Fig. 1. Lymph flow type.

alone,[13,14] but patients are exposed to a radioisotope. Indocyanine Green (ICG) fluoresces when bound to proteins under physiologic conditions, and it can be used to monitor lymph flow using a charge-coupled device.[6] ICG is currently used clinically in cardiovascular surgery,[8,9] organ transplantation,[10] and stomach and intestine surgery.[11] In this paper, we examined the performance characteristics of ICG in combination with a radioisotope or dye for the selection of targets for SNB in patients with breast cancer, and we further classified the patterns of lymphatic flow in these patients.

Patients and methods

Patients

One hundred and forty one patients with clinically node-negative breast cancer were examined from August 2006 to December 2008 at National Cancer Center Hospital.

Methods

SNB Identification Method 1: A 1% solution of patent blue dye (2 ml) was injected into the sub-areolar region and skin overlying the tumor after the induction of anesthesia. The whole breast was compressed and massaged for about 5 min. ICG (2 ml) was injected into the skin overlying the tumor and the sub-areolar region immediately prior to the surgical procedure. Fluorescence in the draining lymphatic duct was visualized with the PDE, and the incision line was determined. All blue-stained SLNs were harvested. After the initial lymph node resection guided by dye labeling, the remaining nodes were re-evaluated using the PDE and harvested if fluorescence was detected.

SNB identification method 2: lymphatic mapping was performed using a combination of ICG and 30–80 megabecquerels of technetium-99 m-labeled Phytate (Daiichi RI Laboratory, Tokyo, Japan). One day prior to surgery, the radiotracer was intradermally injected into the area overlying the tumor and sub-areolar region, while ICG (2 ml) was injected into the skin overlying the tumor and the sub-areolar region immediately prior to the surgical procedure. Lymph nodes containing radioactivity were identified with a scintillation detector, and harvested. The remaining lymph nodes were then examined with the PDE for fluorescence, and harvested as described above.

Depending on the method used, SLNs were identified as blue stained, radioactive, and/or fluorescent. SNB was then followed by standard level 1/2 axillary lymph node dissection.

All SLNs were histologically evaluated by 3 mm serial sectioning and staining with hematoxylin and eosin (H&E). Lymph nodes as part of the axillary dissection were submitted in their entirety and evaluated using standard H&E staining.

Lymphatic flow was defined as follows: Type C (confluent) lymphatic drainage converged to one lymph duct as assessed by the PDE imaging system, and Type S (separate) lymphatic drainage did NOT converge to one lymph duct (Fig. 1).

Results

The average age of the subjects examined in this study was 59.4 years old (range: 31–83 years). The primary tumor was located in the upper-outer quadrant for 58 cases (41.1%), the upper-inner quadrant for 35 (24.8%), the lower-outer quadrant for 16 (11.3%), the lower-inner for 13 (9.2%), and the central portion for 12 cases (8.5%), while 8 cases had multiple masses (5.7%) (Table 1). All lymph nodes containing dye, radioisotope, or fluorescence were collected, and this was considered the total population of SLNs. In the patients who underwent Method 1, only 92.9% (105/113) of SLNs were dye-positive, but all of the identified nodes were fluorescent (113/113). In patients undergoing Method 2, 100% (29/29) of SLNs were identified by radioisotope detection, and 93.1% of these (27/29) were fluorescent. Overall, 3 SLNs per patient were identified by fluorescence, but only 1.9 and 2.0 were identified by dye or radio-isotope, respectively (Median) (Table 2)

We further analyzed the patterns of lymph flow in all 142 patients. In most patients, the lymphatic drainage flowed from the lower-inner quadrant to the upper-outer quadrant, and only one patient had lymphatic drainage to the internal mammary lymph node. Overall, 113 patients had Type C (79.6%) lymph flow while 29 patients had Type S (20.4%). (Table 3) There was no correlation between the number of SLNs identified and the type of lymphatic drainage.

Of those patients examined using Method 1, 31 had metastases detected in the SLN, and, of these patients, 5 were fluorescence-positive and dye-negative (Table 4). Four of these cases were without metastases at non-SLNs that were subsequently resected. The number of SLNs in these 4 cases was one or zero, but there was no relationship to lymph drainage type (Type C or Type S).

Table 1
Patient demographics.

	Number of patients		
	Method 1	Method 2	Total
Age			
Mean	57.6	60	59.4
Range	34–83	31–82	31–83
Tumor classification			
Tis	29 (25.7%)	4 (14.3%)	33 (23.4%)
T1	51 (45.1%)	6 (21.4%)	57 (40.4%)
T2	33 (29.2%)	18 (64.3%)	51 (36.2%)
Tumor location			
A	29 (26%)	6 (21%)	35 (24.8%)
B	11 (10%)	2 (7%)	13 (9.2%)
C	42 (37%)	16 (57%)	58 (41.1%)
D	15 (13%)	1 (4%)	16 (11.3%)
E	10 (9%)	2 (7%)	12 (8.5%)
Other	6 (5%)	2 (7%)	8 (5.7%)
Pathological node status			
Negative	82 (72.6%)	19 (67.9%)	101 (71.6%)
Positive	31 (27.4%)	9 (32.1%)	40 (28.4%)

Table 2
Detection rate.

State of SLN	Number of SLNs (average)	Detection rate
Flu-positive	3.8	99.3% (140/141)
Dye-positive	1.9	92.9% (105/113)
RI-positive	2.0	100% (28/28)

Flu: Fluorescence, SLN: Sentinel lymph node, RI: Radio isotope

Discussion

Identification of SLNs for biopsy during the initial staging of breast cancer is very important, and highly accurate methods are needed to limit the number of nodes and patients incorrectly identified as metastasis free. This has historically relied upon two methods, the dye- or gamma probe-guided methods, or their combination. The gamma probe-guided method is more accurate than the dye method alone, but requires patient exposure to radioisotope. Thus, we wished to determine if use of the fluorescent dye ICG could improve upon these methods. In the present study, we demonstrated the accuracy of two different combined methods using the fluorescent compound ICG paired with either patent blue dye or a radioisotope tracer. Both methods had high SLN identification rates, but fluorescence was particularly beneficial in difficult cases where SLN were not readily identified using the dye method.

In addition to exposing the patient to radiation, the gamma probe-guided method cannot be used in hospitals that are not equipped to handle radioactivity. However, it is able to identify lymph vessels before incision, and can guide the surgical approach. An ICG-based fluorescent detection method can also identify lymphatic vessels preoperatively with similar sensitivity to gamma probe-guided methods. Moreover, ICG does not require any special licensing, storage, or handling procedures. Therefore, the use of ICG should be particularly attractive to hospitals unable to work with radioactive isotopes. ICG can be a visible dye, and, when used in this capacity, it only identifies 71%[2]–84%[3] of SLNs, and indigo carmine is 94% sensitive. This increases to 94%[6] and 100%[7] when ICG is used as a fluorophore. In this study, ICG fluorescence identified 99% of SLNs, consistent with a previous report, and its use identified 3.8 SLNs per patient. In contrast, use of the dye- or gamma probe-guided method alone identified only 1.9 and 2 SLNs per patient, respectively, consistent with published results.[4,5] The larger number of SLNs identified with ICG could be due to the low molecular weight and high degree of diffusion of ICG allow it to spread beyond the SLN to secondary draining LN. Spread of the radioisotope and patent dye may be more limited. Because SNB by a fluorescence method is sensitive, second or third SLN is detected. As a result, the lymph node which we resected increased. We think that this is a weak point of these methods.

We labeled these as Type C and Type S. More patients had Type C drainage, and some patients originally identified as Type S were found to have Type C drainage after incision. Type S drainage flows directly into each SLN, and, in patients with Type S drainage, more SLNs were identified (Table 3). The fact that when lymph flow was type S, there is much number of SLN leads to performing biopsy carefully. We think that a thought such as superscription helps prevention of false negative.

Table 3
Type of lymph flow and SLN.

Type of lymph flow	No. of patients	No. of SLNs
Type C	113 Cases (79.6%)	3.4
Type S	29 Cases (20.4%)	4.1

Table 4
Characteristics of the five patients whose SLN metastases were confirmed only by fluorescence.

Case	State of SLN		Number of metastasis LNs	Location of tumor
	Dye positive	Flu positive		
1		3	1	A and C
2	0	1	1	B
3	1	6	1	C
4	1	4	1	D
5	1	4	5	C

Forty cases (28.4%), including four cases of micro-metastases, had metastases in the SLN and underwent ALN resection. The lymph nodes containing metastases in five of these 40 cases were missed by the dye method alone but were detected by the fluorescence method (Table 4). Metastases were present in only the SLN in four of these five cases. More thorough analysis could not be performed because of the small number of affected patients, but the inability of the dye method to detect SLNs in these cases is consistent with reports of poor detection by the dye method alone. Thus, it is our view that the dye method should be combined with the fluorescence method in difficult cases.

Suami et al.[12] suggested that nearly all of the lymph drainage of the breast travels to one SLN in the axilla, but some lymph flow drains to more than one SLN in some cases. Thus, there is an anatomic basis to the observed decreased detection of SLNs by the dye method. When considered together, the data suggests that use of a highly sensitive SLN identification technique is indicated, and, for institutions that cannot handle radioactivity, ICG fluorescence offers great promise.

Conflict of interest statement

None declared.

Ethical approval

All patients provided written informed consent to be examined in this study.

References

1. Masakuni N. Sentinel lymph node biopsy for breast cancer in Japan. Geka 2003;65:1701–8.
2. Motomura K, Inaji H, Komoike Y, Kasugai T, Noguchi S, Koyama H. Sentinel node biopsy guided by indocyanine green dye in breast cancer patients. Jpn J Clin Oncol 1999;29:604–7.
3. Noguchi M, Motomura K, Imoto S, Miyauchi M, Sato K, Iwata H, et al. A multicenter validation study of sentinel lymph node biopsy by the Japanese Breast Cancer Society. Breast Cancer Res Treat 2000;63:31–40.
4. Duncan M, Cech A, Wechter D, Moonka R. Criteria for establishing the adequacy of a sentinel lymphadenectomy. Am J Surg 2004;187:639–42. discussion 642.
5. Kennedy RJ, Kollias J, Gill PG, Bochner M, Coventry BJ, Farshid G. Removal of two sentinel nodes accurately stages the axilla in breast cancer. Br J Surg 2003;90:1349–53.
6. Kitai T, Inomoto T, Miwa M, Shikayama T. Fluorescence navigation with indocyanine green for detecting sentinel lymph nodes in breast cancer. Breast Cancer 2005;12:211–5.
7. Tagaya N, Yamazaki R, Nakagawa A, Abe A, Hamada K, Kubota K, et al. Intraoperative identification of sentinel lymph nodes by near-infrared fluorescence imaging in patients with breast cancer. Am J Surg 2008;195(6):850–3.
8. Vogt PR, Bauer EP, Graves K. Novadaq Spy Intraoperative Imaging System—current status. Thorac Cardiovasc Surg 2003;51:49–51.
9. Balacumaraswami L, Abu-Omar Y, Anastasiadis K, Choudhary B, Pigott D, Yeong SK, et al. Does off-pump total arterial grafting increase the incidence of intraoperative graft failure? J Thorac Cardiovasc Surg 2004;128:238–44.
10. Sekijima M, Tojimbara T, Sato S, Nakamura M, Kawase T, Kai K, et al. An intraoperative fluorescent imaging system in organ transplantation. Transplant Proc 2004;36:2188–90.

