Table 4 Multivariate analyses for tumor recurrence and tumor-related death in UICC pTNM stage III invasive ductal carcinoma patients

Factors	*Tumor recurrence*		*Tumor-related death*	
	HR (95% CI)	*P-value*	*HR (95% CI)*	*P-value*
p53 Allred score risk classes of tumor-stromal fibroblasts forming and not forming a fibrotic focus				
Low-risk	Referent		Referent	
Intermediate-risk	2.9 (1.3–6.3)	0.009	5.2 (1.6–17.2)	0.007
High-risk	6.0 (2.6–13.9)	<0.001	20.1 (5.8–69.0)	<0.001
Grading system for lymph vessel tumor emboli				
Grade 0	Referent		Referent	
Grade 1	0.6 (0.2–1.8)	0.340	0.5 (0.1–3.1)	0.480
Grade 2	0.6 (0.2–1.6)	0.281	1.7 (0.5–5.8)	0.426
Grade 3	6.5 (2.9–14.4)	<0.001	2.6 (1.0–6.7)	0.045
UICC pN category				
pN0	Referent		Referent	
pN1	6.3 (0.5–81.3)	0.166	8.8 (0.4–203.7)	0.171
pN2	6.9 (0.6–70.2)	0.108	5.0 (0.3–80.1)	0.256
pN3	2.8 (1.5–5.3)	0.001	3.3 (1.4–7.8)	0.005
Fibrotic focus, diameter				
Absent	Referent		Referent	
≤8 mm	1.6 (0.6–4.3)	0.383	1.3 (0.2–8.6)	0.777
>8 mm	2.8 (1.3–6.2)	0.009	2.1 (0.5–9.5)	0.337
The Allred scores for estrogen receptor in tumor cells				
0 or 2	Referent		Referent	
3–6	0.7 (0.3–1.9)	0.488	1.2 (0.3–5.0)	0.836
7 or 8	0.6 (0.2–1.5)	0.257	0.4 (0.2–0.9)	0.033

HR, hazard rate; CI, confidence interval; pN, pathological regional lymph node; N0, no nodal metastasis; N1, 1–3 nodal metastases; N2, 4–9 nodal metastases; N3, 10 or more nodal metastases.
The multivariate analysis for tumor recurrence was performed using the p53 Allred score risk classes in tumor-stromal fibroblasts forming and not forming a fibrotic focus, grading system for lymph vessel tumor emboli, UICC pN category, fibrotic focus diameter, the Allred scores for estrogen receptors in tumor cells, the Allred scores for progesterone receptors in tumor cells, the Allred scores for p53 in tumor cells, invasive tumor size, tumor necrosis, and histological grade.
The multivariate analysis for tumor death was performed using the p53 Allred score risk classes in tumor-stromal fibroblasts forming and not forming a fibrotic focus, grading system for lymph vessel tumor emboli, UICC pN category, fibrotic focus diameter, the Allred scores for estrogen receptors in tumor cells, the Allred scores for p53 in tumor cells, HER2 category in tumor cells, age, invasive tumor size, and histological grade.

and an intermediate-risk or high-risk classification significantly increased the hazard rates for tumor recurrence and tumor-related death independent of the UICC pTNM stage in multivariate analyses that included well-known prognostic factors. Thus, we can conclude that the Allred score risk classification based on the Allred score for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci appears to be an excellent histological predictor of outcome among patients with IDC with or without fibrotic foci. However, as we could not analyze the outcome predictive power of the Allred score risk classification for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci among patients with IDC according to the types of adjuvant therapy (chemotherapy, endocrine therapy, and chemoendocrine therapy) in detail, the predictive power of the Allred score risk classification for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci should be analyzed separately among IDC patients treated with chemotherapy, endocrine therapy, and chemoendocrine therapy in the future.

In this study, we did not investigate the associations of the Allred scores for p53 with the presence of *p53* gene abnormalities in tumor-stromal fibroblasts. Although *p53* mutations in tumor-stromal fibroblasts are relatively common among primary breast cancers and other cancers and have been reported to exert a positive effect on cancer growth,[12–15] some studies have not shown any p53 mutations in the tumor-stroma of breast cancer.[16–18] We have already reported that fibroblasts forming fibrotic foci show significantly higher proliferative activities than those not forming fibrotic foci and found that no significant association exists between the proliferative activity of fibroblasts forming fibrotic foci and the fibrotic foci diameter.[7] In contrast, the Allred scores for p53 in tumor-stromal fibroblasts forming fibrotic foci were significantly lower than the Allred scores for p53 in tumor-stromal fibroblasts not forming fibrotic foci, and a significant association between the increase in the Allred scores for p53 in tumor-stromal fibroblasts forming fibrotic foci and the fibrotic foci diameter was observed in this study. Thus, although the mechanism that increases the malignant potential of IDCs through the expression of p53 in tumor-stromal fibroblasts should be investigated from the viewpoint of

p53 gene abnormalities, p53 immunoreactivity in tumor-stromal fibroblasts produced by tumor cell–stromal cell interactions inside and outside fibrotic foci might in fact reflect specific reactive changes other than the proliferative activity of fibroblasts forming fibrotic foci within the stroma that might be correlated with the prognosis.

