assay (sensitivity, 88.3%; 95% CI, 77.4–95.2%). Of the 242 nodes identified as negative for metastasis on pathological examination, 222 nodes were identified as negative on the OSNA assay (specificity, 91.7%; 95% CI, 87.5–94.9%).

Chemotherapy-induced histological changes and performance of the OSNA assay. Of the 302 lymph nodes, 66 (21.9%) displayed chemotherapy-induced histological changes. The accuracy, sensitivity, and specificity of the OSNA assay relative to the reference pathology were 90.9% (60 out of 66), 88.9% (32 out of 36), and 93.3% (28 out of 30), respectively, among lymph nodes with chemotherapy-induced histological changes and 91.1% (215 out of 236), 87.5% (21 out of 24), and 91.5% (194 out of 212), respectively, among lymph nodes without histological changes (Table 3). There were no differences in accuracy, sensitivity, or specificity between the two groups ($P = 0.96$, 0.87, and 0.73, respectively).

Lymph nodes with discordant results. Of the 302 lymph nodes, 27 (8.9%) showed discordant results between the pathological examination and the OSNA assay (Table 4). Of these 27 nodes, 20 were negative on pathological examination and positive on the OSNA assay (false positive when using pathology as the gold standard), whereas 7 were positive on pathological examination and negative on the OSNA assay (false negative when using pathology as the gold standard).

Of the 20 nodes with false-positive results, ITCs were identified in five nodes during the original pathological assessment. Moreover, cancer cells were identified in two nodes (one with micrometastasis and one with ITC) during the additional pathological assessment. In contrast, no cancer cells were identified in 13 nodes during the additional pathological examination; the median CK19 mRNA copy number was 450 (range, 280–250 000).

Of the seven nodes with false-negative results, six nodes displayed micrometastasis and one node exhibited macrometastasis. The median size of metastasis on pathology was 0.8 mm (range, >0.2–12.0 mm). In all seven nodes, CK19 protein expression was detected by immunohistochemistry.

Table 2. Comparison of the results of the OSNA assay with pathological examination

	Pathology			
	Positive		Negative	
	Macro	Micro	ITC	None
OSNA				
Positive				
(++)	32	3	2	4
(+)	10	8	3	11
Negative	1	6	1	221

Abbreviations: ITC = isolated tumour cells; OSNA = one-step nucleic acid amplification.

DISCUSSION

To the best of our knowledge, this prospective multicentre trial is the first study to evaluate the performance of a molecular assay in detecting lymph node metastasis in patients with breast cancer who were treated with preoperative systemic therapy. The OSNA assay can detect the residual tumour burden in lymph nodes after chemotherapy as accurately as conventional pathology. The overall performance of the OSNA assay in this study is almost equivalent to the results of two pooled analyses of previous trials in which similar protocols were used in patients who did not receive preoperative systemic therapy (accuracy, 93.6–96.1%; sensitivity, 87.9–91.7%; specificity, 94.8–97.0%; Cserni, 2012; Tamaki, 2012). Moreover, chemotherapy-induced histological changes did not affect the performance of the OSNA assay. The performance of the assay for lymph nodes with chemotherapy-induced histological changes was similar to that for lymph nodes without histological changes as well as that reported in the aforementioned pooled analyses.

The main reason for the discordant results between the OSNA assay and conventional pathology may be tissue allocation bias. As per the protocol of this study, small metastases localised in only one slice inevitably result in discordant findings. Of the 20 nodes displaying false-positive results, 7 nodes showed cancer cells on the original or additional pathological assessment slides. In addition, 10 nodes had a low tumour burden of no >1000 copies. Thus, in

Table 3. Chemotherapy-induced histological changes and performance of the OSNA assay

	Pathology							
	Presence of histological changes ($n = 66$)				Absence of histological changes ($n = 236$)			
	Positive		Negative		Positive		Negative	
	Macro	Micro	ITC	None	Macro	Micro	ITC	None
OSNA								
Positive								
(++)	17	1	1	0	15	2	1	4
(+)	8	6	1	0	2	2	2	11
Negative	0	4	0	28	1	2	1	193
Accuracy (95% CI)	90.9% (0.81–0.97)				91.1% (0.87–0.94)			
Sensitivity (95% CI)	88.9% (0.74–0.97)				87.5% (0.68–0.97)			
Specificity (95% CI)	93.3% (0.78–0.99)				91.5% (0.87–0.95)			

Abbreviations: CI = confidence interval; ITC = isolated tumour cells; OSNA = one-step nucleic acid amplification.

