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 an 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-

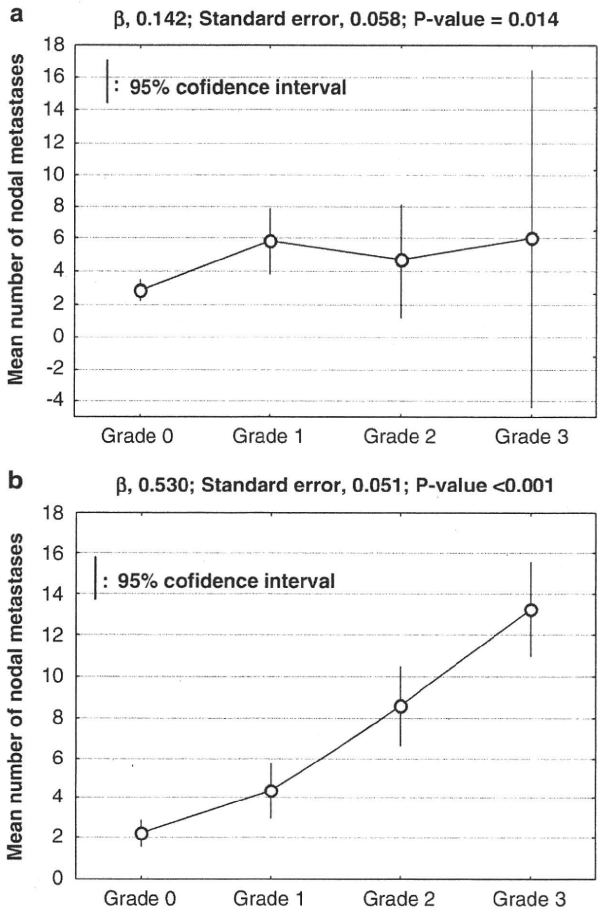

Figure 2 Graphs showing associations between mean nodal metastasis values and the grades of lymph vessel tumor emboli in the biopsy specimens (**a**) and in the surgical specimens (**b**). The mean number of nodal metastases increases significantly with the grade of lymph vessel tumor emboli in the biopsy 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				
Case ($n=271$)	*Grade 0* 191	*Grade 1* 42	*Grade 2* 22	*Grade 3* 16	*P-value*
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