In conclusion, this is the first study to show clearly that p53 expression in tumor-stromal fibroblasts forming and not forming fibrotic foci is strongly associated with the outcome of IDC patients. Because p53 expression in tumor-stromal fibroblasts forming and not forming fibrotic foci might be important in tumor progression in IDCs, p53 expression could be a very important target for tumor gene therapy for IDCs, suppressing tumor cell–stromal cell interactions arising from *p53* gene abnormalities or p53-related tumor microenvironment reactions.

Acknowledgement

This study was supported in part by a Grant-in-Aid for Scientific Research (KAKENHI) (C) (21590393) from the Japan Society for the Promotion of Science and was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health, Labor and Welfare of Japan (20-16, H21-006).

Disclosure/conflict of interest

The authors declare no conflict of interest.

References

1 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.
2 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;15:502–516.
3 Colpaert C, Vermeulen PB, van Beest P, *et al.* Intratumoral hypoxia resulting in the presence of a fibrotic focus is an independent predictor of early distant relapse in lymph node-negative breast cancer patients. Histopathology 2001;39:416–425.
4 Baak JP, Colpaert CG, van Diest PJ, *et al.* Multivariate prognostic evaluation of the mitotic activity index and fibrotic focus in node-negative invasive breast cancers. Eur J Cancer 2005;41:2093–2101.
5 Van den Eynden GG, Colpart CG, Couveland A, *et al.* A fibrotic focus is a prognostic factor and a surrogate marker for hypoxia and (lymph) angiogenesis in breast cancer: review of the literature and proposal on the criteria of evaluation. Histopathology 2007;51:440–451.
6 Hasebe T, Sasaki S, Imoto S, *et al.* Proliferative activity of intratumoral fibroblasts is closely correlated with lymph node and distant organ metastases of invasive ductal carcinoma of the breast. Am J Pathol 2000;156:1701–1710.
7 Hasebe T, Sasaki S, Imoto S, *et al.* Highly proliferative fibroblasts forming fibrotic focus govern metastasis of invasive ductal carcinoma of the breast. Mod Pathol 2001;14:325–337.
8 Finak G, Bertos N, Pepin F, *et al.* Stromal gene expression predicts clinical outcome in breast cancer. Nat Med 2008;14:518–527.
9 Singer CF, Gschwantler-Kaulich D, Fink-Retter A, *et al.* Differential gene expression profile in breast cancer-derived stromal fibroblasts. Breast Cancer Res Treat 2008;110:273–281.
10 Hasegawa M, Furuya M, Kasuya Y, *et al.* CD151 dynamics in carcinoma–stroma interaction: integrin expression, adhesion strength and proteolytic activity. Lab Invest 2007;87:882–892.
11 Studebaker AW, Storci G, Werbeck JL, *et al.* Fibroblasts isolated from common sites of breast cancer metastasis enhance cancer cell growth rates and invasiveness in an interleukin-6-dependent manner. Cancer Res 2008;68:9087–9095.
12 Kurose K, Gilley S, Matsumoto PH, *et al.* Frequent somatic mutations in PTEN and TP53 are mutually exclusive in the stroma of breast carcinoma. Nat Genet 2002;32:355–357.
13 Hill R, Song Y, Cardiff RD, *et al.* Selective evolution of stromal mesenchyme with p53 loss in response to epithelial tumorigenesis. Cell 2006;123:1001–1011.
14 Bierie B, Moses HL. Under pressure: stromal fibroblasts change their ways. Cell 2005;123:985–987.
15 Patocs A, Zhang L, Xu Y, *et al.* Breast-cancer stromal cells with TP53 mutations and nodal metastases. N Engl J Med 2007;357:2543–2551.
16 Allinen M, Beroukhim R, Cai L, *et al.* Molecular characterization of the tumor microenvironment in breast cancer. Cancer Cell 2004;6:17–32.
17 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.
18 Campbell IG, Qiu W, Polyak K, *et al.* Breast-cancer stromal cells with *TP53* mutations. N Engl J Med 2008;10:1634–1635.
19 Hasebe T, Okada N, Tamura N, *et al.* p53 expression in tumor-stromal fibroblasts is closely associated with the outcome of patients with invasive ductal carcinoma. Cancer Sci 2009;100:2101–2108.
20 Hasebe T, Tamura N, Okada N, *et al.* p53 expression in tumor-stromal fibroblasts is closely associated with the nodal metastasis and outcome of patients with invasive ductal carcinoma who received neoadjuvant therapy. Hum Pathol 2010;41:262–270.
21 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;39:427–436.
22 Hasebe T, Okada N, Iwasaki M, *et al.* Grading system for lymph vessel tumor emboli: significant outcome predictor for invasive ductal carcinoma of the breast. Hum Pathol 2010 (in press).
23 Sobin LH, Wittekind Ch, (eds). TNM Classification of Malignant Tumors 6th edn. Wiley-Liss: Geneva, 2002, pp 131–141.
24 Bloom HJG, Richardson WW. Histological grading and prognosis in breast cancer. Br J Cancer 1957;11:359–377.
25 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: 1929–1935.

