19	
pN0(i+) ^a	ITC	2+	0	0 (0)	0	1/13 (8)
		1+	5	1 (20)	4	
		–	8	0 (0)	8	
pN0 (mol+) ^b	–	2+	4	1 (25)	3	1/44 (2)
		1+	35	0 (0)	35	
		+1	5	0 (0)	5	
			206	53	154	53/206 (26)

Five patients who did not receive ALND were excluded from the calculation.

^a Axillary macro- or micrometastases absent but ITC present as per histology, regardless of OSNA results.

^b Axillary metastases absent as per histology but present as per OSNA (2+, 1+, and/or +1).

Table 5
Predictive factors for non-SLN metastasis by multivariate logistic regression model analysis.

Parameter	Odds Ratio	95% confidence interval	p Value
pT factor			
PT1	1		
pT2	1.29	0.59–2.83	0.52
pT3	2.64	0.89–7.85	0.081
Hormone receptor status			
Positive	1		
Negative	2.47	0.88–6.96	0.088
SLN status by histology			
No malignant cell	1		
ITC	4.99	0.26–95.17	0.285
Micrometastasis	8.98	0.99–81.76	0.052
Macrometastasis	12.17	1.45–102.34	0.02
SLN status by OSNA			
–	1		
1+	2.27	0.59–8.66	0.232
2+	4.75	1.23–17.35	0.018

We did not include patients with pTis and OSNA + 1 in the analysis because there were no patients with non-SLN metastases in these groups.

However, histological corroboration of cancer volume is impossible if entire SLN tissues are used for OSNA assays. We considered that comparison of intraoperative OSNA results with intraoperative histology results was necessary for several patients before complete substitution of intraoperative histological diagnosis by OSNA. In this study, the SLN metastasis detection rate using combined OSNA and histology was 27.1% higher than that by histology only and 25.6% higher than that by OSNA only.

Among the SLN⁺ 211 patients using either method, 88 showed discordant results. Only 123 (58.3%) patients were both OSNA⁺ and histology⁺. Such discordances were especially common among patients who were OSNA 1⁺ (50%, 36 of 72), OSNA + 1 (83%, five of six), and those with histology⁺ micrometastases (50%, 23 of 46) and histology⁺ ITC (69%, 11 of 16) in SLNs. In contrast, the discordance rate was only 4% (four of 90) among OSNA 2⁺ patients and 9% (nine of 104) among patients with histology⁺ macrometastases.

Reportedly, most discrepancies occur because of uneven distribution of minuscule metastases [6,7]. Vegue et al. showed that histological examination of a single SLN section misclassified 41.8% patients as SLN⁻ compared OSNA data ($P = 0.007$) [17]. Although we histologically assessed multiple slices of SLN samples (2-mm intervals, two to seven slices per node) to ensure accurate comparison of SLN⁺ rates between the two methods, uneven distribution of metastatic foci in SLNs appeared to occur in >40% SLN⁺ patients. Tamaki et al. examined SLN metastases using both OSNA and histology methods similar to those used in the present study and reported that discordant results due to uneven distribution occurred in 38% of patients with OSNA⁺ SLNs and 11% of patients with histology⁺ SLNs [7].

Another possible explanation for this discordance may lie in the false-negative results exhibited by tumors with low CK19 expression. Low CK19 protein expression is reported in approximately 2–3% of breast cancers [6,18]. In the present study, we performed CK19 immunostaining for 16 of 31 patients with histology⁺ but OSNA⁻ tumors; however, we found that only one of 16 patients with positive residual SLN metastases was CK19⁻. Therefore, most discordant results were attributed to uneven distribution of tumor cell foci in each SLN.

In the present study, the non-SLN metastasis detection rate was high in OSNA 2⁺ patients (43%, 39 of 90) and patients with SLN micrometastases (40%, 42 of 104). In addition, the incidences of non-SLN metastases among OSNA 1⁺ patients and patients

with histology⁺ micrometastases were 14% (10 of 71) and 20% (nine of 45), respectively. The present data concurs with those reported by Castellano et al., in which OSNA 2⁺ patients had a 42% chance of non-SLN metastases while OSNA 1⁺ patients had a 22% chance of non-SLN metastases [6]. When both OSNA and histology were combined, the rate of non-SLN metastasis detection was extremely low in patients with histology⁺ ITC in SLNs (regardless of OSNA status; 8%; 1 of 13), OSNA 1⁺ patients without histological metastases (0%; 0 of 35), and OSNA + I patients without histological metastases (0%; 0 of five). Both histology⁺ metastasis size and semiquantitative OSNA SLN data were significant independent predictors of non-SLN metastases according to logistic regression analysis. Moreover, combined use of OSNA and histological examination could identify patients whose SLN statuses, – specifically, ITC, OSNA 1⁺, and OSNA + I, without histology⁺ tumor deposits –, correlated with low risk of non-SLN metastasis.