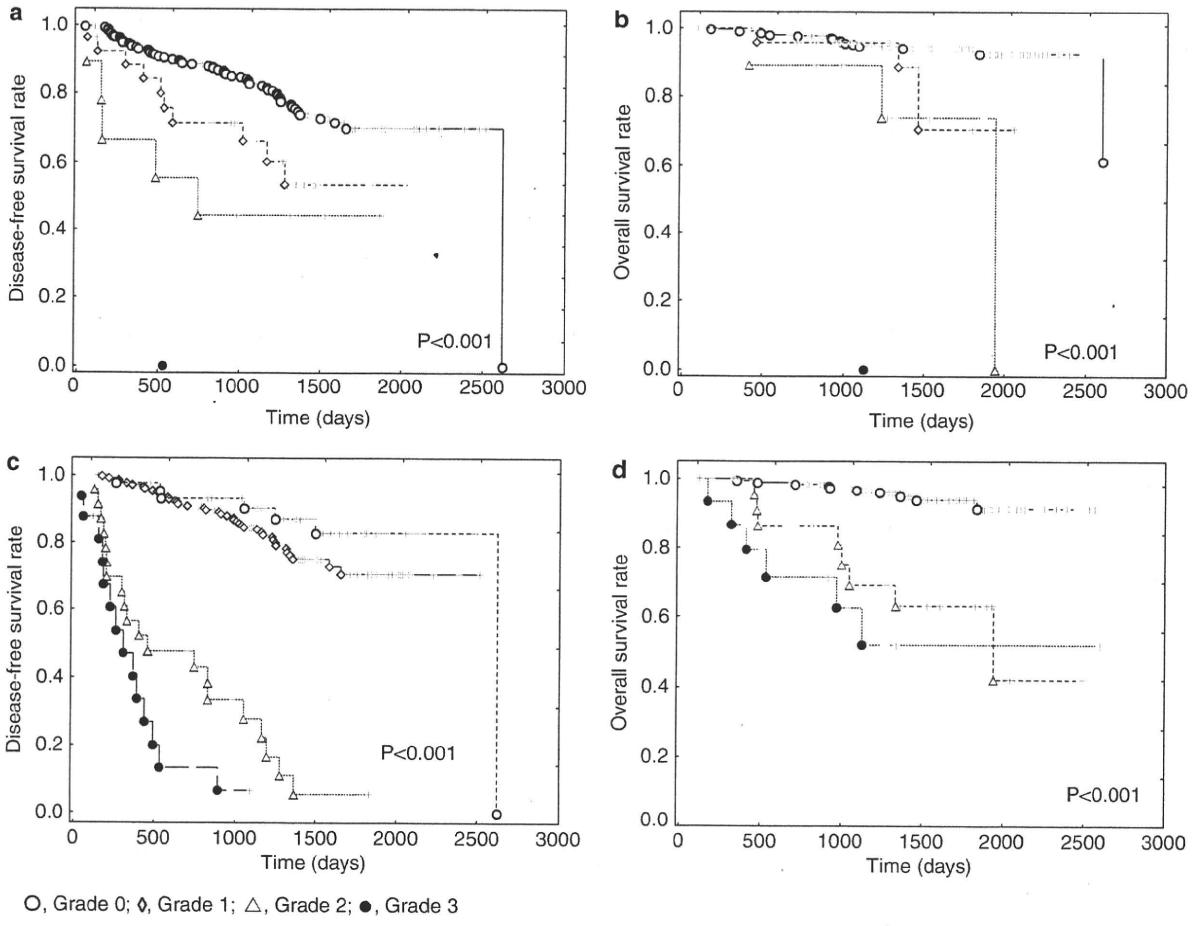

Figure 3 Disease-free survival curves (**a** and **c**) and overall survival curves (**b** and **d**) of all of the invasive ductal carcinoma (IDC) patients who received neoadjuvant therapy as a whole. (**a** and **b**) The disease-free survival time and the overall survival time of the IDC patients 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

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 multivariate 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

Table 3 Multivariate analysis for tumor recurrence in invasive ductal carcinoma patients who did not have nodal metastasis

Factors	*Cases*	*Number of patients (%) Tumor recurrence*			
		Present	*HRs*	*95% CI*	*P-value*
Model 1					
Grading system for lymph vessel tumor emboli					
Grade 0	113	10 (9)	Referent		
Grades 1 and 2	12	4 (33)	4.2	1.3–13.0	0.014
The Allred scores for p53 in tumor-stromal fibroblasts					
0 or 2	40	0	Referent		
3	27	3 (11)	Referent		
4–8	57	12 (21)	3.9	1.1–14.3	0.037
Age (years)					
⩽39	21	7 (33)	Referent		
>39	114	11 (10)	0.3	0.1–0.9	0.040
Model 2					
Grading system for lymph vessel tumor emboli					
Grade 0	90	12 (13)	Referent		
Grade 1	7	0	Referent		
Grade 2	3	3 (100)	10.2	2.5–40.9	0.001
The Allred scores for p53 in tumor-stromal fibroblasts					
0 or 2	64	3 (5)	Referent		
3	12	4 (33)	6.4	1.4–29.6	0.017
4–8	19	7 (37)	9.8	2.2–34.9	0.002

CI, confidence interval; HR, hazard rate.
Model 1: Tumor recurrence was adjusted for grading system for lymph vessel tumor emboli and the Allred scores for p53 in tumor-stromal fibroblasts assessed in biopsy specimen obtained before neoadjuvant therapy, and age.
Model 2: Tumor recurrence was adjusted for grading system for lymph vessel tumor emboli and the Allred scores for p53 in tumor-stromal fibroblasts assessed in surgical specimen obtained after neoadjuvant therapy, and age.

for tumor recurrence, and Allred scores of 3–6 for estrogen receptors in tumor cells significantly decreased the hazard rate for tumor death in the multivariate analyses (Table 4). UICC pN3 category significantly increased the hazard rate for tumor recurrence, and HER2 category 3 in tumor cells, histological grade 3, and absence of adjuvant therapy significantly increased the hazard rates for tumor-related death in the multivariate analyses (Table 4). In model 2, lymph vessel tumor embolus grade 2, lymph vessel tumor embolus grade 3, Allred scores of 4–8 for p53 in tumor-stromal fibroblasts, and histological grade 3 significantly increased the hazard rates for tumor recurrence and tumor-related death in the multivariate analyses (Table 4). Residual invasive tumor size >50 mm and Allred scores of 7 or 8 for p53 in tumor cells significantly increased the hazard rates for tumor recurrence, and the presence of skin invasion significantly increased the hazard rate for tumor-related death in the multivariate analysis (Table 4).