11. Parungo CP, Ohnishi S, Kim SW, Kim S, Laurence RG, Soltesz EG, et al. Intraoperative identification of esophageal sentinel lymph nodes with near-infrared fluorescence imaging. *J Thorac Cardiovasc Surg* 2005; **129**:844–50.

12. Suami Hiroo, Wei-Ren Pan G, Mann Bruce, Ian Taylor G. The lymphatic anatomy of the breast and its implications for sentinel lymph node biopsy: a human cadaver study. *Ann Surg Oncol* 2008;**15**(3):863–71.

13. Noguchi M, Motomura K, Imoto S, Miyauchi M, Sato K, Iwata H, et al. A multicenter validation study of sentinel lymph node biopsy by the Japanese Breast Cancer Society. *Breast Cancer Res Treat* 2000;**63**:31–40.

14. McMasters KM, Tuttle TM, Carlson DJ, Brown M, Noyes RD, Glaser RL, et al. Sentinel lymph node biopsy for breast cancer: a suitable alternative to routine axillary dissection multi-institutional practice when optimal technique is used. *J Clin Oncol* 2000;**18**:2560–6.

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Original contribution

Grading system for lymph vessel tumor emboli: significant outcome predictor for invasive ductal carcinoma of the breast⋆

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Summary The purpose of this study was to confirm that the grading system for lymph vessel tumor emboli is a significant histologic outcome predictor for patients with invasive ductal carcinoma. The subjects of this study were 1042 invasive ductal carcinoma patients who did not receive neoadjuvant therapy. We classified all invasive ductal carcinomas according to the grading system for lymph vessel tumor emboli we devised, and performed multivariate analyses with well-known prognostic factors. Of 1042 carcinomas, 666, 250, 97, and 29 were classified according to the grading system for lymph vessel tumor emboli as grade 0 (no lymph vessel invasion), grade 1, grade 2, and grade 3, respectively. The univariate analyses showed that the difference in outcome between the group with grade 0 and the group with grade 1 was not significant, but that survival time was significantly shorter in the group of patients with grade 2 carcinomas than in the group with grade 1 carcinomas and significantly shorter in the group of patients with grade 3 carcinomas than in the group with grade 2 carcinomas. Multivariate analyses demonstrated that having a grade 2 or grade 3 carcinoma significantly increased the hazard rates for tumor recurrence and tumor-related death in the patients as a whole as well as in both the group of patients with nodal metastasis and the group without nodal metastasis. The grading system for lymph vessel tumor emboli is an excellent histologic grading system for predicting the outcome of patients with invasive ductal carcinoma of the breast.
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1. Introduction

Lymphatic invasion in breast cancer patients with invasive ductal carcinoma (IDC) has been reported to have prognostic significance [1-5]. We have already reported that the grading system for lymph vessel tumor emboli (LVTEs) that we devised is a very useful histologic grading system for accurately predicting the outcome of patients with IDC who had not received neoadjuvant therapy and that the grading system can be used to classify IDC patients with lymph vessel invasion into low-, intermediate-, and high-risk groups for outcome [6].

The purpose of this study was to confirm that the grading system for LVTEs is a significant outcome predictor for IDC patients, by multivariate analysis with well-known clinico-pathologic factors, in the different IDC groups. In addition, because p53 protein expression in tumor-stromal fibroblasts, but not in tumor cells, in IDCs has recently been clearly demonstrated to be a very important outcome predictor for IDC patients [7], we tried comparing the outcome predictive power of the grading system for LVTEs with that of p53 expression in tumor-stromal fibroblasts in IDCs. We also examined correlations between the grading system for LVTEs and expression of p53 in tumor cells in LVTEs as well as in tumor-stromal fibroblasts and in stroma-invasive tumor cells. The results of this study clearly demonstrated that the grading system for LVTEs is an excellent histologic outcome predictive grading system for IDC patients and significant correlations between the grades of LVTEs and p53 expression in tumor cells in LVTEs as well as p53 expression in tumor-stromal fibroblasts and in stroma-invasive tumor cells.

2. Materials and methods

2.1. Cases

The subjects of this study were drawn from the 1042 consecutive patients with IDC of the breast who were surgically treated at the National Cancer Center Hospital between January 2000 and December 2005 (the same patient series in our previous study [7]). Their IDCs had been diagnosed preoperatively by needle biopsy, aspiration cytology, mammography, or ultrasonography. Clinical information was obtained from the patients' medical records after complete histologic examination of all IDCs. All patients were Japanese women, and they ranged in age from 23 to 75 years old (median, 55 years). All had a solitary lesion; 473 patients were premenopausal, and 887 were postmenopausal. Partial mastectomy had been performed in 571, and modified radical mastectomy had been performed in 789. Level I and level II axillary lymph node dissection had been performed in all patients, and level III axillary lymph node dissection had been performed in some of IDC the patients.

Of the 1042 patients, 873 received adjuvant therapy, which consisted of chemotherapy in 209 patients, endocrine therapy in 294 patients, and chemoendocrine therapy in 370 patients. The chemotherapy regimens used were anthracycline based with or without taxane and non–anthracycline based, and the endocrine therapy regimens consisted of tamoxifen with or without a gonadotropin-releasing-hormone agonist, tamoxifen followed by an aromatase inhibitor, an aromatase inhibitor alone, or a gonadotropin-releasing-hormone agonist alone. None of the patients received radiotherapy before surgery. There were no cases of inflammatory breast cancer in this series. All tumors were classified according to the pathologic International Union Against Cancer (UICC) tumor-node-metastasis classification system [8]. The protocol of this study was reviewed by the institutional review board of the National Cancer Center (20-112), and all patients provided written informed consent.

For pathologic examination, the surgically resected specimens were fixed in 10% formalin; and the size and gross appearance of the tumors were recorded. Their size was confirmed by comparison with tumor size on histologic slides; and if there was more than one invasive focus, the size of the largest invasive focus was recorded as the invasive tumor size in this study.

2.2. Histologic examination

Serial sections of each tumor area were cut from paraffin blocks. One section from each tumor was stained with hematoxylin and eosin and examined histologically to confirm the diagnosis, and another section was subjected to immunohistochemistry. The following 9 histologic factors were evaluated: (1) invasive tumor size (≤ 20, >20 to ≤ 50, >50 mm), (2) histologic grade (1, 2, 3) [9], (3) tumor necrosis (absent, present) [10], (4) fibrotic focus (FF) (absent, FF diameter ≤ 8 mm, FF diameter >8 mm) [11,12], (5) blood vessel invasion (absent, present), (7) adipose tissue invasion (absent, present), (8) skin invasion (absent, present), and (9) muscle invasion (absent, present).

2.3. Grading system for LVTEs in IDCs

We have already stated the histologic criteria of the grading system for LVTEs in our previous study [6]. Briefly, we first examined all slides of IDCs that contained both tumor areas and nontumor areas to identify LVTEs. Next, we selected the LVTEs, for example, large LVTEs far from the stroma-invasive tumor margin to examine for the number and number of mitotic figures and apoptotic figures of LVTEs in an IDC (Fig. 1A). We classified all IDCs into the following 4 grades according to the number of mitotic and apoptotic figures in tumor cells in lymph vessels in 1 per high-power field: grade 0—no lymph vessel invasion; grade 1—absence of mitotic and/or apoptotic figures, or presence of any number of mitotic figures and absence of apoptotic
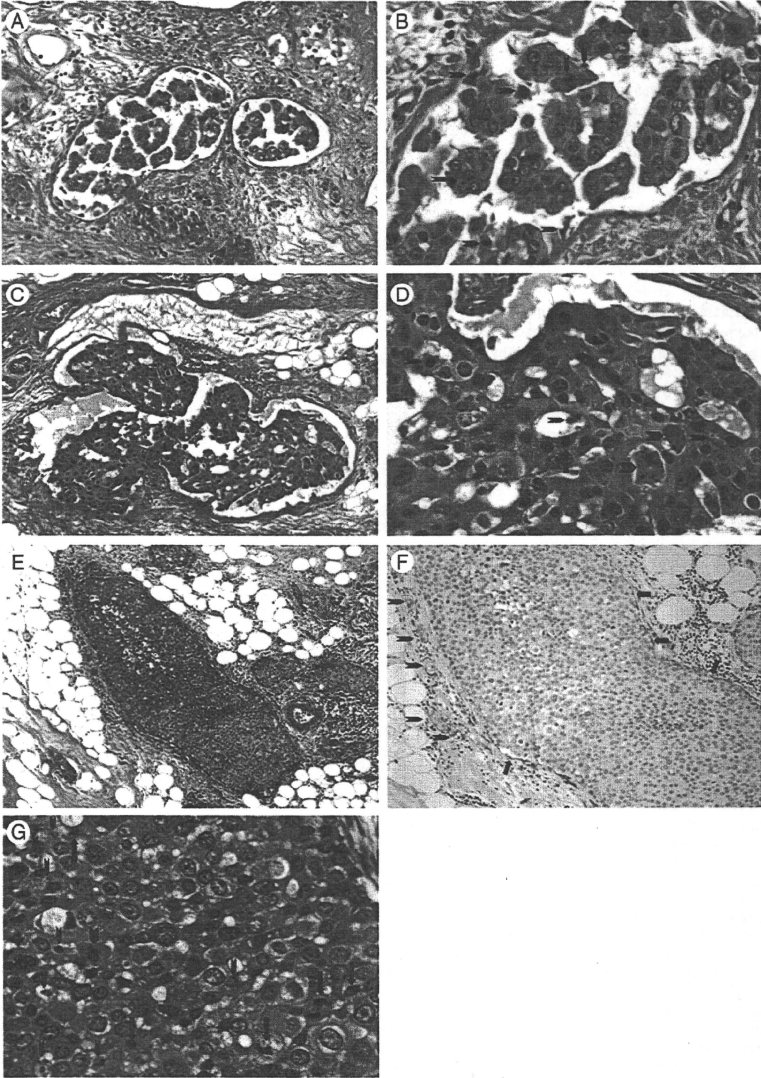
figures, or absence of mitotic figure and presence of any number of apoptotic figures; grade 2—1 to 4 mitotic figures and 1 or more apoptotic figures, or 1 or more mitotic figures and 1 to 6 apoptotic figures; and grade 3—more than 4 mitotic figures and more than 6 apoptotic figures (Fig. 1). We selected the LVTEs in which to count the mitotic figures and apoptotic figures and then recorded the numbers of mitotic figures and apoptotic figures among the tumor cells making up the LVTEs of the IDC in the high-power field that contained the largest number of mitotic figures and/or the largest number of apoptotic figures. These were recorded as the number of mitotic figures and apoptotic figure in the LVTEs of the IDC. Some IDCs contained large LVTEs, especially in IDCs containing a grade 2 or grade 3 LVTEs; and it was difficult to determine whether they were true LVTEs or a noninvasive ductal carcinoma component by hematoxylin and eosin staining alone. We therefore performed immunohistochemical staining with D2-40 antibody (monoclonal mouse antibody; Signet, Dedham, MA, 1:200) to confirm that the LVTEs identified by hematoxylin and eosin staining were true tumor emboli in some IDCs with grade 2 or grade 3 LVTEs (Fig. 1F). The D2-40 antibody was generated against an O-linked sialoglycoprotein having a molecular weight of 40 kd and had been demonstrated to be a selective marker of lymphatic endothelium [13,14].