Non-SLN metastases would have been overlooked in maximum 10% (four of 39) of SLN[–] patients if OSNA alone had been used. Similarly, non-SLN metastases would have been overlooked in 2% (one of 44) of SLN[–] patients if histological examination alone had been used. These estimations imply that ALND can be omitted in patients with SLN⁺ detected by OSNA only in combined OSNA and histological examination. The non-SLN metastasis detection rate was much lower in histology[–] SLNs than for OSNA[–] SLNs when combined examination was used.

OSNA tended to detect SLN metastases more frequently than histological examination in primary tumors with non-invasive histology, histological grade 1, and lack of LVI. Although Osako et al. reported that OSNA could detect metastases more frequently than histological frozen-section examination in elderly or post-menopausal patients [15], we could not find such an interaction on using OSNA.

In the present study, there were 21 discordant diagnoses between intraoperative frozen section examination and permanent section examination. These included three cases of macrometastases, 11 of micrometastases, and seven of ITC. OSNA detected more than half of the metastases missed by frozen section diagnosis: two of three cases of macrometastasis samples, six of 11 cases of micrometastasis, and four of seven cases of ITC samples. An advantage of using the OSNA assay is that it confirms histological results and identifies patients who require ALND. However, OSNA may also lead to unnecessary ALNDs. Given these circumstances, combining OSNA and histology can prevent physicians from overlooking SLN and non-SLN metastases that can be missed when either method is used alone.

ALND was recently shown to have no significant influence on clinical outcomes of patients with micrometastases or ITC [19,20]. Osako et al. showed that routine histological examination of non-SLN metastases could overlook many occult metastases that can be detected by combined OSNA and histological examination [21]. Our previous study found that SLN and non-SLN occult metastases that were not detected routinely but detected by serial-step sections at 85- μ m intervals did not have significant prognostic implications [22].

The OSNA assay is a promising alternative or additional tool for intraoperative detection of SLN metastases. Because of the low rate of metastases to non-SLNs, ALND may be omitted in patients with OSNA 1⁺/histology[–] SLNs or OSNA[–]/histology⁺ ITC⁺ SLNs when OSNA and histological examination are combined.

To date, however, there is no evidence of whether or not metastases evaluated only by molecular analysis require ALND. Further data on tumor recurrence and patient survival will clarify how SLN metastases detected by molecular methods can be optimally managed.

Conclusions

Intraoperative SLN metastasis detection may be more accurate using a combination of OSNA and histological examination than with OSNA or histological examination alone. This combination technique may prevent physicians from overlooking patients with non-SLN metastases. Although stratification of non-SLN⁺ and non-SLN[–] patients according to the present OSNA categories (2⁺, 1⁺, and +I) is not perfect, more complete predictions of non-SLN metastases using OSNA may only be possible if stratification of these categories is improved in the near future.

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Conflict of interest statement

None declared.

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Keywords: breast cancer; lymph node metastasis; molecular diagnostic technique; preoperative chemotherapy; OSNA

Molecular detection of lymph node metastasis in breast cancer patients treated with preoperative systemic chemotherapy: a prospective multicentre trial using the one-step nucleic acid amplification assay

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Background: For patients with breast cancer treated with preoperative chemotherapy, residual tumour burden in lymph nodes is the strongest prognostic factor. However, conventional pathological examination has limitations that hinder the accurate and reproducible measurement. The one-step nucleic acid amplification (OSNA) assay is a novel molecular method for detecting nodal metastasis. In this prospective multicentre trial, we assessed the performance of the OSNA assay in detecting nodal metastasis after chemotherapy.

Methods: In total, 302 lymph nodes from 80 breast cancer patients who underwent axillary dissection after chemotherapy were analysed. Each node was cut into two or four slices. One piece or alternate pieces were evaluated by pathology, and the other(s) were examined using the OSNA assay. The results of the two methods were compared. Stromal fibrosis, histiocytic aggregates, and degenerated cancer cells were regarded as chemotherapy-induced histological changes.

Results: The overall accuracy, sensitivity, and specificity of the OSNA assay compared with the reference pathology were 91.1%, 88.3%, and 91.7%, respectively. Of the 302 lymph nodes, 66 (21.9%) exhibited chemotherapy-induced histology. For these nodes, the accuracy, sensitivity, and specificity were 90.9%, 88.9%, and 93.3%, respectively.

Conclusion: The OSNA assay can detect the residual tumour burden as accurately as conventional pathology, although chemotherapy-induced histological changes are present.

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