Discussion

The results of this study clearly showed significant associations between increases in grade of lymph vessel tumor embolus assessed in the biopsy specimens and surgical specimens and the number of nodal metastases. We have also found a significant association between grade of lymph vessel tumor embolus and number of nodal metastases in a different no-neoadjuvant therapy IDC group in another study.[6] Thus, the grading system for lymph vessel tumor embolus can be concluded to be a very useful histological grading system for accurately predicting lymph node metastasis by IDCs in the no-neoadjuvant therapy group and in the neoadjuvant therapy group.

In a previous study, we found that the grading system for lymph vessel tumor emboli can be used to classify IDC patients with lymph vessel invasion into a low-, intermediate-, and high-risk groups for outcome, and that IDCs with grade 0 lymph vessel tumor embolus and IDCs with grade 1 lymph vessel tumor emboli were almost equally malignant in a different no-neoadjuvant therapy IDC group.[6] Although those findings were clearly confirmed in this study again, the results of this study clearly showed that lymph vessel tumor embolus grade 2 in the surgical specimens was an important outcome-predictive factor for IDC patients independent of nodal status. It can be therefore concluded that lymph vessel tumor embolus grade 2 is an

Table 4 Multivariate analyses for tumor recurrence and tumor-related death in invasive ductal carcinoma patients who had nodal metastasis

Factors	*Cases*	*Number of patients (%)*			
		Tumor recurrence		*Tumor-related death*	
		Present	*HRs (95% CI) P-value*	*Present*	*HRs (95% CI) P-value*
Model 1					
Grading system for lymph vessel tumor emboli					
Grade 0	147	51 (35)	Referent	13 (9)	Referent
Grade 1	14	6 (43)	1.8 (0.7–5.0) 0.249	3 (21)	4.3 (1.1–17.7) 0.044
Grades 2 and 3	8	5 (63)	3.6 (1.4–9.5) 0.008	3 (38)	0.5 (0.1–3.3) 0.458
The Allred scores for p53 in tumor-stromal fibroblasts					
0 or 2	47	7 (15)	Referent	0	Referent
3	38	14 (36)	(1.6–9.1) 0.003	6 (16)	Referent
4–8	81	39 (48)	2.4 (1.4–4.1) 0.002	11 (14)	1.8 (0.6–5.7) 0.324
The Allred scores for estrogen receptors in tumor cells					
0 or 2	62	28 (45)	Referent	14 (23)	Referent
3–6	22	11 (50)	1.4 (0.5–3.7) 0.550	3 (14)	0.1 (0.02–0.6) 0.009
7 or 8	82	21 (26)	0.4 (0.2–0.6) <0.001	0	
UICC pN category					
N1	97	27 (28)	Referent	6 (6)	Referent
N2	55	25 (45)	1.7 (0.8–3.5) 0.158	11 (20)	—
N3	31	17 (55)	2.6 (1.4–4.7) 0.002	4 (13)	—
HER2 category in tumor cells					
0 or 1	104	35 (34)	Referent	5 (5)	Referent
2	28	8 (29)	0.6 (0.3–1.4) 0.244	2 (7)	0.6 (0.06–7.1) 0.703
3	35	18 (51)	1.5 (0.7–3.4) 0.317	11 (31)	14.5 (3.9–53.1) <0.001
Histological grade					
1	43	7 (16)	Referent	0	Referent
2	104	46 (44)	2.3 (0.9–5.9) 0.082	13 (13)	Referent
3	22	9 (41)	2.0 (0.6–6.6) 0.281	6 (27)	6.2 (1.8–21.0) 0.003
Adjuvant therapy					
No	36	16 (44)	Referent	7 (19)	Referent
Yes	147	53 (36)	— —	14 (10)	0.2 (0.06–0.7) 0.014
Model 2					
Grading system for lymph vessel tumor emboli					
Grade 0	109	30 (28)	Referent	8 (7)	Referent
Grade 1	36	7 (19)	0.3 (0.1–0.9) 0.027	0	Referent
Grade 2	20	17 (85)	5.7 (2.9–11.0) <0.001	6 (30)	4.2 (1.4–12.6) 0.010
Grade 3	16	14 (88)	6.8 (3.1–14.8) <0.001	6 (38)	8.1 (2.5–25.7) <0.001
The Allred scores for p53 in tumor-stromal fibroblasts					
0 or 2	80	16 (20)	Referent	4 (5)	Referent
3	27	9 (33)	1.4 (0.5–4.0) 0.484	0	Referent
4–8	69	41 (59)	2.5 (1.5–4.3) 0.001	16 (23)	5.2 (1.9–14.4) 0.002