26 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;17:1474–1481.

27 Mohsin S, Weiss H, Havighurst T, *et al.* Progesterone receptor by immunohistochemistry and clinical outcome in breast cancer: a validation study. Mod Pathol 2004;17:1545–1554.

28 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;85:200–206.

29 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.

The Breast 19 (2010) 210–213

Contents lists available at ScienceDirect

The Breast

journal homepage: www.elsevier.com/brst

Original article

Evaluation of sentinel node biopsy by combined fluorescent and dye method and lymph flow for breast cancer

Takashi Hojo*, Tomoya Nagao, Mizuho Kikuyama, Sadako Akashi, Takayuki Kinoshita

National Cancer Center Hospital, Department of Surgery and Division of Breast Cancer, 5-1-1 Tsukiji Chuo-ku, Tokyo, Japan

ARTICLE INFO

Article history:
Received 9 July 2009
Received in revised form
10 September 2009
Accepted 19 January 2010
Available online 13 February 2010

Keywords:
Breast cancer
Sentinel lymph node biopsy
Lymph flow

ABSTRACT

Background: Conservative breast resection with subsequent sentinel lymph node biopsy (SNB) is an increasingly popular initial approach for the treatment of breast cancer due to decreased invasiveness. SNB is a shorter procedure with fewer side effects than more substantial surgical procedures, but it sometimes fails to identify metastatic disease. Therefore, a highly sensitive and convenient method is needed to identify sentinel lymph nodes (SLN) with a high probability of containing disease in SNB. We compared the combination of radioisotope or dye with a fluorescence compound to analyze lymph flow to identify targets for SNB.

Materials and methods: We examined patients with breast cancer lacking metastases in the axillary lymph node (ALN). Two methods for targeted SNB were developed: (1) Indocyanine Green (ICG) and Patent blue were injected into the skin overlying the tumor and sub-areolar region just before the surgical procedure. (2) ICG and radiocolloid were injected into the skin overlying the tumor and sub-areolar region. The draining fluorescent lymphatic duct was visualized using a Photodynamic Eye (PDE). We removed the SLNs that were identified by the dye and fluorescence imaging methods. Method 1 was applied to 113 patients undergoing SNB, and 29 patients were treated with Method 2. In our study, patients were grouped by lymph flow into two types: Type C demonstrated convergence to one lymph duct. Type S demonstrated separate lymph ducts.

Results: Using the fluorescence imaging method, 99.3% of SLNs were identified, and 3.8 SLNs per patient were seen. The SLN identification rates for Patent blue dye and radiocolloid were 92.9% and 100%, respectively, while 1.9 and 2.0 SLNs per patient, respectively, were seen with these methods. We classified two types of lymph flow based on the pattern of lymphatic drainage. Type C converged to a single lymph duct, while Type S drained to separate ducts. Type S lymph drainage was seen in 29/142 patients (20.4%), and Type C drainage was found in 113/141 patients (79.6%). Of the patients with Type S drainage, there were 4.1 SLNs per patient, but only 3.4 SLNs per patient were seen in individuals with Type C drainage. Forty cases had metastases found in the ALNs, and five of these cases were dye-negative and fluorescence-positive. Among these cases, the average number of SLNs identified was one.

Conclusion: The combination of fluorescence with a visible dye is a highly sensitive method for SLN identification. When SNB is guided by only the dye method, there is a risk of missing appropriate SLNs in patients with Type S lymph drainage or weak dye staining. The use of a fluorescence method together with dye could increase sensitivity of detection in these cases. Furthermore, fluorescent methods are ideal for hospitals that cannot use conventional radioactive measures.