2.4. Immunohistochemistry

Immunohistochemical staining for estrogen receptors (ERs), progesterone receptors (PRs), p53, HER2 products, and D2-40 was performed with an autoimmunostainer (Optimax Plus; BioGenex, San Ramon, CA). The antigen retrieval device used for the Optimax Plus was an autoclave, and each specimen was immersed in citrate buffer and incubated at 121°C for 10 minutes. Immunoperoxidase staining was performed by using a labeled streptavidin biotin staining kit (BioGenex) according to the manufacturer's instructions. The antibodies used were an anti-ER mouse monoclonal antibody (mAb), ER88 (BioGenex), an anti-PR mAb, PR88 (BioGenex), and an anti-HER2 mAb, CB11 (BioGnex), and a p53 mAb, DO7 (Dako, Glostrup, Denmark). ER88, PR88, and CB11 were already diluted; and DO7 was applied at a 1:100 dilution. After immunostaining, the sections were counterstained with hematoxylin. Sections of IDCs

positive for ER, PR, p53, HER2, and D2-40 were used each time as positive controls. As for a negative control, the primary antibody was replaced with normal mouse immunoglobulin.

Slides immunostained for ER, PR, and p53 in stroma-invasive tumor cells and for p53 in tumor-stromal fibroblasts were scored by the Allred scoring system as described previously [7,15-19]. As for the assessment for p53 in tumor-stromal fibroblasts, the highest intensity score, not the average intensity score, was recorded as the score for nuclear expression of p53 assigned for the tumor-stromal fibroblasts; and the highest p53 nuclear expression proportion score and intensity score were then to evaluated in 1 high-power field (×40 objective and ×10 ocular) [7]. The Allred scores for ER, PR, and p53 expression in stroma-invasive tumor cells and tumor-stromal fibroblasts (Fig. 2A) were classified into the following 3 categories [7]: (a) Allred score for ER in stroma-invasive tumor cells: 0 or 2, 3 to 6, and 7 or 8; (b) Allred score for PR in stroma-invasive tumor cells: 0 or 2, 3 to 6, and 7 or 8; (c) Allred scores for p53 in stroma-invasive tumor cells: 0 or 2 or 3, 4 to 6, and 7 or 8; and (d) Allred scores for p53 in tumor-stromal fibroblasts: 0 or 2, 3, and 4 to 8. HER2 expression in stroma-invasive tumor cells was classified into 3 categories: 0 or 1, 2, and 3 [20]. In addition, we also assigned Allred scores for ER, PR, and p53 (Fig. 2B), and HER2 category in LVTEs in the same manner as in stroma-invasive tumor cells in 109 of the 376 IDCs with lymph vessel invasion. In other IDCs with lymph vessel invasion, we could not assess Allred scores for ER, PR, and p53, and HER2 category because immunohistochemistry for their antibodies was performed in tumor tissue sections that did not contain an LVTE.

One author (TH) assessed all of the immunohistochemical parameters, and 1 of 3 other authors (HT, TS, or YS) reviewed the immunohistochemical parameters to confirm the IDC immunohistochemical characteristics recorded by TH. Discordant results were reevaluated jointly until a consensus was reached. The histologic examination and immunohistochemical examination were performed without knowledge of the patient's outcome.

### 2.5. Patient outcome and statistical analysis

Survival was evaluated by follow-up for a median period of 62 months (range, 38-105 months) until February 2009. As of the end of February 2009, 911 patients were alive and

Fig. 1 Grades 1, 2, and 3 LVTEs. A and B, Grade 1 LVTEs. Two LVTEs, one small and one of medium size, are present (A; ×10 objective and ×10 ocular); and several apoptotic tumor cells (arrowheads) are visible in one LVTE (B; ×40 objective and ×10 ocular). Apoptotic tumor cells were identified as tumor cells that contained eosinophilic or amphophilic cytoplasm and an irregularly shaped pyknotic nucleus. No mitotic tumor cells are visible in the tumor embolus. C and D, Grade 2 LVTE. One large lymph vessel is present (C; ×5 objective and ×10 ocular), and one mitotic tumor cell (arrow) and several apoptotic tumor cells or apoptotic bodies (arrowheads) are seen in the embolus (D; ×40 objective and ×10 ocular). Two clumps of apoptotic bodies (wide arrowheads) are also visible. Apoptotic bodies are small, variously shaped pyknotic bodies that resemble sesame seeds. E, F, and G, Grade 3 LVTE. One very large LVTE located adjacent to one medium-sized artery is present, and stroma-invasive tumor cell nests can be seen in the area surrounding the tumor embolus (E; ×2.5 objective and ×10 ocular). The wall of the tumor lymph vessel containing the embolus is positive for D2-40 (arrows), and D2-40–positive small lymph vessels are seen in the vicinity of the tumor embolus (F; ×10 objective and ×10 ocular). Five mitotic tumor. cells, several apoptotic bodies (arrowheads), and 2 apoptotic tumor cells (wide arrowheads) are seen in the tumor embolus (G; ×40 objective and ×10 ocular).

well, 131 had developed tumor recurrence, and 58 had died of their disease. The measurements of tumor-recurrence-free survival and overall survival started at the time of surgery. Tumor relapse was considered to have occurred whenever there was evidence of metastasis.

Multiple regression analysis was used to perform the statistical analyses for associations between LVTE grade and number of lymph vessel invasion and between LVTE grade and number of lymph node metastases, and the correlation analyses were performed by the correlation statistics of Cochran-Mantel-Haenszel statistics.

We analyzed the outcome predictive power of the 9 histologic factors; grading system for LVTEs; Allred scores
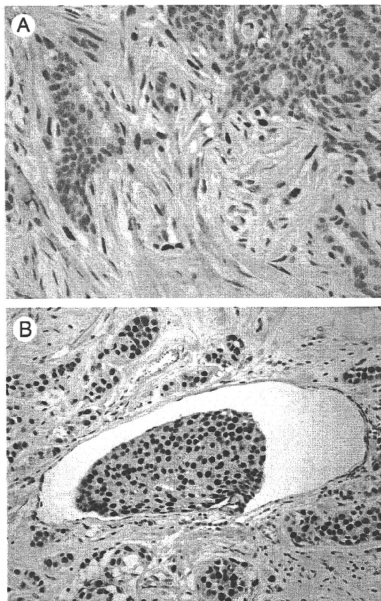
Fig. 2 Tumor-stromal fibroblasts positive for p53 and tumor embolus positive for p53. A, The tumor stroma contains several tumor-stromal fibroblasts with scores of 1, 2, or 3 for intensity of p53 expression; and the Allred score for p53 in the tumor-stromal fibroblasts is 7. The tumor cells have scores of 1 or 2 for intensity of p53 expression, and the Allred score of p53 in tumor cells is 5. More than 60% of the tumor-stromal fibroblasts show positive nuclear staining for p53, and more than 60% of the tumor cells also show positive nuclear staining for p53. B, An LVTE with an Allred score of 8 for p53 and stroma-invasive tumor cells with an Allred score of 8 for p53 can be seen. More than 90% of the tumor cells comprising the LVTE and more than 90% of the stroma-invasive tumor cells show intense nuclear staining for p53.

Table 1 Outcome rates of patients with IDC according to grade of LVTEs and according to nodal status

IDC patients as a whole					
	Cases	TRR (%)	P value	MR (%)	P value
Grade of LVTEs					
Grade 0	666	51 (8)		18 (3)	
Grade 1	250	25 (10)	.442	11 (4)	.628
Grade 2	97	35 (36)	<.001	15 (16)	.017
Grade 3	29	20 (69)	<.001	14 (48)	<.001
Total	1042	131		58	
IDC patients who did not have nodal metastasis					
Grade 0	465	25 (5)		6 (1)	
Grade 1	111	7 (6)	.982	1 (1)	.540
Grade 2	14	5 (36)	.006	0	NA
Grade 3	1	1 (100)	.022	1 (100)	NA
Total	591	38		8	
IDC patients who had nodal metastasis					
Grade 0	201	26 (13)		12 (6)	
Grade 1	139	18 (13)	.872	10 (7)	.891
Grade 2	83	30 (36)	.002	15 (18)	.008
Grade 3	28	19 (68)	<.001	13 (46)	.002
Total	451	93		50	

Abbreviations: TRR, tumor recurrence rate; MR, mortality rate; NA, not available.

for ER, PR, and p53 in stroma-invasive tumor cells; category of HER2 expression in stroma-invasive tumor cells; Allred scores for p53 in tumor-stromal fibroblasts; adjuvant therapy (no and yes); age (≤39 and >39 years); and UICC pathologic nodal status (N factor; ie, no nodal metastasis, N0; 1-3 nodal metastases, N1; 4-9 nodal metastases, N2; and ≥10 nodal metastases, N3) [8] for tumor recurrence and tumor death in the univariate analyses using the Cox proportional hazard regression model [21]. The factors significantly associated with outcome in the univariate analyses were then entered together into the multivariate analyses using the Cox proportional hazard regression model [21] according to nodal status. The casewise and step-down method was applied until all of the remaining factors were significant at a P value < .05. Because there were fewer than 10 tumor deaths among the patients who did not have nodal metastasis (Table 1), it was impossible to perform multivariate analyses for tumor death in this group. Survival curves were drawn by the Kaplan-Meier method [22]. All analyses were performed with Statistica/Windows software (StatSoft, Tulsa, OK).