Table 4. Lymph nodes with discordant results and the possible cause

	OSNA		Pathology			
Lymph node	Result	CK19 mRNA (copy μl^{-1})	Original assessment (size, mm)	Additional assessment (size, mm)	CK19 protein	Possible cause
False positive						
JC28-4	(+ +)	32 000	ITC (≤0.2)	NA	(+)	Allocation bias
SL07-4	(+ +)	6300	ITC (≤0.2)	NA	(+)	Allocation bias
CR02-3	(+)	2300	ITC (≤0.2)	NA	(+)	Allocation bias
JC07-4	(+)	460	ITC (≤0.2)	NA	(+)	Allocation bias
JC26-4	(+)	300	ITC (≤0.2)	NA	(+)	Allocation bias
SL02-4	(+)	280	None	Micro (0.8)	(+)	Allocation bias
CR02-2	(+ +)	13 000	None	ITC (≤0.2)	(+)	Allocation bias
SL07-2	(+ +)	250 000	None	None	NA	Human error
SL12-4	(+ +)	6300[a]	None	None	NA	Allocation bias
CR17-4	(+ +)	5600	None	None	NA	Allocation bias
JC11-4	(+)	1000	None	None	NA	Allocation bias
CR07-2	(+)	960	None	None	NA	Allocation bias
JC18-1	(+)	710[a]	None	None	NA	Allocation bias
SL06-4	(+)	450[a]	None	None	NA	Allocation bias
CR13-3	(+)	410	None	None	NA	Allocation bias
JC10-2	(+)	400	None	None	NA	Allocation bias
SL09-1	(+)	400	None	None	NA	Allocation bias
SL14-1	(+)	330	None	None	NA	Allocation bias
SL06-1	(+)	300	None	None	NA	Allocation bias
SL13-2	(+)	280	None	None	NA	Allocation bias
False negative						
SL07-3	(−)	<250	Macro (12.0)	NA	(+)	Human error
JC19-2	(−)	ND	Micro (1.0)	NA	(+)	Allocation bias
JC35-2	(−)	ND	Micro (1.0)	NA	(+)	Allocation bias
SL09-4	(−)	<250	Micro (0.8)	NA	(+)	Allocation bias
JC21-1	(−)	ND	Micro (0.8)	NA	(+)	Allocation bias
JC04-3	(−)	<250	Micro (0.5)	NA	(+)	Allocation bias
JC21-3	(−)	ND	Micro (0.2[b])	NA	(+)	Allocation bias

Abbreviations: CK19 = cytokeratin 19; ITC = isolated tumour cells; NA = not available; ND = not detected; OSNA = one-step nucleic acid amplification.
[a]CK19 mRNA copy numbers in the diluted sample.
[b]Just over 0.2 mm in size.

these 17 nodes, tissue allocation bias could have resulted in discordant findings. In addition, two nodes (#SL12-4 and #CR17-4) had metastasis with 5000–6000 copies; these copy numbers suggest that the tumours are approximately 2 mm in size (Tsujimoto *et al*, 2007). Although the metastatic status of these two nodes is indeterminate, tissue allocation bias is also suspected as the cause of the discordant results. Furthermore, all seven nodes with false-negative results were positive for CK19 protein expression. Although the OSNA assay may miss metastases that do not express CK19 mRNA in principle, the false-negative results in this study did not appear to be caused by the absence or low expression of CK19 mRNA. Of the seven nodes, six had micrometastasis of ⩽1.0 mm in size. Therefore, tissue allocation bias is a possible cause of the discordant results for these six nodes.

The discordant results of the remaining two nodes (#SL07-2 and #SL07-3) may be due to human error. In lymph node #SL07-2, the CK19 mRNA copy number was high, but no cancer cells were detected during the original or additional pathological examination. In contrast, in lymph node #SL07-3, the CK19 mRNA copy number was low, whereas a large metastatic lesion expressing CK19 was observed during the pathological examination. Lymph nodes #SL07-2 and #SL07-3 were sampled from the same patient. Therefore, the pieces for the OSNA assay or the pathological samples of the two nodes may have been switched during the handling of the samples.

In clinical practice, the OSNA assay can contribute to the accurate, reproducible, and standardised evaluation of the residual tumour burden after preoperative chemotherapy. When a whole lymph node or a large amount of a node is examined using the OSNA assay, more micrometastases can be detected than by the use of routine pathological examinations (Osako *et al*, 2011a, b, 2012; Remoundos *et al*, 2013). This is reasonable considering that routine pathology analyses only limited a part of the lymph node, whereas the OSNA assay can thoroughly evaluate the entire lymph node. Patients with negative nodes or micrometastases who were not treated with preoperative chemotherapy had identical survival rates, whereas the survival rate of patients with micrometastases in lymph nodes after chemotherapy was similar to that of patients with macrometastases and significantly worse than that of patients with negative nodes (Fisher *et al*, 2002). Thus, the OSNA assay facilitates prediction of the prognosis of patients treated with preoperative chemotherapy more accurately than conventional pathological examinations. Although further chemotherapy may potentially not be delivered after neoadjuvant chemotherapy plus surgery, adjuvant therapies including radiation, hormone, and molecular-target therapies can be considered for these patients. Therefore, this more accurate diagnosis of lymph node status can enable to personalise the adjuvant therapy for each of the patients.

In conclusion, the OSNA assay can detect residual tumour burden in lymph nodes after chemotherapy as accurately as

conventional pathology even when chemotherapy-induced histological changes are present. The main cause of discordant results may be tissue allocation bias. Therefore, the OSNA assay can contribute to the accurate, reproducible, and standardised evaluation of lymph node status after preoperative chemotherapy.