Table 4 Continued

Factors	*Cases*	*Number of patients (%)*			
		Tumor recurrence		*Tumor-related death*	
		Present	*HRs (95% CI)* P-*value*	*Present*	*HRs (95% CI)* P-*value*
Histological grade					
1	43	5 (12)	Referent	0	Referent
2	86	32 (37)	1.9 (0.7–5.7) 0.232	5 (6)	Referent
3	52	31 (60)	2.2 (3.1–14.8) <0.001	15 (29)	5.4 (1.9–15.9) 0.002
Residual invasive tumor size (mm)					
⩽20	42	8 (19)	Referent	3 (7)	Referent
>20–⩽50	92	29 (32)	1.0 (0.4–2.4) 0.943	8 (9)	—
>50	47	31 (66)	3.0 (1.8–5.3) <0.001	9 (19)	—
The Allred scores for p53 in tumor cells					
0 or 2	48	12 (25)	Referent	2 (4)	Referent
3–6	79	25 (32)	1.9 (0.8–4.6) 0.142	6 (8)	3.0 (0.5–17.5) 0.224
7 or 8	48	29 (60)	2.1 (1.1–4.2) 0.023	12 (25)	1.2 (0.2–8.0) 0.817
Skin invasion					
Absent	131	40 (31)	Referent	9 (7)	Referent
Present	50	28 (56)	1.9 (0.9–3.6) 0.068	11 (22)	2.9 (1.1–7.5) 0.025

CI, confidence interval; HR, hazard rate; —, not significant in univariate analysis.
Model 1: Tumor recurrence was adjusted for grading system for lymph vessel tumor emboli, the Allred scores for p53 in tumor-stromal fibroblasts, histological grade, the Allred scores for estrogen receptors in tumor cells, HER2 category in tumor cells, the Allred scores for progesterone receptors, and the Allred scores for p53 in tumor cells assessed in biopsy specimens obtained before neoadjuvant therapy, and UICC pN category assessed in surgical specimens obtained after neoadjuvant therapy and type of neoadjuvant therapy. Tumor-related death was adjusted for grading system for lymph vessel tumor emboli, the Allred scores for p53 in tumor-stromal fibroblasts, histological grade, the Allred scores for estrogen receptors in tumor cells, HER2 category in tumor cells, and the Allred scores for progesterone receptors in tumor cells assessed in biopsy specimens obtained before neoadjuvant therapy, and adjuvant therapy.
Model 2: Tumor recurrence was adjusted for grading system for lymph vessel tumor emboli, the Allred scores for p53 in tumor-stromal fibroblasts, histological grade, residual invasive tumor size, the Allred scores for p53 in tumor cells, skin invasion, the Allred scores for estrogen receptors in tumor cells, HER2 category in tumor cells and UICC pN category assessed in surgical specimens obtained after neoadjuvant therapy, and type of neoadjuvant therapy. Tumor-related death was adjusted for grading system for lymph vessel tumor emboli, the Allred scores for p53 in tumor-stromal fibroblasts, histological grade, residual invasive tumor size, the Allred scores for p53 in tumor cells, skin invasion, the Allred scores for estrogen receptors in tumor cells, and HER2 category in tumor cells assessed in surgical specimens obtained after neoadjuvant therapy, and adjuvant therapy.

important outcome predictor for IDC patients who have received neoadjuvant therapy, the as same as lymph vessel tumor embolus grade 3 is. The results of this study also clearly showed that lymph vessel tumor embolus grades based on biopsy specimens or surgical specimens are a very important outcome-predictive factor for IDC patients who have received neoadjuvant therapy independent of nodal status, but the outcome-predictive power of lymph vessel tumor embolus grade in the surgical specimens was superior to that of lymph vessel tumor embolus grade in the biopsy specimens. Thus, we can conclude that evaluation of lymph vessel tumor embolus grade in surgical specimens should be used to predict outcome.