3. Results

3.1. Associations between the LVTE grades and factors

The LVTE grades were significantly associated with the increase in mean number of lymph vessels invaded (β, 0.492;
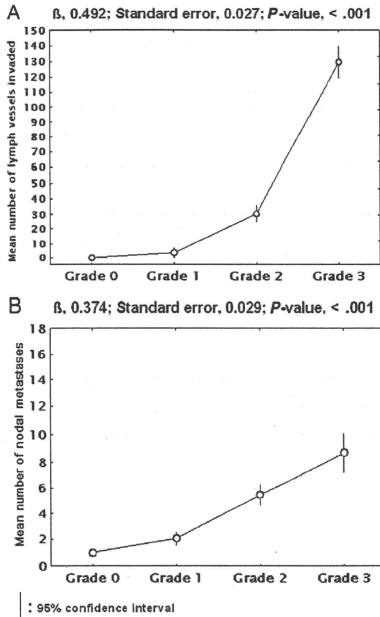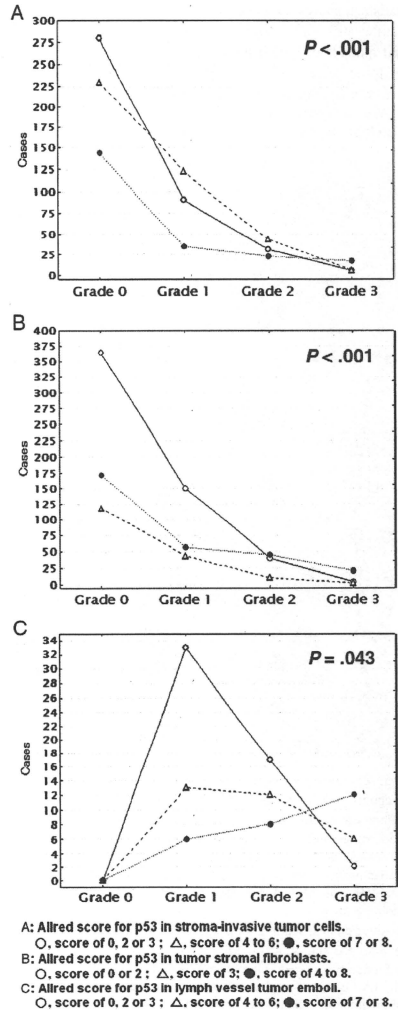
A ß, 0.492; Standard error, 0.027; *P*-value, < .001

B ß, 0.374; Standard error, 0.029; *P*-value, < .001

: 95% confidence interval

Fig. 3 Graphs showing associations between numbers of lymph vessels invaded and grades of LVTEs (A) and between mean nodal metastasis values and the grades of LVTEs in the no-neoadjuvant therapy group (B). The mean numbers of lymph vessels invaded and of nodal metastases increase significantly with the grade of LVTEs.

standard error, 0.027; *P* value < .001; Fig. 3A) and with the increase in nodal metastases (ß, 0.374; standard error, 0.029; *P* value < .001; Fig. 3B). The results of the correlation analyses showed that the LVTE grades were significantly associated with the Allred scores for p53 in stroma-invasive tumor cells and in tumor-stromal fibroblasts (Fig. 4A and B) and that they were also significantly inversely associated with Allred scores for ER and PR in stroma-invasive tumor cells
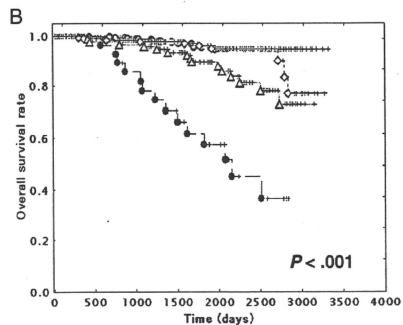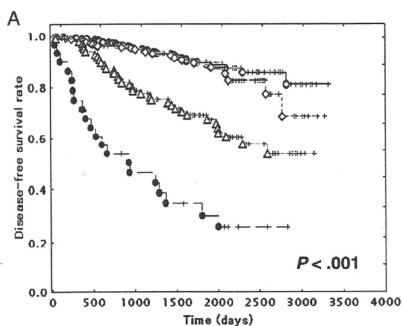
Fig. 4 Graphs showing associations between the Allred scores for p53 in tumor cells (A) and in tumor-stromal fibroblasts (B), and the grades of LVTEs. The number of cases with Allred scores of 7 or 8 for p53 in tumor cells significantly increases with the grade of LVTEs (A), and number of cases with Allred scores of 4 to 8 for p53 in tumor-stromal fibroblasts also significantly increases with the grade of LVTEs (B). C, Graphs showing associations between Allred scores for p53 in LVTEs. The number of cases with Allred scores of 7 or 8 for p53 in LVTEs significantly increases with the grade of LVTEs.

(data not shown). In addition, the Allred scores for p53 (Fig. 4C) and HER2 category in the LVTEs were significantly associated with the grades of the LVTEs; and the All red scores for ER in LVTE were significantly inversely associated with the grades of the LVTEs (data not shown).

A

P < .001

B

P < .001

C

P = .043

A: Allred score for p53 in stroma-invasive tumor cells.
  O, score of 0, 2 or 3 ; △, score of 4 to 6; ●, score of 7 or 8.
B: Allred score for p53 in tumor stromal fibroblasts.
  O, score of 0 or 2 ; △, score of 3; ●, score of 4 to 8.
C: Allred score for p53 in lymph vessel tumor emboli.
  O, score of 0, 2 or 3 ; △, score of 4 to 6; ●, score of 7 or 8.

3.2. Factors significantly associated with outcome

In the IDC patients as a whole, although no significant difference in intervals to tumor recurrence or to tumor-related death was observed between IDC patients with grade 0 LVTEs and those with grade 1 LVTEs in the univariate analyses, among the IDC patients with grades 1, 2, and 3 LVTEs, the intervals to tumor recurrence and tumor-related death became significantly shorter as grades of LVTEs increased (Table 1, Fig. 5). Similar findings were also observed in the IDC patients who had nodal metastasis; and in the IDC patients who did not have nodal metastasis, the interval to tumor recurrence became significantly shorter as the grades of LVTEs increased (Table 1).

In the IDC patients as a whole, the multivariate analyses showed that grade 3 LVTEs, Allred scores of 4 to 8 for p53 in tumor-stromal fibroblasts, FF diameter greater than 8 mm, and UICC pN3 category significantly increased the hazard rates (HRs) for tumor recurrence and tumor-related death (data not shown). Grade 2 LVTEs and HER2 category 3 in tumor cells significantly increased the HRs for tumor recurrence, and UICC pN1 and pN2 categories and the presence of skin invasion significantly increased the HRs for tumor-related death in the multivariate analyses (data not shown). Allred scores of 7 or 8 for PRs in tumor cells and Allred scores of 7 or 8 for ERs in tumor cells significantly decreased the HRs for tumor-related death in the multivariate analyses (data not shown).

In the IDC patients who did not have nodal metastasis, grades 2 and 3 LVTEs, Allred scores of 4 to 8 for p53 in tumor-stromal fibroblasts, HER2 category 3, and histologic grade 3 significantly increased the HRs for tumor recurrence in the multivariate analysis (Table 2).

In the IDC patients who had nodal metastasis, grade 3 LVTEs, Allred scores of 4 to 8 for p53 in tumor-stromal fibroblasts, and FF diameter greater than 8 mm significantly increased the HRs for tumor recurrence and tumor-related death in the multivariate analyses (Table 3). Grade 2 LVTEs and UICC pN3 category significantly increased the HRs for tumor recurrence, and the presence of skin invasion significantly increased the HR for tumor-related death in the multivariate analyses (Table 3). Allred scores of 7 or 8 for

A

$P < .001$

B

$P < .001$

○, Grade 0; ◇, Grade 1; △, Grade 2; ●, Grade 3

Fig. 5   Disease-free survival curves and overall survival curves of IDC patients (A and B) according to the grade of LVTEs. A, The disease-free survival time of the IDC patients without nodal metastasis significantly decreases with the grade of LVTEs. B, The overall survival time of IDC patients with nodal metastasis significantly decreases with the grade of LVTEs.

Table 2   Multivariate analysis for tumor recurrence in IDC patients who did not have nodal metastasis

Factors	Cases	No. of patients (%)			
		Tumor recurrence			
		Present	HRs	95% CI	P value
Grading system for LVTEs					
Grade 0	465	25 (5)	Referent		
Grade 1	111	7 (6)	1.0	0.4-2.5	.987
Grades 2 and 3	15	6 (40)	3.2	1.2-8.4	.017
The Allred scores for p53 in tumor-stromal fibroblasts					
0 or 2	323	7 (2)	Referent		
3	102	5 (5)	1.7	0.5-5.6	.363
4 to 8	155	23 (15)	3.8	1.8-7.8	<.001
HER2 category in tumor cells					
0 or 1	414	16 (4)	Referent		
2	103	6 (6)	1.3	0.5-3.4	.654
3	62	12 (19)	2.1	1.1-5.0	.023
Histologic grade					
1	173	3 (2)	Referent		
2	251	11 (4)	1.2	0.3-4.4	.821
3	167	24 (14)	2.1	1.0-4.4	.043

NOTE. Tumor recurrence: adjusted for grading system for LVTEs, the Allred scores for p53 in tumor-stromal fibroblasts, HER2 category in tumor cells, histologic grade, the Allred scores for PRs in tumor cells, and the Allred scores for ERs in tumor cells.
Abbreviation: CI, confidence interval.