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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)

Locoregiocal 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

	Clinicopathological group			
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	
	Primary invasive tumor cell-related group			
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	
	Primary tumor-stromal fibroblast-related group			
1	Fibrotic focus, dimension (mm)			
	Absent	≤8	>8	
2	Types of invasive ductal carcinoma			
	Type 1	Type 2	Type 3	Type 4
	Tumor embolus-related group			
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

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

1 *Histological features of atypical tumor-stromal fibroblasts and the type of invasive ductal carcinoma*

(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 *Grading system for lymph vessel tumor embolus*

Grade 0 Invasive ductal carcinomas with no lymph vessel tumor embolus

Grades 1–3 Invasive ductal carcinomas with lymph vessel tumor embolus or emboli

	No. of mitotic figures	No. of apoptotic figures
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 *Grade of stromal fibrosis in metastatic carcinomas to the lymph node*

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 *Extranodal blood vessel tumor embolus or emboli*

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 *Mitotic figures in metastatic carcinomas to the lymph node*

(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

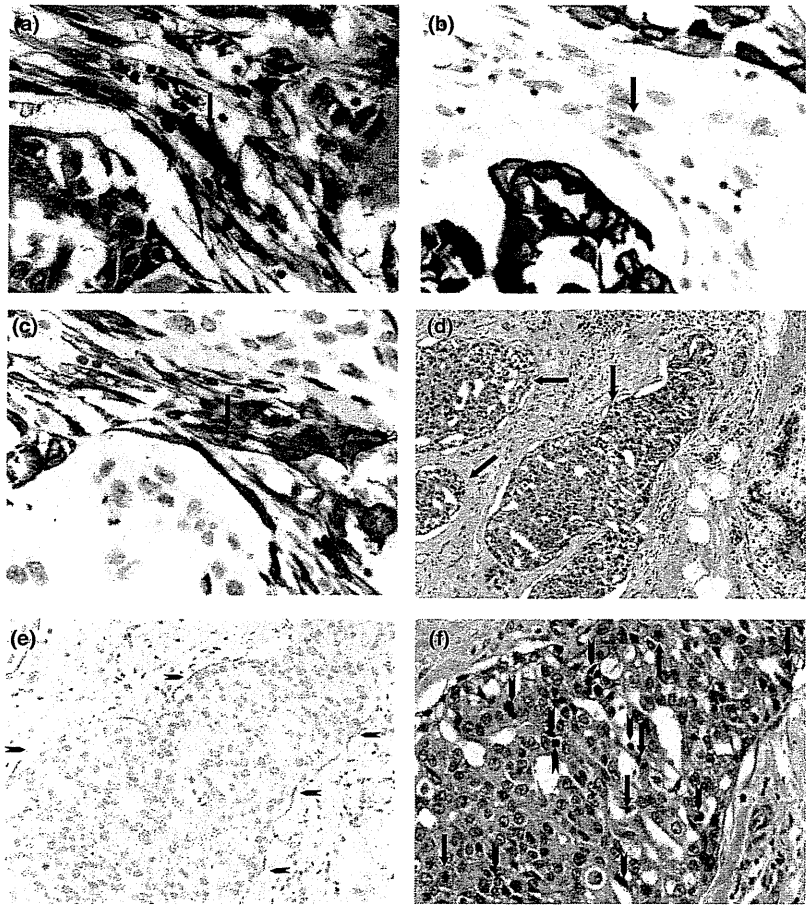

Fig. 1. (a–c) Type 2 invasive ductal carcinoma. One atypical tumor-stromal fibroblast with a large spindle nucleus is visible in the tumor stroma (arrow). The fibroblast was stained negative for AE1/3 (arrow, b) and positive for smooth muscle actin (arrow, c). The invasive tumor cells were stained positive 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

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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.

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.

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 (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-

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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 G2	G3 G2	G3 G2	G3	G3 G2	G3	G3	G3 G2	G3	G3	•
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.