Introduction

Recent efforts in the surgical treatment of breast cancer have focused on breast conserving procedures, and ALN resection has become progressively less invasive with the implementation of sentinel lymph node biopsy (SNB). However, SNB can fail to identify lymph node metastases, and it is important to identify the optimal sentinel lymph node (SLN) for biopsy. This is a critical step in the evaluation of ALN status in patients with early breast cancer. Several methods are currently used to identify sentinel nodes including the dye method, the gamma probe-guided method, or a combination of these two, and there are many reports describing the successful use of these methods.[1] The combined use of a dye and gamma probe is more accurate compared to the dye method

* Corresponding author.
E-mail address: tahojo@ncc.go.jp (T. Hojo).

0960-9776/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.breast.2010.01.014

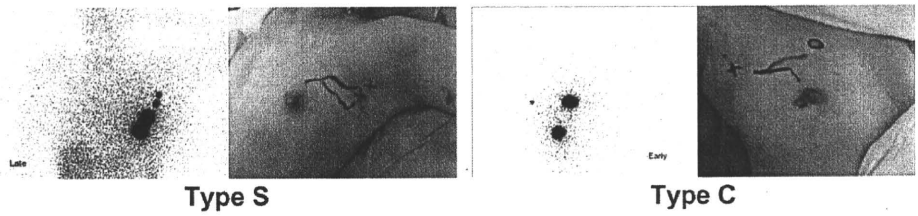


Fig. 1. Lymph flow type.

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 radioisotope, 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 radiosotope 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 identified two patterns of lymph flow in our patient group; 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	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.

Human Pathology (2010) 41, 706–715

ELSEVIER

Human PATHOLOGY

www.elsevier.com/locate/humpath

Original contribution

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

Takahiro Hasebe MD, PhD[a,*], Nao Okada MD[b], Motoki Iwasaki MD, PhD[c], Sadako Akashi-Tanaka MD, PhD[b], Takashi Hojo MD, PhD[b], Tatsuhiro Shibata MD, PhD[d], Yuko Sasajima MD, PhD[e], Histoshi Tsuda MD, PhD[e], Takayuki Kinoshita MD, PhD[b]

[a]*Pathology Consultation Service, Clinical Trials and Practice Support Division, Center for Cancer Control and Information Services, National Cancer Center, Tokyo 104-0045, Japan*
[b]*Department of Breast Surgery, National Cancer Center Hospital, Tokyo 104-0045, Japan*
[c]*Epidemiology and Prevention Division, Research Center for Cancer Prevention and Screening, National Cancer Center, Tokyo 104-0045, Japan*
[d]*Cancer Genomics Project, National Cancer Center Research Institute, Tokyo 104-0045, Japan*
[e]*Clinical Laboratory Division, National Cancer Center Hospital, Tokyo 104-0045, Japan*

Received 19 August 2009; revised 22 October 2009; accepted 23 October 2009

Keywords:
Lymph vessel;
Grade;
Breast cancer;
Prognosis;
p53

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.

☆ This study was supported in part by a Grant-in-Aid for Scientific Research (KAKENHI) (C) (19590378, 21590393) from the Japan Society for the Promotion of Science and was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health, Labor, and Welfare of Japan (20-16, H21-006).
* Corresponding author.
E-mail address: thasebe@ncc.go.jp (T. Hasebe).

0046-8177/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.humpath.2009.10.015

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 clinicopathologic 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.