Table 3 Multivariate analyses for tumor recurrence and tumor-related death in IDC patients who had nodal metastasis

Factors	Cases	No. of patients (%)			
		Tumor recurrence		Tumor-related death	
		Present	HRs (95% CI) P value	Present	HRs (95% CI) P value
Grading system for LVTEs					
Grade 0	201	26 (13)	Referent	12 (6)	Referent
Grade 1	139	18 (13)	1.1 (0.6-2.1) .713	10 (7)	1.2 (0.5-2.9) .717
Grade 2	83	30 (36)	1.8 (1.1-2.9) .023	15 (18)	1.2 (0.5-2.8) .685
Grade 3	28	19 (68)	3.6 (2.0-6.6) <.001	13 (46)	2.3 (1.1-4.8) .028
The Allred scores for p53 in tumor-stromal fibroblasts					
0 or 2	236	20 (8)	Referent	8 (3)	Referent
3	71	8 (11)	1.6 (0.7-3.6) .292	3 (4)	1.6 (0.4-6.4) .514
4 to 8	139	64 (46)	4.3 (2.7-7.0) <.001	38 (27)	7.2 (3.5-14.8) <.001
FF diameter (mm)					
Absent	252	45 (18)	Referent	19 (8)	Referent
≤8	107	15 (14)	0.6 (0.3-1.1) .112	9 (8)	1.0 (0.4-2.4) .930
>8	92	33 (36)	2.2 (1.4-3.3) <.001	22 (24)	2.7 (1.5-4.8) <.001
UICC pN category					
N1	317	50 (16)	Referent	25 (8)	Referent
N2	86	18 (21)	0.8 (0.5-1.5) .548	10 (12)	1.1 (0.5-2.5) .841
N3	48	25 (52)	2.1 (1.3-3.4) .002	15 (31)	2.1 (0.9-4.7) .071
Skin invasion					
Absent	400	78 (20)	Referent	38 (10)	Referent
Present	51	15 (29)	–	12 (24)	3.4 (1.7-7.1) <.001
The Allred scores for PRs in tumor cells					
0 or 2	77	24 (31)	Referent	11 (14)	Referent
3 to 6	134	33 (25)	0.5 (0.2-1.2) .103	23 (17)	0.8 (0.4-2.1) .712
7 or 8	235	35 (15)	0.5 (0.3-1.1) .067	15 (6)	0.4 (0.2-0.8) .006

NOTE. Tumor recurrence: adjusted for grading system for LVTEs, the Allred scores for p53 in tumor-stromal fibroblasts, FF diameter, UICC pN category, skin invasion, the Allred scores for PR in tumor cells, age, the Allred scores for ERs in tumor cells, the Allred scores for p53 in tumor cells, invasive tumor size, histologic grade, tumor necrosis, and blood vessel invasion. Tumor-related death: adjusted for grading system for LVTEs, the Allred scores for p53 in tumor-stromal fibroblasts, FF diameter, UICC pN category, skin invasion, the Allred scores for PR in tumor cells, age, the Allred scores for ERs in tumor cells, the Allred scores for p53 in tumor cells, invasive tumor size, histologic grade, and blood vessel invasion. "–" indicates not significant in univariate analysis.

PR significantly decreased the HR for tumor-related death in the multivariate analysis (Table 3).

4. Discussion

The results of this study clearly demonstrated significant associations between increases in grades of LVTE and number of nodal metastases. Because we have also found a significant association between grades of LVTE and number of nodal metastases in a different IDC group in another study [6], the grading system for LVTEs can be concluded to be a very useful histologic grading system for accurately predicting lymph node metastasis by IDCs.

In a previous study, we found that the grading system for LVTEs can be used to classify IDC patients with lymph vessel invasion into low-, intermediate-, and high-risk groups for outcome and that IDCs with grade 0 LVTEs and IDCs with grade 1 LVTEs are almost equally malignant

in a different IDC group [6]. Those findings were clearly confirmed in the present study again; and more than half of IDCs with LVTEs were IDCs with grade 1 LVTEs, and the IDCs with grade 2 or grade 3 LVTEs accounted for a relatively small proportion of the total number of IDCs with LVTEs. Thus, the LVTE grading system enables pathologists to identify many low–malignant-grade IDCs with LVTEs as similar as IDCs without LVTE from the IDC group with LVTEs. The grading system for LVTEs is therefore concluded not only to be an excellent histologic grading system for IDCs that makes it possible to accurately predict the outcome of IDC patients, but to enable pathologists to accurately determine the probability of IDCs having lymph vessels to be invaded by tumor cells independent of nodal status.

The grading system for LVTEs was superior to histologic grade in terms of accurately predicting the outcome of the IDC patients in this study. This finding strongly suggests that tumor cells that invade the tumor stroma and enter lymph vessels are more aggressive than tumor cells that invade the tumor stroma but are unable to enter lymph vessels. However, histologic grade reflects only the number of mitotic figures among stroma-invasive tumor cells; and it does not reflect the number of apoptotic figures among them. Apoptotic figures in tumors have been reported to be a useful histologic predictor for accurately differentiating between carcinoma and noncarcinoma and between different types of tumors [23,24], and de Jong et al [25] reported that apoptotic figures in invasive tumor cells are useful in making a prognosis in breast cancer patients. Thus, a histologic grade for mitotic figures and apoptotic figures in stroma-invasive tumor cells may be superior to the ordinary histologic grade as a basis for accurately predicting the outcome of IDC patients.

The results of this study clearly demonstrated that the LVTE grades are significantly associated with both Allred scores for p53 in LVTEs and Allred scores for p53 in stroma-invasive tumor cells and in tumor-stromal fibroblasts; this strongly suggests that p53 protein expression in LVTEs, in tumor-stromal fibroblasts, and in stroma-invasive tumor cells is a very important key factor for evaluating the malignant potential of IDCs with LVTEs. Especially, because the LVTE grades are based on the numbers of mitotic figures and apoptotic figures in tumor cells in lymph vessels, p53 protein expression in LVTEs probably accelerates the turnover rate of tumor cells comprising LVTEs and increases the malignancy of IDCs as the LVTE grade rises. Although p53 mutations in tumor-stromal fibroblasts are relatively common in primary breast cancer and other cancers and they have a positive effect on cancer growth [26-30], some studies have found no p53 mutations in the tumor stroma of breast cancer [31-33]. Thus, p53 immunoreactivity in tumor-stromal fibroblasts as well as p53 gene abnormality may in fact reflect specific reactive changes within the stroma that are related to the outcome of IDC patients. The mechanism responsible for the increases in

malignant potential of IDCs according to grade of LVTE should therefore be investigated from the standpoint of p53 gene abnormalities or specific reactive change in LVTEs as well as in tumor-stromal fibroblasts or in stroma-invasive tumor cells. In addition, because some studies have reported several genes that closely regulate the cell cycle of tumors [34-36], such genes should be investigated to determine whether they are candidates for p53 in regulating tumor cell cycle of LVTEs.

Although no cases of inflammatory breast cancer cases were included in this study, a significant association between increases in grade of LVTEs and number of lymph vessels invaded was observed in this study, especially in grade 3 LVTEs; and IDCs with grade 2 and grade 3 LVTEs had a poor outcome. Their biological features were very similar to those of inflammatory breast cancer. These findings strongly suggest that some inflammatory ductal carcinomas originate from IDCs with grade 2 or 3 LVTEs. Because it has been reported that some stem cell markers play a very important role in tumor progression by inflammatory breast cancers [37], they are probably also very important key regulators of the cell cycle of tumor cells in lymph vessels, thereby increasing the biological malignancy of IDCs according to the grades of LVTEs.

In conclusion, the grading system for LVTEs is significantly associated with nodal metastasis and is an excellent histologic grading system for accurately predicting the outcome of patients with IDC of the breast. Pathologists can most accurately assess the true malignant potential of IDCs by using this grading system as a histologic prognostic classification for IDCs of the breast.

References

[1] Lauria R, Perrone F, Carlomagno C, et al. The prognostic value of lymphatic and blood vessel invasion in operable breast cancer. Cancer 1995;76:1772-8.
[2] Nime FA, Rosen PP, Tzvi Thaler H, et al. Prognostic significance of tumor emboli intramammary lymphatics in patients with mammary carcinoma. Am J Surg Pathol 1977;1:25-30.
[3] Hasebe T, Sasaki S, Imoto S, et al. Characteristics of tumors in lymph vessels play an important role in the tumor progression of invasive ductal carcinoma of the breast: a prospective study. Mod Pathol 2002; 15:904-13.
[4] Hoshida T, Isaka N, Hagendoorn J, et al. Imaging steps of lymphatic metastasis reveals that vascular endothelial growth factor-C increases metastasis by increasing delivery of cancer cells to lymph nodes: therapeutic implications. Cancer Res 2006;66:8065-75.
[5] Yamauchi C, Hasebe T, Iwasaki M, et al. Accurate assessment of lymph vessel tumor emboli in invasive ductal carcinoma of the breast according to tumor areas, and their prognostic significance. HUM PATHOL 2007;38:247-59.
[6] Hasebe T, Yamauchi C, Iwasaki M, et al. Grading system for lymph vessel tumor emboli for prediction of the outcome of invasive ductal carcinoma of the breast. HUM PATHOL 2008;39:427-36.
[7] Hasebe T, Okada N, Tamura N, et al. p53 expression in tumor-stromal fibroblasts is associated with the number of nodal metastasis and the outcome of patients with invasive ductal carcinoma independent of the breast. Cancer Sci 2009;100:2101-8.

[8] Sobin LH, Wittekind CH. Breast tumours (ICD-O C50). In: Sobin LH, Wittekind CH, editors. TNM classification of malignant tumors. 6th ed. Geneva: Wiley-Liss; 2002. p. 131-41.

[9] Bloom HJG, Richardson WW. Histological grading and prognosis in breast cancer. Br J Cancer 1957;11:359-77.

[10] Gilchrist KW, Gray R, Fowble B, et al. Tumor necrosis is a prognostic predictor for early recurrence and death in lymph node-positive breast cancer: a 10-year follow-up study of 728 eastern cooperative oncology group patients. J Clin Oncol 1993;11:1929-35.

[11] Hasebe T, Tsuda H, Hirohashi S, et al. Fibrotic focus in infiltrating ductal carcinoma of the breast: a significant histopathological prognostic parameter for predicting the long-term survival of the patients. Breast Cancer Res Treat 1998;49:195-208.

[12] Hasebe T, Sasaki S, Imoto S, et al. Prognostic significance of fibrotic focus in invasive ductal carcinoma of the breast: a prospective observational study. Mod Pathol 2002;15:502-16.

[13] Fukunaga M. Expression of D2-40 in lymphatic endothelium of normal tissues and in vascular tumours. Histopathology 2005;46: 396-402.

[14] Niakosari F, Kahn HJ, Marks A, et al. Detection of lymphatic invasion in primary melanoma with monoclonal antibody D2-40: a new selective immunohistochemical marker of lymphatic endothelium. Arch Dermatol 2005;141:440-4.

[15] Allred DC, Harvey JM, Berardo MD, et al. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. Mod Pathol 1998;11:155-68.

[16] Harvey JM, Clark GM, Osborne K, et al. Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. J Clin Oncol 1999;17:1474-81.

[17] Mohsin S, Weiss H, Havighurst T, et al. Progesterone receptor by immunohistochemistry and clinical outcome in breast cancer: a validation study. Mod Pathol 2004;17:1545-54.

[18] Badve SS, Baehner FL, Gray RP, et al. Estrogen- and progesterone-receptor status in ECOG2197: comparison of immunohistochemistry by local and central laboratories and quantitative reverse transcription polymerase chain reaction by central laboratory. J Clin Oncol 2008;26: 2473-81.