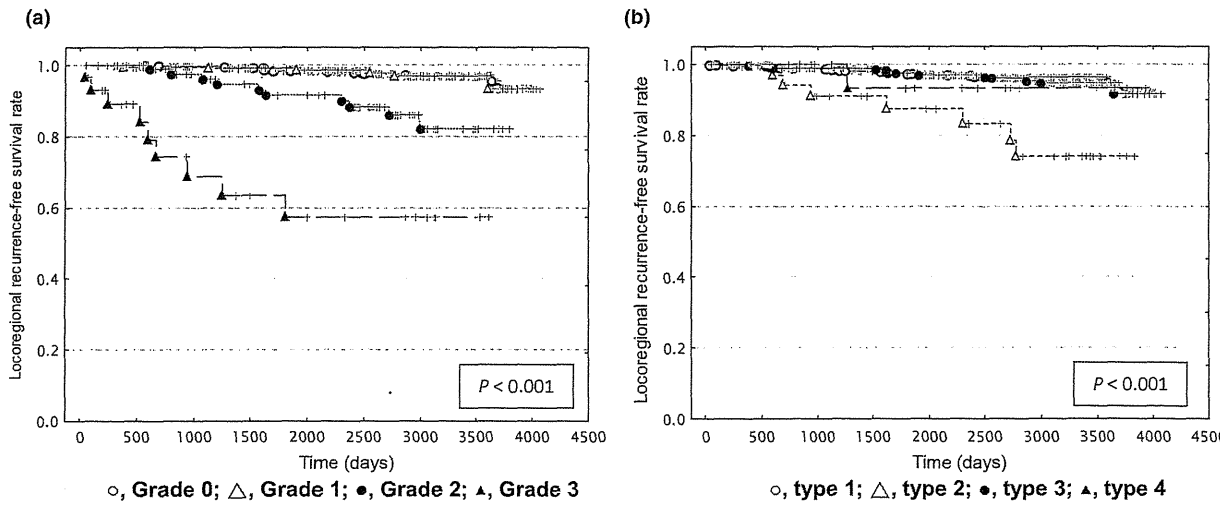

Fig. 2. First locoregional recurrence-free survival curves for overall patients with invasive ductal carcinoma (a and b). (a) Patients with grade 3 lymph vessel tumor emboli had the shortest locoregional recurrence-free survival curve. Patients with grade 2 lymph vessel tumor emboli also had a significantly 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 radiotherapy; 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

Use of the neo-adjuvant exemestane in post-menopausal estrogen receptor-positive breast cancer: A randomized phase II trial (PTEX46) to investigate the optimal duration of preoperative endocrine therapy

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ABSTRACT

Purpose: The optimal treatment duration time and the causal relationship between neoadjuvant endocrine therapy and clinical response are not clear. Therefore, we conducted the present study to investigate the potential benefits of neoadjuvant exemestane therapy with the goal of identifying the optimal treatment duration.

Methods: This study was conducted at three hospitals, as a multicenter, randomized phase II trial(UMIN000005668) of pre-operative exemestane treatment in post-menopausal women with untreated primary breast cancer. Fifty-one post-menopausal women with ER-positive and/or PgR-positive invasive breast cancer were randomly assigned to exemestane for 4 months or 6 months. Clinical response, pathological response, and decisions regarding breast-conserving surgery were the main outcome measures.

Results: Of the 52 patients that enrolled, 51 patients underwent surgery. Of those, 26 and 25 patients had been treated with exemestane for 4 and 6 months, respectively. Treatments were performedat 3 hospitals in Japan between April 2008 and August 2010. The response rates as assessed by clinical examination were 42.3% and 48.0% for 4 and 6 months of treatment, respectively. Pathological responses (minimal response or better) were observed in 19.2% and 32.0% of patients, and breast-conserving surgery was performed on 50.0% and 48.0% of patients from the 4 and 6 month treatment groups, respectively.

Conclusion: The results of this study demonstrate that responses were equal to 4 or 6 months of exemestane treatment. Therefore, we propose that the rates of breast-conserving surgery could be maximized by 4 months of treatment. Furthermore, in addition to using exemestane as a preoperative treatment in post-menopausal women with ER-positive breast cancer, we envision administering the drug over the long term under careful clinical supervision.

Introduction

Since the 1990s, primary endocrine therapy has been considered the gold standard in the adjuvant and metastatic treatment settings for estrogen (ER) and/or progesterone (PR) receptor-positive breast cancer. The NSABP B-18 clinical trial[1] in 1988 demonstrated that neoadjuvant chemotherapy yielded the same survival rate as adjuvant chemotherapy, with an improved rate of breast-conserving surgery, indicating that neoadjuvant therapy could have important clinical ramifications. With that in mind, neoadjuvant endocrine therapy for hormone receptor-positive breast cancer was also assessed, and was shown to be effective in a number of clinical trials (Table 1). Recently, clinical interest has shifted from tamoxifen to third-generation aromatase inhibitors. A few trials[2–8] have indicated that anastrozole led to improved response rates as compared to tamoxifen, but the results were not statistically significant. The PROACT trial reported that anastrozole treatment allowed for breast-conserving surgery in significantly

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Table 1
Neoadjuvant endocrine trials.

Author or trial name	Number of patients	Design	Duration (month)	Clinical ORR[e]
IMPACT[2]	330	ANA[a] vs TAM[b] vs ANA + TAM	3	37%, 36%, 39%
PROACT[3]	451	ANA vs TAM	3	49.7%, 39.7%
PO24 Trial[4]	337	LET[c] vs TAM	4	55%, 36%
GENARI Trial[5]	29	EXE[d]	4	37.0%
French study[6]	45	EXE	14–27 weeks	70.6%
Gil Gil (Spain)[7]	55	EXE	6	50%
Mustacchi[8]	44	EXE	6	66%

[a] ANA = Anastrozole.
[b] TAM = Tamoxifen.
[c] LET = Letrozole.
[d] EXE = Exemestane.
[e] ORR = objective response rates.

more patients than did tamoxifen. The neoadjuvant drug, exemestane, has been evaluated in several small studies. The results have been promising and warrant further evaluation to determine the optimal therapeutic conditions for hormone receptor-positive patients. Specifically, the optimal treatment duration time and the causal relationship between neoadjuvant endocrine therapy and clinical response are not clear (Table 1). In addition, there are studies that have reviewed the optimal duration time of hormone treatments. Here, we investigated the benefits of 4 and 6 month long neoadjuvant exemestane therapy.