Although we have already reported that lymph vessel tumor embolus grade is an important outcome predictor for IDC patients who have received neoadjuvant therapy, the outcome-predictive power of the lymph vessel tumor embolus grade for IDC patients who received neoadjuvant therapy and did not have nodal metastasis could not be assessed.[7] This study clearly showed that lymph vessel tumor embolus grades based on biopsy specimens and surgical specimens are very important outcome predictors for IDC patients who have received neoadjuvant therapy and do not have nodal metastasis. Furthermore, the outcome-predictive power of lymph vessel tumor embolus grade is almost the same as that of p53 expression in tumor-stromal fibroblasts, and superior to that of histological grade.

The lymph vessel tumor embolus grading system is therefore concluded to be an excellent histological grading system for accurately predicting the outcome of IDC patients who have received neoadjuvant therapy that is independent of their nodal status.

The results of this study clearly showed that lymph vessel tumor embolus grades are significantly associated with both the Allred scores for p53 in lymph vessel tumor emboli, as well as the Allred scores for p53 in stroma-invasive tumor cells, and in tumor-stromal fibroblasts, this strongly suggesting that p53 protein expression in lymph vessel tumor emboli, in tumor-stromal fibroblasts, and in stroma-invasive tumor cells is a very important key factor for evaluating the malignant potential of IDCs with lymph vessel tumor emboli. Especially, as lymph vessel tumor embolus grades are based on the numbers of mitotic figures and apoptotic figures in tumor cells in lymph vessels, p53 protein expression in lymph vessel tumor embolus probably accelerates the turnover rate of tumor cells comprising lymph vessel tumor emboli, and increases the malignancy of IDCs as lymph vessel tumor embolus grade rises. As we did not investigate for the presence of p53 gene abnormalities, the mechanism that is responsible for the increase in the malignant potential of IDCs according to grades of lymph vessel tumor embolus from the standpoint of p53 gene abnormalities in lymph vessel tumor emboli, as well as in tumor-stromal fibroblasts, or in stroma-invasive tumor cells should be investigated. In addition, as some studies have reported some identifying genes that closely regulate the cell cycle of tumors,[24–26] such genes should be investigated to determine whether they are candidates for p53 in regulating tumor cell cycle of lymph vessel tumor emboli.

In conclusion, the grading system for lymph vessel tumor emboli is significantly associated with nodal metastasis, and is an excellent histological grading system for accurately predicting the outcome of patients with IDC of the breast who received neoadjuvant therapy. Pathologists can most accurately assess the true malignant potential of IDCs by using this grading system as a histological prognostic classification for IDCs of the breast.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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Immunohistochemical Expression of HER1, HER3, and HER4 in HER2-Positive Breast Cancer Patients Treated With Trastuzumab-Containing Neoadjuvant Chemotherapy

KAN YONEMORI, MD,[1]* KOJI TSUTA, MD, PhD,[2] CHIKAKO SHIMIZU, MD,[1] YUTAKA HATANAKA, PhD,[3] AKIHIRO HIRAKAWA, MSc,[4] MAKIKO ONO, MD,[1] TSUTOMU KOUNO, MD,[1] NORIYUKI KATSUMATA, MD,[1] MASASHI ANDO, MD,[1] KENJI TAMURA, MD, PhD,[1] TADASHI HASEGAWA, MD, PhD,[5] TAKAYUKI KINOSHITA, MD,[6] AND YASUHIRO FUJIWARA, MD, PhD[1]

[1]*Breast and Medical Oncology Division, National Cancer Center Hospital, Chuo-ku, Tokyo, Japan*
[2]*Clinical Laboratory Division, National Cancer Center Hospital, Chuo-ku, Tokyo, Japan*
[3]*Medical Science Division, Dako Japan, Inc., Bunkyo-ku, Tokyo, Japan*
[4]*Department of Management Science, Graduate School of Engineering, Tokyo University of Science, Shinjuku-ku, Tokyo, Japan*
[5]*Department of Surgical Pathology, Sapporo Medical School of Medicine, Chuo-ku, Sapporo, Hokkaido, Japan*
[6]*Breast Surgery Division, National Cancer Center Hospital, Chuo-ku, Tokyo, Japan*

Background and Objectives: The aim of the present study was to examine the association between the expression of human epidermal receptor (HER) 1, HER3, and HER4 and pathologic complete response (pCR) in HER2-positive patients treated with trastuzumab-containing neo-adjuvant chemotherapy.