[19] Allred DC, Clark GM, Elledge R, et al. Association of p53 protein expression with tumor cell proliferation rate and clinical outcome in node-negative breast cancer. J Natl Cancer Inst 1993;85:200-6.

[20] Wolff AC, Hammond ME, Schwartz JN, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. Arch Pathol Lab Med 2007;131:18-43.

[21] Cox DR. Regression models and life-tables. J R Stat Soc 1972;34: 187-220.

[22] Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. J Am Stat Assoc 1958;53:457-81.

[23] Charles VB, William H. Apoptotic bodies: a consistent morphologic feature of endocervical adenocarcinoma in situ. Am J Surg Pathol 1998;22:434-9.

[24] Bishop EF, Badve S, Morimiya A, et al. Apoptosis in spermatocytic and usual seminomas: a light microscopic and immunohistochemical study. Mod Pathol 2007;20:1036-44.

[25] de Jong JS, van Diest PV, Baak JPA. Number of apoptotic cells as a prognostic marker in invasive breast cancer. Br J Cancer 2000;82: 368-73.

[26] Hill R, Song Y, Cardiff RD, et al. Selective evolution of stromal mesenchyme with p53 loss in response to epithelial tumorigenesis. Cell 2006;123:1001-11.

[27] Bierie B, Moses HL. Under pressure: stromal fibroblasts change their ways. Cell 2005;123:985-7.

[28] Moinfar F, Man YG, Arnould L, et al. Concurrent and independent genetic alterations in the stromal and epithelial cells of mammary carcinoma: implication for tumorigenesis. Cancer Res 2000;60: 2562-6.

[29] Patocs A, Zhang L, Xu Y, et al. Breast-cancer stromal cells with TP53 mutations and nodal metastases. N Engl J Med 2007;357:2543-51.

[30] Kiaris H, Chatzistamou I, Trimis G, et al. Evidence for nonautonomous effect of p53 tumor suppressor in carcinogenesis. Cancer Res 2005;65:1627-30.

[31] Allinen M, Beroukhim R, Cai L, et al. Molecular characterization of the tumor microenviroment in breast cancer. Cancer Cell 2004;6: 17-32.

[32] Lebret SC, Newgreen DF, Thompson EW, et al. Induction of epithelial to mesenchymal transition in PMC42-LA human breast carcinoma cells by carcinoma-associated fibroblast secreted factors. Breast Cancer Res 2007;9:R19.

[33] Campbell IG, Qiu W, Polyak K, et al. Breast-cancer stromal cells with TP53 mutations. N Engl J Med 2008;10:1634-5.

[34] Westbrook L, Manuvakhova M, Kern FG, et al. Cks1 regulates cdk1 expression: a novel role during mitotic entry in breast cancer cells. Cancer Res 2007;67:11393-401.

[35] Nakamura Y, Tanaka F, Haraguchi N, et al. Clinicopathological and biological significance of mitotic centromere-associated kinesin over-expression in human gastric cancer. Br J Cancer 2007;97:543-9.

[36] Budhram-Mahadeo VS, Irshad S, Bowen S, et al. Proliferation-associated Brn-3b transcription factor can activate cyclin D1 expression in neuroblastoma and breast cancer cells. Br J Cancer 2008;27:145-54.

[37] Xiao Y, Ye Y, Yearsley K, et al. The lymphovascular embolus of inflammatory breast cancer expresses a stem cell-like phenotype. Am J Pathol 2008;173:561-74.

Grading system for lymph vessel tumor emboli: significant outcome predictor for patients with invasive ductal carcinoma of the breast who received neoadjuvant therapy

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The purpose of this study was to confirm that the grades of lymph vessel tumor emboli in biopsy specimens obtained before neoadjuvant therapy and in the surgical specimens obtained after neoadjuvant therapy according to the grading system we devised are significant histological outcome predictor for invasive ductal carcinoma (IDC) patients who received neoadjuvant therapy. The subjects of this study were the 318 consecutive IDC patients who had received neoadjuvant therapy in our institution. The lymph vessel tumor embolus grades in the biopsy specimens and in the surgical specimens were significantly associated with the increases in mean number of nodal metastases. Multivariate analyses with well-known prognostic factors and p53 expression in tumor-stromal fibroblasts clearly showed that the lymph vessel tumor embolus grade based on the biopsy specimens and based on the surgical specimens significantly increased the hazard rates for tumor recurrence and tumor-related death in all the IDC patients as a whole, in the IDC patients who did not have nodal metastasis, and in the IDC patients who had nodal metastasis, and the outcome-predictive power of the lymph vessel tumor embolus grades based on the surgical specimens was superior to that of the lymph vessel tumor embolus grades based on the biopsy specimens. The grades in the grading system for lymph vessel tumor emboli were significantly associated with nodal metastasis, and the histological grading system is an excellent system for accurately predicting the outcome of patients with IDC of the breast who have received neoadjuvant therapy.
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Keywords: lymph vessel; histology; breast cancer; prognosis; p53

Lymphatic invasion in breast cancer patients with invasive ductal carcinoma (IDC) has been reported to have prognostic significance.[1–5] We have already

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reported that the grading system for lymph vessel tumor emboli that we devised is a very useful histological grading system for accurately predicting the outcome of patients with IDC who did not receive neoadjuvant therapy, and that the grading system can be used to classify IDC patients with lymph vessel invasion into a low-, intermediate-, and high-risk groups for outcome.[6] Furthermore, we have recently reported finding that the lymph vessel tumor embolus grades based on the biopsy specimens and based on the surgical specimens are also

an important outcome-predictive factor for IDC patients who received neoadjuvant chemotherapy and had nodal metastasis.[7]

The purpose of this study was to confirm that the grading system for lymph vessel tumor embolus is a significant outcome predictor for IDC patients who received neoadjuvant therapy according to nodal status, by multivariate analysis with well-known clinicopathological factors and p53 protein expression in tumor-stromal fibroblasts in IDCs.[8] p53 protein expression in tumor-stromal fibroblasts, but not in tumor cells, in IDCs has been recently demonstrated to be a very important outcome predictor for IDC patients who received neoadjuvant therapy.[8] The results of this study clearly showed that the grading system for lymph vessel tumor emboli is an excellent histological outcome predictive of the histological grading system for IDC patients who received neoadjuvant therapy independent of nodal metastasis.

Materials and methods

Cases

The subjects of this study were the 318 consecutive patients with IDC of the breast who received neoadjuvant therapy and were surgically treated at the National Cancer Center Hospital between January 2000 and December 2005 (the same series of patients as in an earlier study we conducted[8] and 88 patients of the 318 patients in this series were included among the subjects of our previous study[7]). Their IDCs were diagnosed preoperatively by needle biopsy, aspiration cytology, mammography, or ultrasonography. Clinical information was obtained from the patients' medical records after complete histological examination of all IDCs. All patients were Japanese women, and they ranged in age from 23 to 77 years old (median, 55 years). All had a solitary lesion; 127 patients were premenopausal, and 191 were postmenopausal. Partial mastectomy had been performed in 152, and modified radical mastectomy in 166. Level I and Level II axillary lymph node dissection had been performed in all patients, and Level III axillary lymph node dissection had been performed in some of the IDC patients.

Of the 318 subjects, 37 (12%) had shown a pathological complete response to neoadjuvant therapy (34, no residual tumor and no nodal metastasis; 3, residual ductal carcinoma *in situ* and no nodal metastasis).

The neoadjuvant therapy consisted of chemotherapy in 235 patients, endocrine therapy in 43 patients, and chemoendocrine therapy in 3 patients, and 214 out of the 281 patients who had received neoadjuvant therapy had also received adjuvant therapy, which consisted of chemotherapy in 47 patients, endocrine therapy in 116 patients, and chemoendocrine therapy in 51 patients. The chemotherapy regimens used were anthracycline-based with or without taxane and non-anthracycline-based, and 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. There were no cases of inflammatory breast cancer in this series. All tumors were classified according to the pathological UICC-TNM (pTNM) classification system.[9] The protocol of this study (20–112) was reviewed by the institutional review board of the National Cancer Center, and all patients provided written informed consent.

For the pathological examination, biopsy specimens obtained before neoadjuvant therapy and surgically resected specimens obtained after neoadjuvant therapy were fixed in 10% formalin and subsequently examined. The size and gross appearance of the surgically resected tumor specimens were recorded as the residual invasive tumor size. The tumor size of the surgically resected specimens was confirmed by comparison with the tumor size on histological slides; if more than one invasive focus was present, the size of the largest invasive focus was recorded as the residual invasive tumor size in this study.

Histological Examination

Serial sections of the biopsy specimens obtained before neoadjuvant chemotherapy and of the tumor area in the surgically resected specimens obtained after neoadjuvant therapy were cut from paraffin wax blocks. One section of each biopsy specimen and surgical specimen was stained with hematoxylin and eosin and examined histologically to confirm the diagnosis, and another section was subjected to immunohistochemistry. The following eight histological features of the primary-invasive tumors were evaluated in the biopsy specimens obtained before neoadjuvant therapy and the surgical specimens obtained after neoadjuvant therapy: (1) residual tumor size (no residual tumor or residual ductal carcinoma *in situ*, residual tumor ≤ 20 mm, > 20 to ≤ 50 mm, > 50 mm), (2) histological grade (1, 2, 3),[10] (3) tumor necrosis (absent, present),[11] (4) fibrotic focus (biopsy specimen: absent, present; surgical specimen: absent, fibrotic focus diameter < 8 mm, fibrotic focus diameter > 8 mm),[12,13] (5) blood vessel invasion (absent, present), (6) adipose tissue invasion (absent, present), (7) skin invasion (absent, present), and (8) muscle invasion (absent, present). We also evaluated the outcome-predictive power of Fisher's neoadjuvant therapy effect classification for surgical specimens obtained after neoadjuvant therapy.[14,15]

Grading System for Lymph Vessel Tumor Emboli in IDCs

We have already stated the histological criteria of the grading system for lymph vessel tumor emboli in