Materials and methods

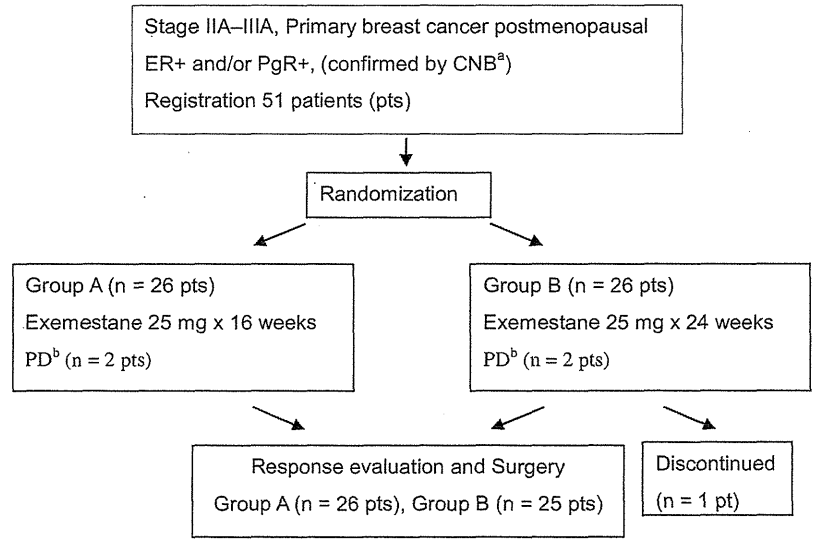
Patients

We enrolled ≥55-year-old post-menopausal women (defined as: no spontaneous menses for > 1 year; LH levels > 30 IU/L; or bilateral oophorectomy prior to breast cancer diagnosis) with stage IIA–IIIA invasive ER- and/or PgR-positive breast carcinoma, as confirmed by immunohistochemical examination of core-needle biopsies (defined as: >10% endocrine receptor + nuclear staining). We further required that tumors be measurable by clinical palpation. Written informed consent was obtained from each patient.

Patients were ineligible if they had any severe coincident medical disease that would prevent them from receiving surgery, place them at unusual risk, or confound the study results; were unwilling or unable to discontinue using drugs affecting sex hormones (including hormone replacement therapy); had suffered from any invasive malignancy within the previous 5 years (other than carcinoma of the skin or carcinoma in situ of the cervix, adequately cone biopsied); had received any previous breast cancer treatment or tamoxifen as part of a breast cancer prevention study; or, had received treatment with non-approved drugs during the 3 months prior to randomization. Criteria for withdrawal from the study included patients who had completed the 5-year treatment course; did not begin randomized therapy; withdrew informed consent; had confirmed clinically significant disease before surgery or confirmed recurrence after surgery; had an adverse event; or, were withdrawn at the investigator's discretion.

Study design and setting

This study was conducted at three hospitals in Japan as a multicenter, open-label, double-arm, randomized, phase II clinical trial of pre-operative exemestane treatment in post-menopausal women with primary breast cancer. In order to optimally balance the patients in the two treatment arms with respect to prognostic factors, the patients were stratified by tumor factor, node factor, and age. The neoadjuvant endocrine treatment regimen consisted of one 25 mg exemestane tablet daily for 4 or 6 months. Fifty-one post-menopausal women with ER-positive and/or PgR-positive invasive breast cancer were randomly assigned to exemestane (25 mg/day) for 4 months (Group A) or exemestane



Setting: Multicenter study involving 3 hospitals in Japan

[a]CNB = core needle biopsy

[b]PD = Progressive Disease

The patient with PD canceled treatment and underwent immediate surgery.

Fig. 1. Study design.

(25 mg/day) for 6 months (Group B; Fig. 1). When antitumor effects were observed with progressive disease (PD), the treatment was canceled and patients underwent surgery immediately. All patient data was collected by UMIN (UMIN000005668) and analyzed at the National Cancer Center in Japan. Tumors were measured by caliper before exemestane treatment began, and again in the eighth week of therapy. Tumor regression by clinical examination, pathological response, decisions regarding breast-conserving surgery, and safety assessments were the main outcome measures. All patients provided written informed consent. This investigational registration period was planned three years from May 2008. The trial was conducted in accordance with the principles of Good Clinical Practice as specified in the Declaration of Helsinki (Edinburgh, 2000). The study protocol was guided by the current regulations governing clinical trials, and was approved by the Ethics Committees of the individual hospitals involved. All patients gave written informed consent before study enrollment.

Study endpoints

The primary endpoints were objective response rates (ORR) by caliper at 4 and 6 months of treatment using an intention to treat analysis. Secondary endpoints were the rates of breast-conserving surgery or mastectomy, and the pathological response rates.

Clinical assessments

The primary study objective was to compare the differences between exemestane treatment for 4 and 6 months, using objective complete responses (CRs) and partial responses (PRs) as defined by the Response Evaluation Criteria in Solid Tumors (RECIST),[9] which is based on caliper measurements of tumor size. Clinical response was assessed by comparing the longest diameter of the target lesions with the baseline measurement based on RECIST criteria. Every 4 weeks, patients underwent a physical examination, toxicity assessment, and tumor assessment using WHO criteria. If tumor progression was suspected, the tumor was further assessed by ultrasound or mammography. At baseline and immediately before surgery, the investigator recorded the extent of the least invasive feasible breast surgery option at that particular time: whether breast-conserving surgery or mastectomy was needed, or whether the tumor was inoperable.