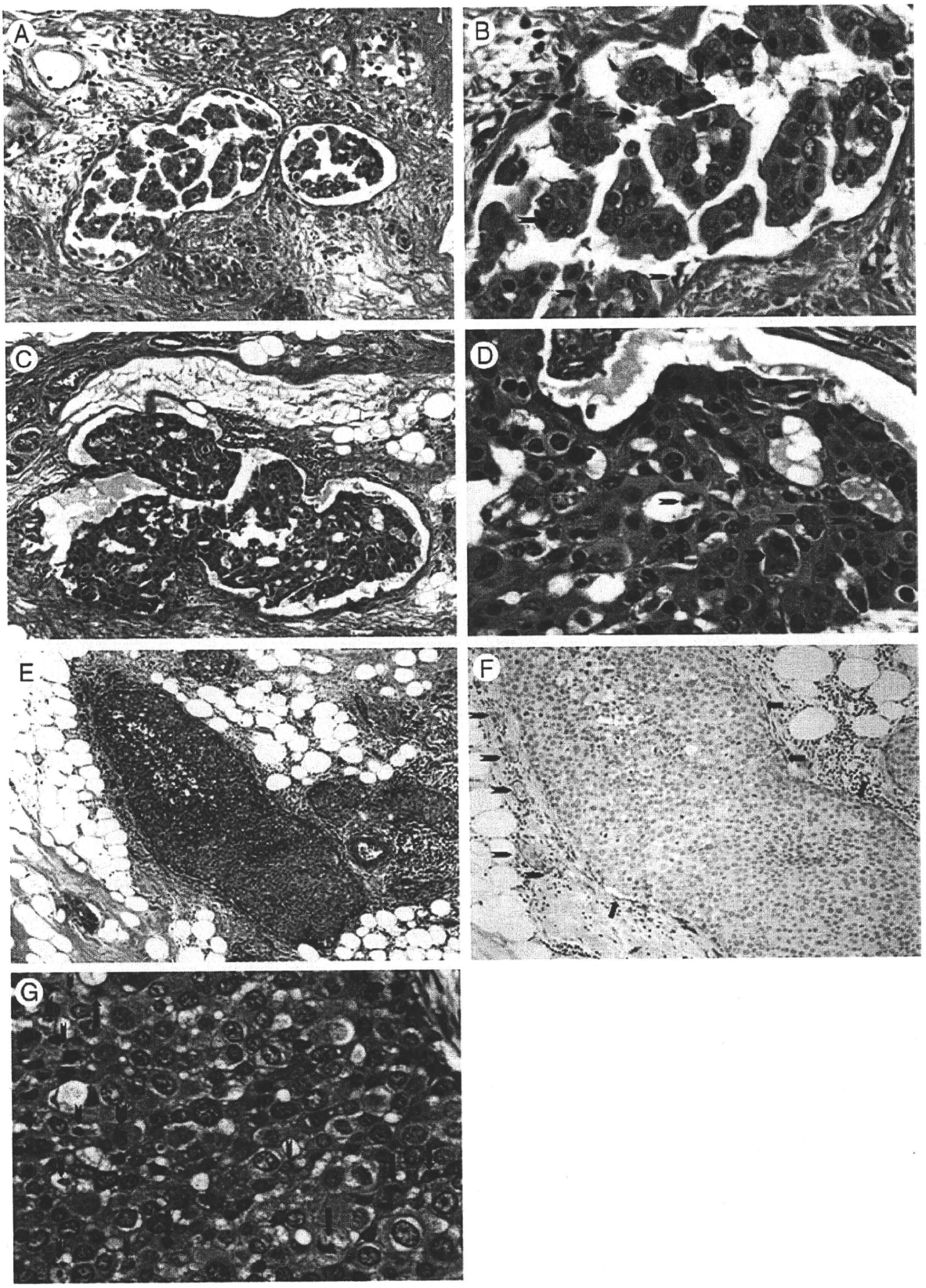
For pathologic examination, the surgically resected specimens were fixed in 10% formalin; and the size and gross appearance of the tumors were recorded. Their size was confirmed by comparison with tumor size on histologic slides; and if there was more than one invasive focus, the size of the largest 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 ($\leq$20, >20 to $\leq$50, >50 mm), (2) histologic grade (1, 2, 3) [9], (3) tumor necrosis (absent, present) [10], (4) fibrotic focus (FF) (absent, FF diameter $\leq$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 presence 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


A
B
C
D
E
F
G

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 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).

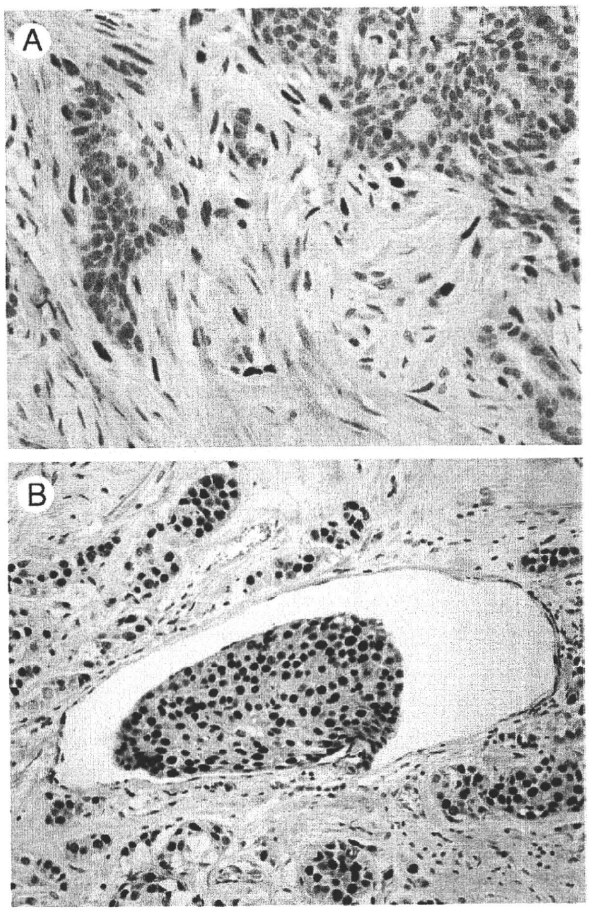


Fig. 2 Tumor-stromal fibroblasts positive for p53 and tumor embolus positive for p53. A, The tumor stroma contains several tumor-stromal fibroblasts with scores of 1, 2, or 3 for intensity of p53 expression; and the Allred score for p53 in the tumor-stromal fibroblasts is 7. The tumor cells have scores 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

	Cases	TRR (%)	P value	MR (%)	P value
IDC patients as a whole					
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.