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our previous study.[6,7] Briefly, we first examined all slides of IDCs that contained both tumor areas and nontumor areas to identify lymph vessel tumor emboli. Next, we selected the lymph vessel tumor emboli, for example, large lymph vessel tumor emboli far from the stroma-invasive tumor margin to examine for the presence and number of mitotic figures and apoptotic figures of lymph vessel tumor emboli in an IDC (Figures 1a and c). We classified all IDCs into the following four grades according to the number of mitotic and apoptotic figures in tumor cells in lymph vessels in one per high-power field: grade 0, no lymph vessel invasion; grade 1, absence of no mitotic and or apoptotic figures, or presence of any number of mitotic figures and absence of but no apoptotic figures, or absence of mitotic figure and presence of any number of apoptotic figures but no mitotic figures; grade 2, 1–4 mitotic figures and ≥1 apoptotic figures, or ≥1 mitotic figures and 1–6 apoptotic figures; and grade 3, >4 mitotic figures and >6 apoptotic figures. We selected the lymph vessel tumor emboli in which to count the mitotic figures and apoptotic figures and then recorded the numbers of mitotic figures and apoptotic figures among the tumor cells making up the lymph vessel tumor emboli of the IDC in the high-power field that contained the largest number of mitotic figures, and/or the largest number of apoptotic figures were recorded as the number of mitotic figures and apoptotic figure in the lymph vessel tumor emboli of the IDC. For the biopsy specimens, we examined the presence or absence of lymph vessel tumor
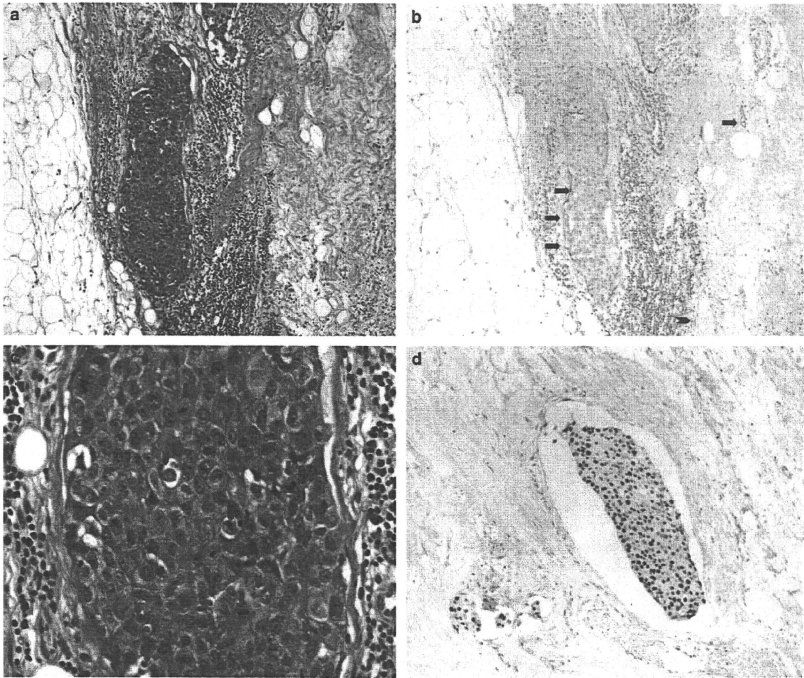
Figure 1 (a) Two lymph vessel tumor emboli are observed. (b) Vessel walls positive for D2-40 around two lymph vessel tumor emboli can be seen (arrows). One small lymph vessel is also positive for D2-40 (arrow), and one artery is negative for D2-40 (arrowhead). (c) Several apoptotic bodies and apoptotic tumor cells are observed (arrowheads), and there are five mitotic tumor cells (arrows) in tumor embolus in the lymph vessel. Apoptotic bodies are small, variously shaped pyknotic bodies that resemble sesame seeds, and apoptotic tumor cells were identified as tumor cells that contained eosinophilic or amphophilic cytoplasm and an irregularly shaped pyknotic nucleus. (d) Three lymph vessel tumor emboli with an Allred score of 8 for p53 can be seen. More than 90% of the tumor cells comprising of the lymph vessel tumor emboli show an intense nuclear staining for p53.

embolus or lymph vessel tumor emboli; when lymph vessel tumor embolus or lymph vessel tumor emboli were observed in the biopsy specimen, an assessment similar to that described above was performed. Some IDCs contained large lymph vessel tumor emboli, especially in IDCs containing a grade 2 or 3 lymph vessel tumor emboli, and it was difficult to determine whether they were true lymph vessel tumor emboli or a non-IDC 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 in some of the IDCs with grade 2 or 3 lymph vessel tumor emboli were true tumor emboli (Figure 1b). The D2-40 antibody was generated against an O-linked sialoglycoprotein having a molecular weight of 40 kDa and had been shown to be a selective marker of the lymphatic endothelium.[16,17]

Immunohistochemistry

Immunohistochemical staining for estrogen receptors, progesterone receptors, p53, HER2 products, and D2-40 was performed with an autoimmunostainer (Optimax Plus: BioGenex, San Ramon, CA, USA). The antigen retrieval device used for the Optimax Plus was an autoclave, and each specimen was immersed in citrate buffer and incubated at 121°C for 10 min. Immunoperoxidase staining was performed by using a labeled streptavidin biotin staining kit (BioGenex) according to the manufacturer's instructions. The antibodies used were an anti-estrogen receptor mouse monoclonal antibody (mAb), ER88 (BioGenex), an anti-progesterone receptor mAb, PR88 (BioGenex), and an anti-HER2 mAb, CB11 (BioGnex), and a p53 mAb, DO7 (Dako, Glostrup, Denmark). ER88, PR88, and CB11 were already diluted, and DO7 was applied at a 1:100 dilution. After immunostaining, the sections were counterstained with hematoxylin. Sections of IDCs positive for estrogen receptor, progesterone receptor, p53, HER2, and D2-40 were used each time as positive controls. As for a negative control, the primary antibody was replaced with normal mouse immunoglobulin.

Slides immunostained for estrogen receptor, progesterone receptor, and p53 in stroma-invasive tumor cells, and for p53 in tumor-stromal fibroblasts were scored by the Allred scoring system as previously described.[8,18–22] The highest intensity score, not the average intensity score, for nuclear expression of p53 was assigned for in tumor-stromal fibroblasts, and the highest p53 nuclear expression proportion score and intensity score were then to be evaluated in one high-power field (× 40 objective and × 10 ocular).[8] The Allred scores for estrogen receptor, progesterone receptor, and p53 expression

in stroma-invasive tumor cells and tumor-stromal fibroblasts were classified into the following three categories: (1) Allred score for estrogen receptor in stroma-invasive tumor cells, 0 or 2, 3–6, and 7 or 8; (2) Allred score for progesterone receptor in stroma-invasive tumor cells, 0 or 2, 3–6, and 7 or 8; (3) Allred scores for p53 in stroma-invasive tumor cells, 0 or 2 or 3, 4–6, and 7 or 8; and (4) Allred scores for p53 in tumor-stromal fibroblasts, 0 or 2, 3, and 4–8. HER2 expression in stroma-invasive tumor cells was classified into the three categories: 0 or 1, 2, and 3.[23] We also assigned Allred scores for estrogen receptor, progesterone receptor, and p53 (Figure 1d), and HER2 category in lymph vessel tumor emboli by the similar manner as in stroma-invasive tumor cells in 26 of the 82 IDCs with lymph vessel invasion. We were unable to assign Allred scores for estrogen receptor, progesterone receptor, and p53, and HER2 category in the other IDCs with lymph vessel invasion, because immunohistochemistry for these was performed in tumor tissue sections that did not containing a lymph vessel tumor embolus.

One author (TH) assessed all the immunohistochemical parameters, and one of four other authors (NT, HT, TS, or YS) reviewed the immunohistochemical parameters to confirm the IDC immunohistochemical characteristics recorded by TH. Discordant results were reevaluated jointly to reach until a consensus was reached. The histological examination and immunohistochemical examination were performed without knowledge of the patient's outcome.

Patient Outcome and Statistical Analysis

Survival was evaluated by follow-up for a median period of 62 months (range: 38–105 months) until February 2009. As of the end of February 2009, 191 of the 281 patients were alive and well, 90 had developed tumor recurrence, and 53 had died of their disease. The measurements of tumor-recurrence-free survival, and overall survival started at the time of surgery. Tumor relapse was considered to have occurred whenever there was evidence of metastasis.

Multiple regression analysis was used to perform the statistical analyses for associations between lymph vessel tumor embolus grade and number of lymph node metastases, and the correlation analyses were performed by the correlation statistics of Cochran–Mantel–Haenszel statistics. We analyzed the outcome-predictive power for tumor recurrence and tumor-related death by the univariate and multivariate analyses using the Cox proportional hazard regression model. The factors analyzed were the mentioned eight factors, age (≤ 39, > 39 years), type of neoadjuvant therapy (endocrine therapy, chemotherapy and chemoendocrine therapy), adjuvant therapy (no, yes), and the factors that were significantly associated with outcome in the uni-

a β, 0.142; Standard error, 0.058; P-value = 0.014

b β, 0.530; Standard error, 0.051; P-value <0.001
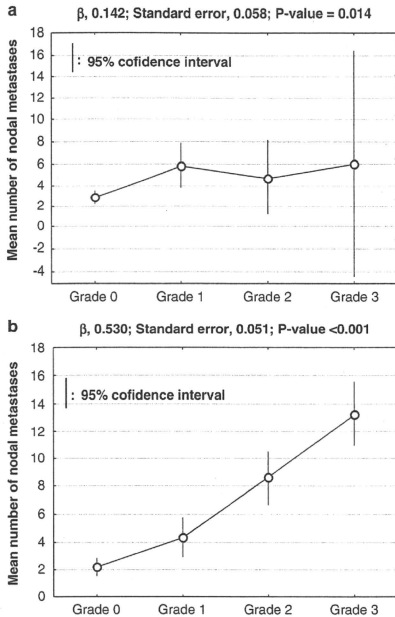
Figure 2 Graphs showing associations between mean nodal metastasis values and the grades of lymph vessel tumor emboli in the biopsy specimens (a) and in the surgical specimens (b). The mean number of nodal metastases increases significantly with the grade of lymph vessel tumor emboli in the biopsy specimens and in the surgical specimens.

variate analyses were then entered together into the multivariate analyses according to nodal status. As the eight factors were examined using both biopsy specimens obtained before neoadjuvant therapy and surgical specimens obtained after neoadjuvant chemotherapy, to accurately assess the prognostic value of each of these factors in multivariate analyses, their mutual influence on outcome was avoided by conducting separate analyses of the prognostic predictive power of the findings in the biopsy specimens obtained before neoadjuvant therapy and the surgical specimens obtained after neoadjuvant therapy (model 1, factors examined based on biopsy specimens obtained before neoadjuvant therapy; model 2, factors examined based on surgical specimens obtained after neoadjuvant therapy). The case-wise and step-down method was applied until all the remaining factors were significant at a P-value below 0.05. As there were fewer than 10 tumor deaths among the patients who did not have nodal metastasis, we were unable to perform multivariate analyses for tumor death in this groups.