Methods: Immunohistochemical analyses of HER1, HER3, and HER4 were performed using tumor specimens obtained from patients treated with trastuzumab-containing neoadjuvant chemotherapy. The staining intensity of each biomarker was evaluated, and the correlations between the immunohistochemical profiles and pCR were examined.

Results: The present study included 44 patients with HER2-positive breast cancer treated with trastuzumab-containing neo-adjuvant chemotherapy. Seventeen patients achieved a pCR. The expressions of HER1, HER3, and HER4 were observed in 18.2%, 27.3%, and 18.2% of the specimens, respectively. A marginally significant negative correlation between the expression of HER1 and pCR was observed, irrespective of the expression of HER3 and HER4, whereas the expressions of HER3 and HER4 were not significantly correlated with pCR.

Conclusion: The expression of HER1 might be an independently negative predictor of pCR in HER2-positive breast cancer patients treated with trastuzumab-containing neoadjuvant chemotherapy.

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KEY WORDS: **breast cancer; neoadjuvant chemotherapy; HER family; pathologic complete response; trastuzumab**

INTRODUCTION

Recent advances in multidisciplinary approaches for treating breast cancer, including both neo-adjuvant chemotherapy and adjuvant chemotherapy, have played important roles in improving the survival rate [1]. A previous study revealed that patients who achieved a pathologic complete response (pCR) had longer relapse-free survival periods than patients without pCR after neo-adjuvant chemotherapy [2]. This previous study suggested that the chemotherapeutic response at the primary lesion may be correlated with the chemotherapeutic response of micrometastases; therefore, the selection of a chemotherapeutic regimen that best enables a pCR may improve both the relapse-free and overall survival rates.

The epidermal growth factor receptor/human epidermal receptor (HER) family is involved in cell proliferation, differentiation, and survival. It is composed of only four members, namely HER1, HER2, HER3, and HER4 [3]. HER2 over-expression and *HER2* amplification are widely known as markers of aggressive tumor behavior and a poor clinical outcome in breast cancer patients and are observed in approximately 20–30% of breast cancer patients [4]. Trastuzumab, a monoclonal antibody against HER2, has been shown to be significantly effective in both adjuvant and metastatic settings [5,6]. Recently, a randomized phase II trial revealed that trastuzumab-containing neo-adjuvant chemotherapy significantly improved the pCR rate, compared with neo-adjuvant chemotherapy alone, in patients with HER2-positive breast cancer [7].

Trastuzumab is ineffective in some HER2-positive breast cancer patients, and progression may still occur. Currently, multiple possible mechanisms for trastuzumab-resistance have been elucidated in both preclinical and clinical research efforts. One possible hypothesis is that the activation of other HER family members, such as HER1, HER3, and HER4, might associate with trastuzumab resistance through the heterodimerization of HER2 with other HER receptors, enhancing cell proliferation and inhibiting apoptosis because trastuzumab does not prevent the ligand-induced formation of heterodimers [3,4].

Additional Supporting Information may be found in the online version of this article.

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*Correspondence to: Dr. Kan Yonemori, MD, Breast and Medical Oncology Division, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan. Fax: +81-3-3542-3815. E-mail: kyonemor@ncc.go.jp

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The present study examined the association between pCR and the immunohistochemical profiles, including those of HER1, HER3, and HER4, of HER2-positive breast cancer patients receiving trastuzumab-containing neo-adjuvant chemotherapy.

MATERIALS AND METHODS

Patients

The present retrospective study investigated HER2-positive breast cancer patients [HER2/neu 3+ or HER2/neu 2+ and fluorescent in situ hybridization (FISH)-positive] who had been treated with trastuzumab-containing neo-adjuvant chemotherapy at the National Cancer Center Hospital and for whom adequate tumor tissue samples were available. Trastuzumab was administered initially using an intravenous loading dose of 4 mg/kg, followed by weekly infusions of trastuzumab (2 mg/kg), in combination with weekly paclitaxel therapy. The duration of trastuzumab administration within the neoadjuvant chemotherapy regimens was 12 weeks. The dosages of the neo-adjuvant chemotherapy regimens were as follows: CEF therapy (cyclophosfamide, 500 mg/m^2, i.v. on day 1; epirubicin, 100 mg/m^2, i.v. on day 1; 5FU, 500 mg/m^2, i.v. on day 1; 21-day cycles), AC therapy (doxorubicin, 60 mg/m^2, i.v. on day 1; cyclophosphamide, 600 mg/m^2, i.v. on day 1; 21-day cycles), AT therapy (doxorubicin, 50 mg/m^2, i.v. on day 1; docetaxel, 60 mg/m^2, i.v. on day 1; 21-day cycles), and weekly paclitaxel therapy (80 mg/m^2, i.v. on day 1; 7-day cycles).