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;

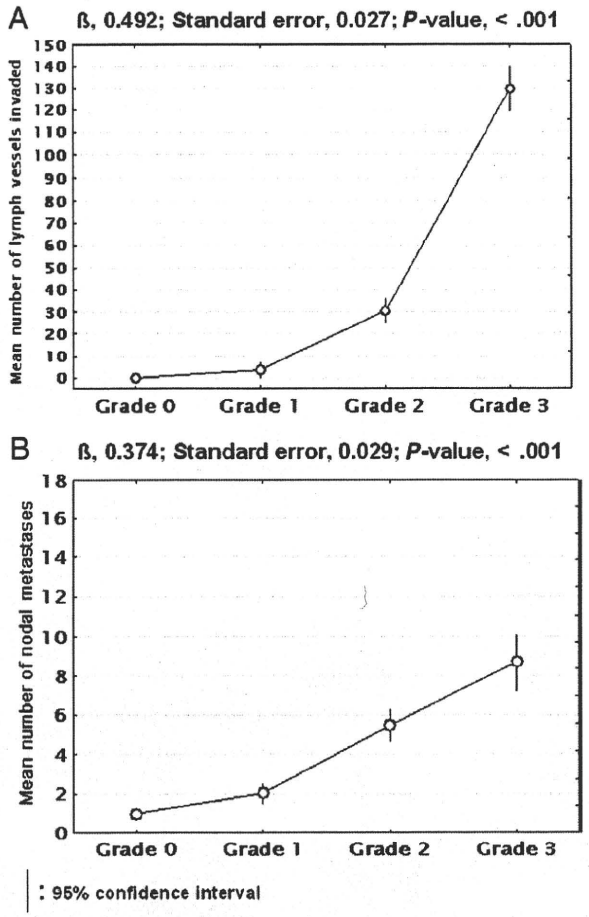
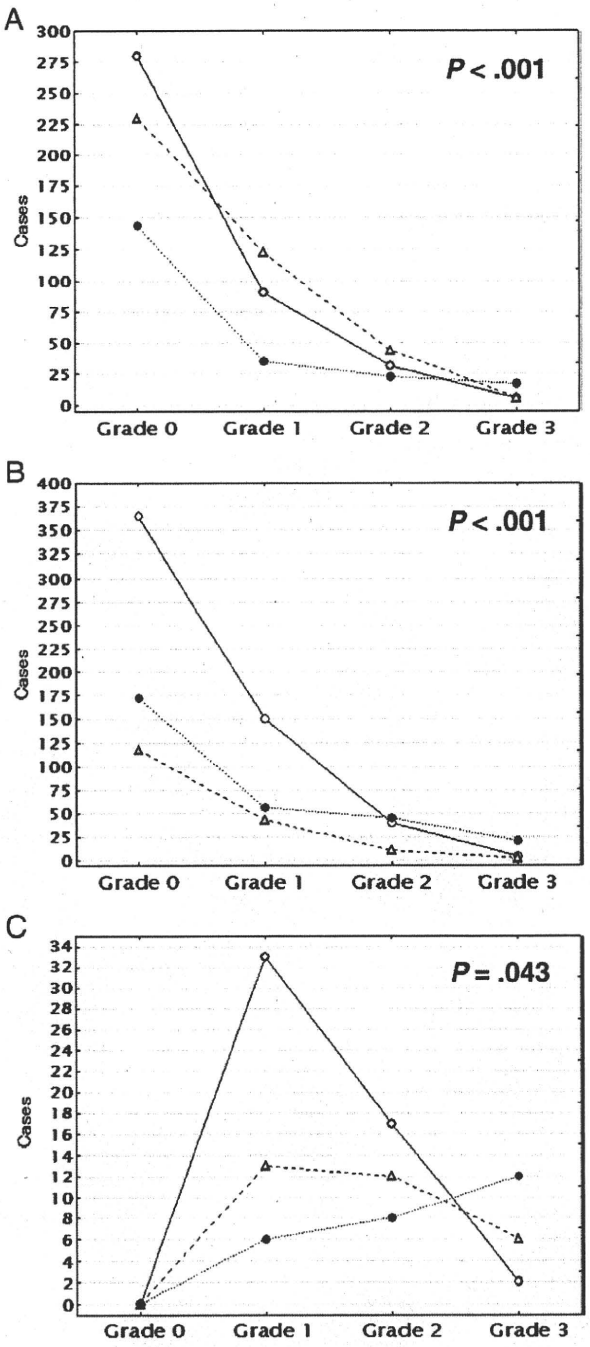