Table 1 Association between grading system for lymph vessel tumor emboli and the Allred scores for p53 in stroma-invasive tumor cells, the Allred scores for p53 in tumor-stromal fibroblasts and the Allred scores for p53 in lymph vessel tumor emboli assessed in the surgical specimens

|	*Grades of lymph vessel tumor emboli*				
	Grade 0	*Grade 1*	*Grade 2*	*Grade 3*	*P-value*
Case (*n* = 271)	191	42	22	16	
Allred scores for p53 in stroma-invasive tumor cells					
0 or 2 or 3	63 (33)	14 (32)	6 (27)	0	0.001
4–6	75 (39)	22 (54)	9 (41)	3 (19)	
7 or 8	53 (28)	6 (14)	7 (32)	13 (81)	
Allred scores for p53 in tumor-stromal fibroblasts					
0 or 2	110 (58)	27 (64)	6 (27)	1 (6)	<0.001
3	26 (13)	6 (14)	2 (9)	5 (31)	
4–8	55 (29)	9 (22)	14 (64)	10 (63)	
Allred scores for p53 in lymph vessel tumor emboli					
0 or 2 or 3		6 (55)	2 (50)	1 (9)	0.005
4–6		2 (18)	0	0	
7 or 8		3 (27)	2 (50)	10 (91)	

Survival curves were drawn by the Kaplan–Meier method. All analyses were performed with Statistica/Windows software (StatSoft, Tulsa, OK, USA).

Results

Associations Between the Lymph Vessel Tumor Embolus Grades and Factors

Although the lymph vessel tumor embolus grades based on the biopsy specimens and based on the surgical specimens were significantly associated with the increases in mean number of nodal metastases (Figure 2), the value of β for the correlation between lymph vessel tumor embolus grades in the surgical specimens and mean number of nodal metastases was higher than between the lymph vessel tumor embolus grades in the biopsy specimens and the mean number of nodal metastases.

The results of the univariate analyses showed that the lymph vessel tumor embolus grades assessed in the surgical specimens were significantly associated with the Allred scores for p53 in stroma-invasive tumor cells and in tumor-stromal fibroblasts assessed in the surgical specimens (Table 1) and that they were also significantly inversely associated with Allred score for estrogen receptor in stroma-invasive tumor cells assessed in the surgical specimens in the univariate analyses (data not shown). There was no significant association between lymph vessel tumor embolus grades in the surgical specimens and progesterone receptor in stroma-invasive tumor cells in the surgical specimens, and between lymph vessel tumor embolus grades in the surgical specimens and HER2 category in tumor cells in the surgical specimens (data not shown). The Allred scores for p53 in lymph vessel tumor emboli

O, Grade 0; ◊, Grade 1; △, Grade 2; ●, Grade 3
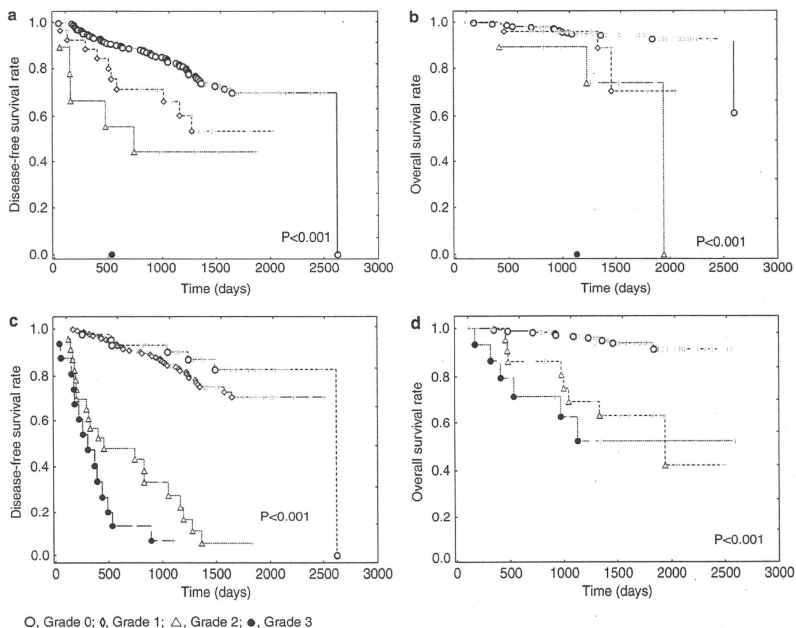
Figure 3 Disease-free survival curves (**a** and **c**) and overall survival curves (**b** and **d**) of all of the invasive ductal carcinoma (IDC) patients who received neoadjuvant therapy as a whole. (**a** and **b**) The disease-free survival time and the overall survival time of the IDC patients classified by grade of lymph vessel tumor emboli in the biopsy specimens obtained before neoadjuvant therapy become significantly shorter as the grades of lymph vessel tumor emboli increased. (**c** and **d**) The disease-free survival time and the overall survival time of the IDC patients classified by the grade of lymph vessel tumor emboli in the surgical specimens become significantly shorter as the grades of lymph vessel tumor emboli increased. None of the IDC patients had grade 1 lymph vessel tumor emboli show tumor-related death.

assessed in the surgical specimens were significantly associated with the grades of the lymph vessel tumor emboli assessed in the surgical specimens (Table 1), but there were no significant associations between the Allred scores for estrogen receptor or progesterone receptor in lymph vessel tumor emboli, or the HER2 categories in the lymph vessel tumor emboli and grades of lymph vessel tumor embolus assessed in the surgical specimens (data not shown).

In our previous study, although the multivariate analysis clearly showed that negative nodal status and HER2 category 3 in tumor cells were significantly associated with pathological complete response,[8] the lymph vessel tumor embolus grades based on the biopsy specimens were not significantly associated with pathological complete response in the univariate analysis (data not shown).

Factors Significantly Associated with Outcome

The univariate analyses of all of the cases as a whole showed that the lymph vessel tumor embolus grades in the biopsy specimens (Figures 3a and b) and the surgical specimens (Figures 3c and d) were significantly associated with tumor recurrence and tumor-related death (Table 2). In the multivariate analyses using model 1, UICC pTNM-pN1, N2, and N3 categories significantly increased the hazard rates for tumor recurrence, and UICC pTNM-pN2 and N3 also significantly increased the hazard rates for tumor-related death (data not shown). The Allred score of 3 for p53 in tumor-stromal fibroblasts and Allred scores of 4–8 for p53 in tumor-stromal fibroblasts significantly increased the hazard rates for tumor recurrence, and lymph vessel tumor embolus grades 2 and 3 also significantly increased the hazard rate

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Table 2 Outcome rates of patients with invasive ductal carcinoma according to grade of lymph vessel tumor emboli, and according to nodal status

	Grade of lymph vessel tumor emboli							
Grade	Assessed in the biopsy specimens				Assessed in the surgical specimens			
	Cases	TRR (%)	MR (%)	P-value	Cases	TRR (%)	MR (%)	P-value
Invasive ductal carcinoma patients as a whole								
0	260	61 (23)	16 (6)	TRR <0.001	199	42 (21)	10 (5)	TRR <0.001
1	26	10 (38)	3 (12)	MR <0.001	43	7 (16)	0	MR <0.001
2	9	5 (56)	3 (33)		23	20 (87)	8 (35)	
3	1	1 (100)	1 (100)		16	14 (88)	6 (38)	
Total	296	77	23		281	83	24	
Invasive ductal carcinoma patients who did not have nodal metastasis								
0	113	10 (9)	3 (3)	TRR 0.003	90	12 (13)	2 (2)	TTR 0.010
1	12	4 (33)	0	MR 0.130	7	0	0	MR 0.006
2	2	1 (50)	1 (50)		3	3 (100)	2 (67)	
3	0				0			
Total	127	15	4		100	15	4	
Invasive ductal carcinoma patients who had nodal metastasis								
0	147	51 (35)	13 (9)	TTR 0.007	109	30 (28)	8 (7)	TTR <0.001
1	14	6 (43)	3 (21)	MR 0.001	36	7 (19)	0	MR <0.001
2	7	4 (57)	2 (29)		20	17 (85)	6 (30)	
3	1	1 (100)	1 (100)		16	14 (88)	6 (38)	
Total	169	62	19		181	68	20	

MR, mortality rate; TRR, tumor recurrence rate.

for tumor recurrence in the multivariate analyses (data not shown). Allred scores of 7 or 8 for estrogen receptors in tumor cells significantly decreased the hazard rate for tumor-related death, and HER2 category 3 in tumor cells significantly increased the hazard rate for tumor-related death in the multi-variate analyses (data not shown). When model 2 was used, lymph vessel tumor embolus grade 2 and lymph vessel tumor embolus grade 3 significantly increased the hazard rates for tumor recurrence and tumor-related death in the multivariate analyses (data not shown). The Allred score of 3 in tumor-stromal fibroblasts, Allred scores of 4–8 for p53 in tumor-stromal fibroblasts, and histological grade 3 significantly increased the hazard rates for tumor recurrence, and the Allred scores of 4–8 in tumor-stromal fibroblasts and histological grade 3 also significantly increased the hazard rate for tumor-related death in the multivariate analyses (data not shown). Residual invasive tumor size >50 mm significantly increased the hazard rate for tumor recurrence and the presence of skin invasion significantly increased the hazard rate for tumor-related death in the multivariate analyses (data not shown).

In the group of IDC patients without nodal metastasis, the univariate analyses showed that the lymph vessel tumor embolus grades in the biopsy specimens were significantly associated with tumor recurrence, but not with tumor-related death, and that the lymph vessel tumor embolus grades in

the surgical specimens were significantly associated with both tumor recurrence and tumor-related death (Table 2). In the multivariate analyses, lymph vessel tumor embolus grades 1 and 2 in the biopsy specimens, Allred scores of 4–8 for p53 in tumor-stromal fibroblasts in the biopsy specimens, and age ≤38 years significantly increased the hazard rates for tumor recurrence in the multivariate analyses (Table 3, model 1), and lymph vessel tumor embolus grade 2 in the surgical specimens, Allred score of 3 for p53 in tumor-stromal fibroblasts in the surgical specimens, and Allred scores of 4–8 for p53 in tumor-stromal fibroblasts in the surgical specimens significantly increased the hazard rates for tumor recurrence in the multivariate analysis (Table 3, model 2).

In the group of IDC patients with nodal metastasis, the univariate analyses showed that the lymph vessel tumor embolus grades in the biopsy specimens and the surgical specimens were significantly associated with tumor recurrence and tumor-related death (Table 2). In the multivariate analyses of model 1, lymph vessel tumor embolus grades 2 and 3, an Allred score of 3 for p53 in tumor-stromal fibroblasts, and Allred scores of 4–8 for p53 in tumor-stromal fibroblasts significantly increased the hazard rate for tumor recurrence, and lymph vessel tumor embolus grade 1 significantly increased the hazard rate for tumor-related death (Table 4). The Allred scores of 7 or 8 for estrogen receptors in tumor cells significantly decreased the hazard rate

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