Tissue Samples and Microscopic and Immunohistochemical Analysis

Tissue samples were obtained from core-needle biopsy specimens before neo-adjuvant chemotherapy. All hematoxylin-and-eosin-stained core-needle biopsy specimens were reviewed by an experienced pathologist (K.T.), and the tissue samples were confirmed to contain adequate amounts of cancer tissue for use in the present study. The present study was approved by the institutional review board at the National Cancer Center Hospital.

After surgical treatment, the pathologist evaluated the pathologic responses in all the specimens using hematoxylin-and-eosin-stained slides. pCR was defined as the complete disappearance of invasive cancer cells in the primary tumor and axilla.

The pathologic and immunohistochemical examinations were conducted by an experienced pathologist (K.T.) who was unaware of the clinical statuses of the patients. Formalin-fixed, paraffin-embedded tissue samples were sectioned (4 μm thick) and mounted on charged slides. Immunohistochemical staining for ER (clone 1D5; Dako, Glostrup, Denmark), and PgR (clone PgR636; Dako) were performed using a conventional detection method (ChemMate EnVision; Dako, Glostrup, Denmark) and were considered positive if 10% or more of the nuclei in the invasive component of the tumor were stained [8]. The HER2/neu status, as assessed using the Dako HercepTest™ (Dako), was scored on a scale of 0–3+ according to the Dako scoring system [9]. HER2 protein overexpression was defined by 3+ complete membrane staining. If HER2 staining on IHC was determined to be 2+, FISH was used to confirm the result. FISH was performed using a PathVysion kit (Abott-Vysis Lab, Abott Park, IL). *HER2* gene amplification was defined as a HER2:chromosome 17 ratio of ≥2.0. The immunohistochemical analysis for HER1 was performed using the Dako EGFR pharmDx kit according to the instruction manual. Clone DAK-H3-IC (Dako) and Rabbit polyclonal (Neomarker, Fremont, CA) were used for the immunohistochemical staining of HER3 and HER4, respectively. For HER3 and HER4, the slides were pretreated using heat-induced epitope retrieval and Target Retrieval Solution, pH 9.0 (S2368; Dako), at 95–99°C for 40 min and then cooled for 20 min at room temperature. Immunohistochemistry was performed using a highly sensitive detection system (CSA II system; Dako). Finally, the slides were counterstained with hematoxylin and mounted. Negative controls, in which the primary antibody was omitted, were also included in each run. Primary HER3/HER4-positive breast cancers confirmed preliminarily for their expressions were used as positive controls for HER3 and HER4 in each run. The HER1 status was defined as positive if distinct membrane staining on the tumor cells was recognized under low power fields, while positive staining for HER3 and HER4 was defined as either membrane or cytoplasmic staining, according to the definitions used in a previous study [9].

Statistical Analysis

We defined cases with a score of 0 as being negative for HER1, HER3, and HER4 in the statistical analysis. The correlations between the expression of one or more members of the HER family and pCR were evaluated using the odds ratios (ORs), 95% confidence intervals (95% CIs), and likelihood ratio tests calculated as part of univariate and multivariate logistic regression analyses. The OR represents the odds of a pCR in patients with positive variables relative to the odds of a pCR in patients with negative variables. All comparisons were two tailed, and $P \leq 0.05$ was considered significant. All the analyses were performed using SAS version 9.1.3 for Windows (SAS Institute, Inc.).

RESULTS

A total of 229 patients with breast cancer were treated with neoadjuvant chemotherapy between January 1999 and January 2006 at the National Cancer Center Hospital. Forty-four patients with adequate tumor tissue samples had been classified as having HER2-positive breast cancer and had received trastuzumab-containing neo-adjuvant chemotherapy. The clinical characteristics of the patients are summarized in Table I. Eighteen patients (40.9%) received CEF therapy followed by weekly paclitaxel/trastuzumab