Fig. 3 Graphs showing associations between numbers of lymph vessels invaded and grades of LVTEs (A) and between mean nodal metastasis values and the grades of LVTEs in the no-neoadjuvant therapy group (B). The mean numbers of lymph vessels invaded and of nodal metastases increase significantly with the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

standard error, 0.027; P value < .001; Fig. 3A) and with the increase in nodal metastases (β, 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 (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: Allred score for p53 in stroma-invasive tumor cells.
○, 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.
○, score of 0 or 2 ; △, score of 3; ●, score of 4 to 8.
C: Allred score for p53 in lymph vessel tumor emboli.
○, score of 0, 2 or 3 ; △, score of 4 to 6; ●, score of 7 or 8.

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.

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).

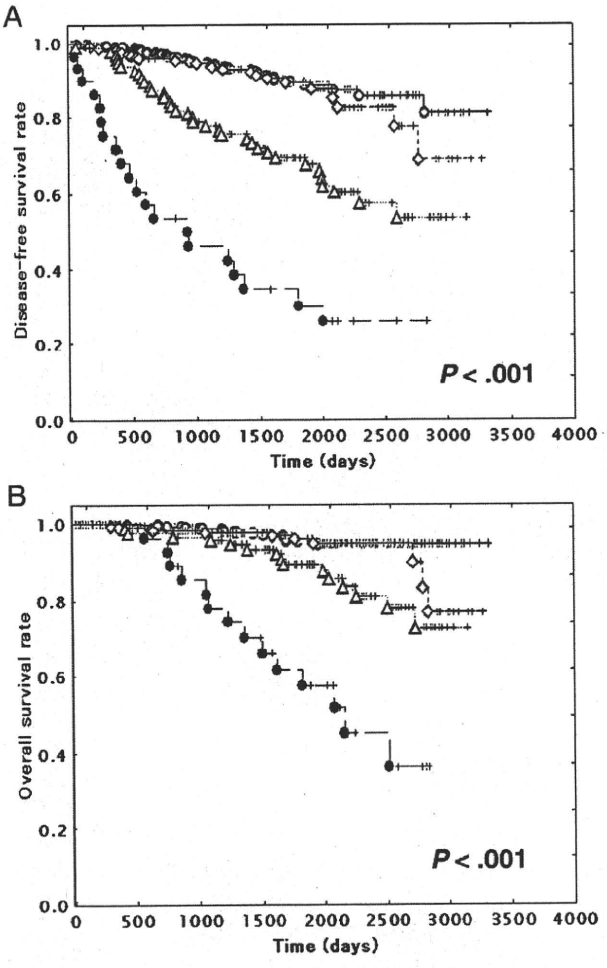


Fig. 5 Disease-free survival curves and overall survival curves of IDC patients (A and B) according to the grade of LVTEs. A, The disease-free survival time of the IDC patients without nodal metastasis significantly decreases with the grade of LVTEs. B, The overall survival time of IDC patients with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

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

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 LVTE.

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, Bachner 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] Bieric 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.

MODERN PATHOLOGY (2010) 23, 581–592

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

Takahiro Hasebe[1], Nobuko Tamura[2], Motoki Iwasaki[3], Nao Okada[2], Sadako Akashi-Tanaka[2], Takashi Hojo[2], Chikako Shimizu[4], Masashi Adachi[4], Yasuhiro Fujiwara[4], Tatsuhiro Shibata[5], Yuko Sasajima[6], Histoshi Tsuda[6] and Takayuki Kinoshita[2]

[1]*Pathology Consultation Service, Clinical Trials and Practice Support Division, Center for Cancer Control and Information Services, National Cancer Center, Tokyo, Japan;* [2]*Department of Breast Surgery, National Cancer Center Hospital, Tokyo, Japan;* [3]*Epidemiology and Prevention Division, Research Center for Cancer Prevention and Screening, National Cancer Center, Tokyo, Japan;* [4]*Division of Breast and Medical Oncology, National Cancer Center Hospital, Tokyo, Japan;* [5]*Cancer Genomics Project, National Cancer Center Research Institute, Tokyo, Japan and* [6]*Clinical Laboratory Division, National Cancer Center Hospital, Tokyo, Japan*

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.
Modern Pathology (2010) **23,** 581–592; doi:10.1038/modpathol.2010.3; published online 29 January 2010