Histological assessments

Histopathological therapeutic response was classified according to the General Rules for the Clinical and Pathological Recording of Breast Cancer 2005.[10] For Grade 0, no response was observed; Grade 1a comprised those tumors with mild changes in cancer cells regardless of the area, or marked changes seen in less than one-third of cancer cells; Grade 1b comprised tumors with marked changes seen in more than one-third but less than two-thirds of tumor cells; Grade 2 tumors contained marked changes in more than two-thirds of tumor cells; and Grade 3 tumors demonstrated a complete response, with no cancerous cells remaining. Mild changes included slight degenerative changes in cancer cells not suggestive of cell death (including cancer cells with vacuolation of the cytoplasm, eosinophilic cytoplasm, swelling of the nucleus, *etc.*). Marked changes include noticeable degenerative changes in cancer cells suggestive of cell death (including liquefaction, necrosis, and disappearance). The pathological response group was defined as tumors with Grade 1b and 2 responses. The non-response group was defined as tumors with Grade 0 and 1a responses.

Statistics

Based upon previous results, we assumed the response rate to be 40% and 60% after 4 and 6 months of exemestane, respectively (Table 1). To achieve an 80% statistical power, 46 examples were required to detect differences in both response rates with a 5% level of significance.[1] To account for attrition, we enrolled 50 patients.[11] Analysis was on an intention to treat (ITT) basis. The chi-squared test was used to compare tumor characteristics and responses, and rates of breast-conserving surgery between groups. Results with $p < 0.05$ were considered to be significant.

Results

Patient baseline characteristics

The study enrolled 52 post-menopausal women at 3 hospitals in Japan between April 25, 2008 and August 12, 2010. Of these, 26 patients were allocated to Group A, and 26 to Group B. One patient withdrew and did not complete the study (Group B). The main characteristics of the eligible patients are described in Table 2. The baseline characteristics were well balanced between the two treatment arms (Table 2).

Efficacy results

Evaluation of the primary efficacy endpoint (overall objective response as determined by clinical palpation) revealed that there was no statistically significant difference in the overall objective response (CR + PR) between the two treatment groups: Group A, 42.3%; Group B, 48.0%; $p = 0.89$ (Table 3). Clinically, 7.7% of Group A and 8.0% of Group B patients progressed while 50.0% and 44.0% of Group A and B patients, respectively, remained stable (not significant). As for the anti-tumor effect assessed by caliper at the eighth week, there were no differences between the two cohorts (Table 3). The pathological response rates of Groups A and B were 19.2% and 32.0%, respectively, a difference that was not statistically significant (Table 4, $p = 0.47$). Pathological CR in the primary breast lesion was only observed in one patient in Group B. Withdrawals from the trial due to side effects did not occur in either Group.

Table 2
Patients' baseline characteristics.

		Group A (4 months)	Group B (6 months)
Age, median (range)		66 (51–80)	64 (57–80)
Tumor stage, number (%)	T2	24 (92.3%)	24 (92.0%)
	T3	2 (7.7%)	2 (8.0%)
Nodal stage, number (%)	N0	21 (80.8%)	24 (92.0%)
	N1	5 (19.2%)	2 (8.0%)
Clinical stage, number (%)	IIA	19 (73.1%)	22 (84.0%)
	IIB	7 (26.9%)	4 (16.0%)
BMI[a]		23.9 (18.5–31.5)	24.5 (17.5–32.3)
Tumor diameter (caliper) Median (range) mm		30.5 (20–60)	30.0 (13–55)
Receptor status			
ER[b] positive/HER2[c] negative		25	22
ER[b] positive/HER2 positive		1	3
PgR[d]	Positive	20	18
	Negative	6	8

There were no differences between Groups A and B in these characteristics.
[a] BMI = body mass index.
[b] ER = estrogen receptor.
[c] HER2 = human epidermal growth factor receptor type 2.
[d] PgR = progesterone receptor.

Table 3
Clinical response (caliper).

Response[a]	Group A (4 months) number (26)		Group B (6 months) number (25)	
	8 weeks	16 weeks	8 weeks	24 weeks
CR	0	1	0	2
PR	7	10	5	10
SD	17	13	20	11
PD	2	2	0	2
Clinical ORR (CR or PR)	26.9%	42.3%	20.0%	48.0%

$p = 0.89$.
Complete Response: CR, Partial Response: PR, Stable Disease: SD, Progressive Disease: PD.
ORR: objective response rates.
[a] The RECIST methodology was used to assess response (Therasse et al., 2000).

Table 4
Clinical response (pathological response).

Pathological response[a]	Group A (4 months) number	Group B (6 months) number
3	0	1
2	0	1
1b	5	6
1a	15	13
0	6	4
Response rate (1b or 2 or 3)	19.2%	32.0%

$p = 0.47$.
0 no response, 1a mild response, 1b moderate response, 2 marked response, 3 complete response.
[a] Pathological response was defined as a Grade 1b, 2, or 3 lesion according to the following criteria.

Breast conservation

Of the 52 randomized patients, 32 would have required a mastectomy at baseline (17 in Group A and 15 in Group B; Table 5). For one of these patients, an operation was not performed. Surgery outcomes with respect to breast conservation improved in 4 of 26 patients in Group A (15.4%), as compared to 1 of 25 patients in Group B (4.0%). As compared to the intent-to-treat population, the increase in patients eligible for breast conserving surgery was numerically higher in Group A than Group B, although this difference did not reach statistical significance.

Discussion

ER-positive tumors are generally less sensitive to chemotherapy than ER-negative tumors.[12,13] Some trials have shown that tamoxifen is an effective primary endocrine agent for the treatment of locally advanced[14] and operable ER-positive breast cancers, especially in the elderly population.[15,16] A combined analysis of the IMPACT and PROACT clinical trials showed a trend toward better objective response rates when patients received aromatase inhibitors, but no statistically significant difference was observed between treatments with aromatase inhibitors or tamoxifen.[2,3]

Table 5
Rate of breast-conserving surgery.

Group A (4 months)			Group B (6 months)		
	Estimation (pre treatment)	Post treatment		Estimation (pre treatment)	Post treatment
Mastectomy	17	13	Mastectomy	14	13
BCS[a]	9	13	BCS	11	12
Rate of BCS	34.6%	50%	Rate of BCS	44.0%	48.0%

[a] BCS = Breast-conserving surgery.

However, in the P024 trial, the objective response rate for treatment with aromatase inhibitors was significantly greater than that for tamoxifen.[4] At present, the optimum duration of treatment for neoadjuvant endocrine treatment is not known. Ideally, the timing would be based on individual patient response. Clinical trials report a common duration period of preoperative endocrine therapy as 4–6 months. Likewise, the duration of many neoadjuvant chemotherapy treatments is 6 months. Therefore, we carried out this study to compare the use of exemestane for 4 and 6 months prior to surgery. We found no significant differences in outcomes between patients who received the drug for 4 or 6 months; however, the latter group tended to have higher anti-tumor responses. It is thought that this observation did not reach statistical significance because we set the significant difference of both groups at 20%.Our study results show that the maximum response of neoadjuvant hormone therapy by exemestane is around four months. These data are consistent with the study by Antonio Llombart-Cussac et al.,[18] in which the maximum response to therapy with letrozole was at 4.2 months. In addition, a randomized phase II study[17] compared 4–8 months of letrozole in a single arm; there tended to have higher anti-tumor responses. We think that these results indicate that the maximum response to neoadjuvant hormone therapy is also around four months. ER- and/or PgR-positive tumors are biologically heterogeneous. It is thought that biologically heterogeneous groups require detailed statistical adjustment. Krainick-Strobel UE et al.[17] found that 4 months of neoadjuvant exemestane therapy improved the rate of breast-conserving surgery. There was not a large difference in response rates for treatments of 3–6 month duration; however, the anti-tumor effects tended to be greater after 6 months of treatment as compared to shorter time points. In our study, neither treatment group experienced severe side effects as a result of the therapy. However, Group B tended to have a higher pathological response rate. It seems that the maximum anti-tumor effect may be reached at different time points for each patient over the course of 24 weeks of treatment. Therefore, we cannot expect a large antitumor effect by treating for longer than 4 months; however, we could extend the treatment period until the time of operation. Furthermore, in addition to using exemestane as preoperative treatment in post-menopausal women with ER-positive breast cancer, due to the mild side effects observed during the 6 month course of treatment, we envision administering the drug over the long term under careful clinical supervision.

Ethical approval

The present work has been approved by the ethical committee of each institutional.

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Conflict of interest statement

All authors declare that they have no conflict of interest.

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ORIGINAL ARTICLE – BREAST ONCOLOGY

Comparison of the Indocyanine Green Fluorescence and Blue Dye Methods in Detection of Sentinel Lymph Nodes in Early-stage Breast Cancer

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ABSTRACT

Purpose. To assess the diagnostic performance of sentinel lymph node (SLN) biopsy using the indocyanine green (ICG) fluorescence method compared with that using the blue dye method, a prospective multicenter study was performed.

Methods. Patients with T1–3 primary breast cancer without clinical lymph node involvement were included in this study. ICG as a fluorescence-emitting source and indigo carmine as blue dye were injected into the subareolar area. Extracted lymph nodes were examined to identify the first, second, and other SLNs. The identified nodes were classified according to the ICG fluorescence signal and blue dye uptake.

Results. Ninety-nine eligible patients were included in this study. The ICG fluorescence method identified an average of 3.4 SLNs (range, 1–8) in 98 of 99 patients (detection rate, 99 %). The number of lymph nodes identified by the fluorescence method was significantly higher than that identified by the blue dye method ($p < 0.001$). SLN involvement was identified in 20 % (20 of 99) of patients, all of whom tested positive for the first SLN. In 16 patients, complete axillary lymph node dissection (ALND) was performed. In 25 % (4 of 16) of these patients, axillary metastases were identified; however, no axillary involvement was found in 8 patients with only one involved node, which was isolated as the first SLN.

Conclusions. High rate of SLN detection was achieved using the ICG fluorescence method. The first SLN identified by fluorescence imaging provides an exact indication of the axillary status. Therefore, the ICG fluorescence method provides precise information required to avoid unnecessary ALND.