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 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

Correspondence: Dr T Hasebe, MD, PhD, Pathology Consultation Service, Clinical Trials and Practice Support Division, Center for Cancer Control and Information Services, National Cancer Center, 5–1–1, Tsukiji, Chuo-ku, Tokyo 104–0045, Japan.
E-mail: thasebe@ncc.go.jp
Received 15 September 2009; revised 27 November 2009; accepted 7 December 2009; published online 29 January 2010

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

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

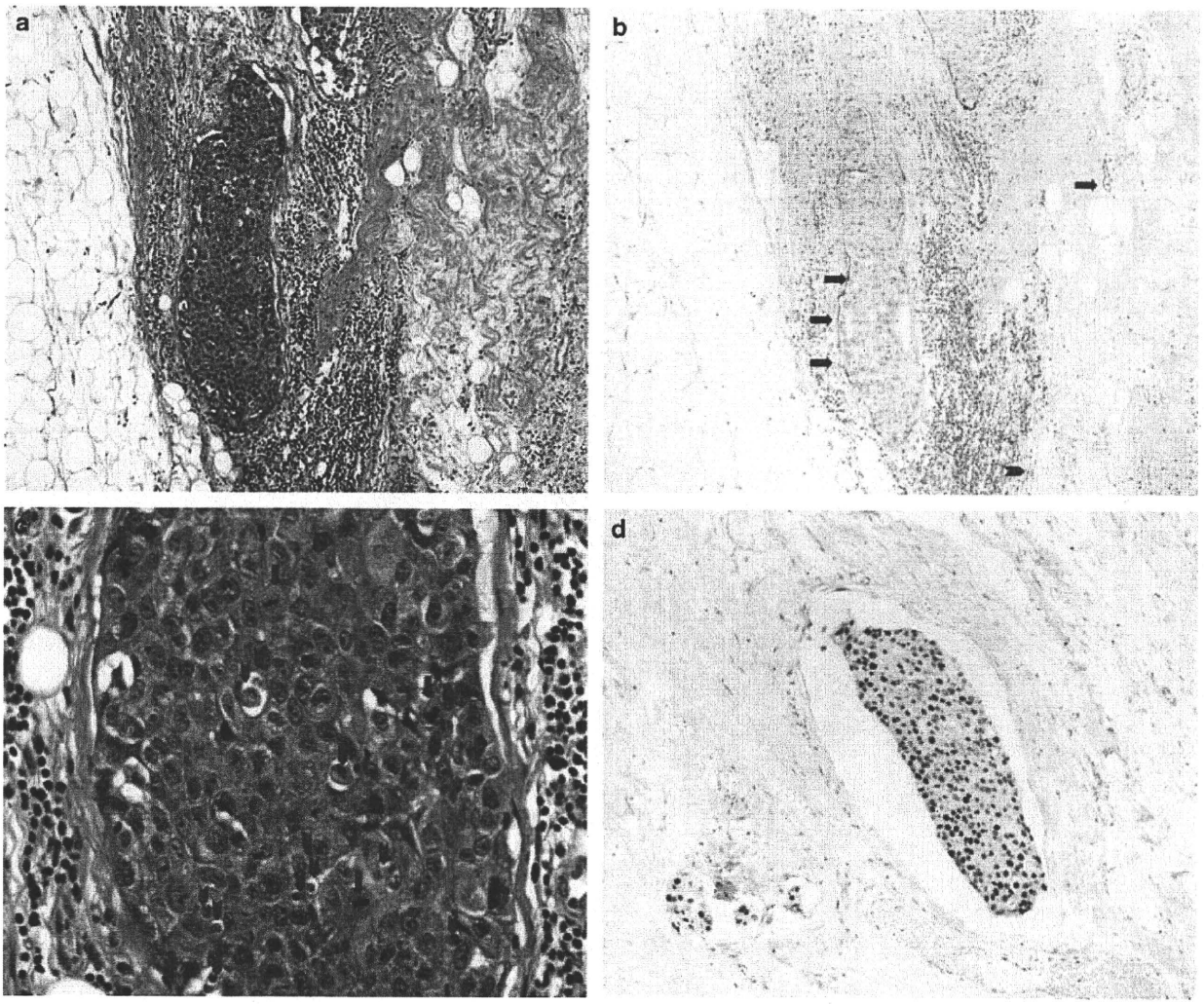


Figure 1 (**a**) Two lymph vessel tumor emboli are observed. (**b**) Vessel walls positive for D2-40 around two lymph vessel tumor emboli can be seen (arrows). One small lymph vessel is also positive for D2-40 (arrow), and one artery is negative for D2-40 (arrowhead). (**c**) Several apoptotic bodies 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.