For many years, axillary lymph node dissection (ALND) has been performed for prevention of lymph node metastasis in patients with breast cancer. However, ALND is associated with a relatively high risk of complications such as edema of the arms (lymphedema), dyskinesia, and pain, which lower quality of life.[1–3] In the 1990s, sentinel lymph node (SLN) biopsy was proposed for the assessment of axillary lymph node involvement to circumvent unnecessary ALND.

Sentinel lymph node is defined as a lymph node that receives lymph flow directly from the primary tumor. Because this concept was first applied to melanoma patients in 1992, SLN biopsy has become a standard method for evaluating the axillary lymph node status in patients with early-stage breast cancer.[4–7] The following two methods are commonly utilized for detecting SLNs: the radioimmunoassay (RI) method, which involves application of radioactive colloids, and injection of blue dye.[8,9] Both methods have their advantages and

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disadvantages.[10–12] The RI method has the advantage of a high SLN identification rate, while disadvantages include the requirement of a radioactive facility, exposure to radiation, and high cost. In contrast, the blue dye method has the advantages of a high prevalence rate, no radiation exposure, and low cost; however, SLN identification rates are lower with this method compared with the RI method.[13] Furthermore, the success of the blue dye method is dependent on the technician's skill and experience.[14]

Indocyanine green (ICG) is a dye on which laser-emitting diodes are centered at 760 nm to collect fluorescence at 830 nm. The fluorescing property of the ICG reagent was first applied to the dye method, followed by the fluorescence method.[15] The ICG fluorescence method requires a photodynamic eye (PDE) camera. It lacks the stringent safety controls of the RI method. Therefore, the fluorescence method is not limited to use in high-volume centers. Lymph flow can be confirmed as a real-time image from outside the body using the ICG fluorescence method; therefore, this method is well suited for performing intraoperative SLN biopsy.

Because the ICG fluorescence method requires little skill and the necessary reagents and apparatus are inexpensive in comparison with the RI method, use of the former method at the physician's discretion has been increasing. Recent clinical results obtained after introduction of the ICG fluorescence method have indicated higher SLN identification rates than those observed with the blue dye method.[16–19] However, operational procedures and experience of the personnel vary among institutions. In addition, no statistical analysis has clearly demonstrated the superiority of the ICG fluorescence method over the blue dye method. The present multicenter, cooperative, prospective analysis using a standardized procedure was performed to demonstrate the efficacy of the ICG fluorescence method in comparison with that of the blue dye method.

METHODS

Patients

Eligible patients were 20–75 years old at registration and diagnosed with T1–3 primary breast cancer without clinical lymph node metastasis (N0). Six participating centers in Japan have been governmentally authorized to perform SLN biopsy. SLN biopsies were performed by ten well-trained physicians according to a standard written procedure. This study was performed in accordance with the Declaration of Helsinki, and all patients provided written informed consent. The study protocol was approved by the local ethics committees at all participating trial sites. Patients in whom previous surgical biopsy or surgery involving the axillary regions had been performed, those in whom preoperative drug therapy (including hormone therapy and chemotherapy) had been administered, and those who had a history of allergy to ICG or indigo carmine dye were excluded from the study.

Surgical Procedure

All surgeons performed SLN biopsy following the standard procedure. In this study, SLNs were categorized as follows: axillary lymph nodes, blue-stained (true SLN); axillary lymph nodes, ICG fluorescence-positive detected by PDE (true SLN); and palpably suspicious, surgically removed lymph nodes in which neither ICG fluorescence nor blue dye was found (para-SLN). The surgeon's goal during the procedure was to remove the blue-stained and/or fluorescent lymph nodes (true SLNs) in the incised region. Palpated lymph nodes in the operative area were also removed as para-SLN.

The ICG fluorescence method has been previously reported.[17] In brief, 0.5–1 ml of 0.5 % ICG as a source of fluorescence and 2–4 ml of indigo carmine as a blue dye were injected in the subareolar area. Lymphatic flow was then traced with a PDE camera (a charge-coupled device; Hamamatsu Photonics Co., Hamamatsu, Japan). Real-time, image-guided surgery was used to identify the fluorescence signals of the SLNs after meticulous dissection. The excised lymph nodes were examined separately according to the order of removal and classified according to detection by ICG fluorescence and/or blue dye.

Study Objectives

The primary endpoint in this study was to determine the number of lymph nodes identified by each method. In each patient, all extracted lymph nodes were classified into four categories on the basis of the two detection methods as follows: SLNs identified by both fluorescence and blue dye (flu^+/dye^+), those identified by fluorescence only (flu^+/dye^-), those identified by dye only (flu^-/dye^+), and those in which neither fluorescence nor dye was observed (para-SLNs; flu^-/dye^-). Secondary endpoints included the SLN identification rate, SLN metastasis rate, and metastasis rate according to the order of SLN detection.

Statistical Methods

The number of lymph nodes identified using the ICG fluorescence method and that using the blue dye method were compared. Differences were calculated by subtracting the number of flu^-/dye^+ SLNs from the number of flu^+/dye^- SLNs for each patient. The sign test was used to test the null hypothesis that the number of identified lymph