TABLE I. Patient Characteristics

Characteristics	Value
Median age (range)	57 (33–78)
Side (right/left)	21/23
Menopausal status	
Pre-menopause	15 (34%)
Post-menopause	29 (66%)
Median clinical tumor size (range)	50 mm (20–120)
Number of patients with clinical lymph node swelling	23 (52%)
Staging	
IIA	15 (34%)
IIB	13 (30%)
IIIA	10 (23%)
IIIB	6 (13%)
Grade	
1	4 (9%)
2–3	40 (91%)
Estrogen receptor	
Negative	39 (89%)
Positive	5 (11%)
Progesterone receptor	
Negative	41 (93%)
Positive	3 (7%)
HER2 status in IHC	
IHC 3+	37 (84%)
IHC 2+ and FISH gene amplification+	7 (16%)
Median FISH gene amplification[a] (range)	5.5 (2.0–7.0)

IHC, immunohistochemistry; FISH, fluorescence in situ hybridization.
[a]Samples from seven patients with IHC 2+ were subjected to FISH.

TABLE II. Frequency Distributions of the Individual Expressions of One or More Members of the HER Family Among Patients With a pCR and Those Without a pCR

Variables	Total (n = 44)	pCR	
		Negative	Positive
HER1			
Negative	36	20	16
Positive	8	7	1
HER3			
Negative	32	18	14
Positive	12	9	3
HER4			
Negative	36	21	15
Positive	8	6	2
HER1 and HER3			
Negative	42	25	17
Positive	2	2	0
HER1 and HER4			
Negative	42	25	17
Positive	2	2	0
HER3 and HER4			
Negative	40	23	17
Positive	4	4	0
HER1, HER3, and HER4			
Negative	43	26	17
Positive	1	1	0

therapy, 11 (25.0%) patients received AC therapy followed by weekly paclitaxel/trastuzumab therapy, 8 (18.2%) patients received AT therapy followed by weekly paclitaxel/trastuzumab therapy, and 7 (15.9%) patients received weekly paclitaxel/trastuzumab therapy. None of the patients had progressive disease during the period of neo-adjuvant chemotherapy. The median time between the last administration of neo-adjuvant chemotherapy and surgery was 5 weeks. Twenty-eight and 16 patients underwent mastectomies and breast-conserving surgeries, respectively. Of the 44 patients, 17 patients achieved a pCR.

Table II shows the frequency distributions of the expressions of one or more members of the HER family among patients with a pCR and among those without a pCR. Overall, HER1, HER3, and HER4 were positive in 18.2%, 27.3%, and 18.2% of the patients, respectively. A pCR was not observed in patients with the co-expression of two or more members of the HER family.

Table III shows the correlations between the expressions of one or more of the HER family members and pCR based on the univariate and multivariate logistic models. According to the results of univariate logistic model, negative correlations were observed between pCR and the individual expressions of HER1, HER3, and HER4. In particular, a marginally significant negative correlation was observed between the individual expression of HER1 and pCR ($P = 0.073$), and this trend was also observed in the multivariate logistic model including HER3 and HER4 ($P = 0.071$). The OR of HER1 in the univariate logistic model was almost same as that in the multivariate logistic model. Thus, the results based on the logistic models indicate that the expression of HER1 might be independently and negatively correlated with pCR, irrespective of the expression of HER3 and HER4. Additionally, the results of multivariate logistic regression analysis using four clinical variables (menopausal status, staging, grade, and hormone status) are shown in Table IV. The relative relationships among the ORs for HER1, HER3, and HER4 are similar to those for the multivariate logistic regression analysis without clinical variables.

TABLE III. Correlations Between Expressions of One or More Members of the HER Family and pCR

Markers	Univariate analysis			Multivariate analysis		
	OR	95% CI	P^a	OR	95% CI	P^a
HER1	0.179	(0.020, 1.605)	0.073	0.171	(0.019, 1.583)	0.071
HER3	0.429	(0.097, 1.886)	0.246	0.430	(0.091, 2.036)	0.275
HER4	0.467	(0.083, 2.638)	0.389	0.611	(0.097, 3.854)	0.593

[a]Likelihood ratio test.

TABLE IV. Results of Exact Multivariate Logistic Regression Analysis With Clinical Variables

Variables	OR	95% CI	P
HER1			0.097
Negative	1		
Positive	0.097	(0.001, 1.313)	
HER3			0.080
Negative	1		
Positive	0.129	(0.003, 1.178)	
HER4			1.000
Negative	1		
Positive	0.680	(0.055, 6.158)	
UICC-TNM staging			0.064
IIA/IIB/IIIA	1		
IIIB	0.148	(0.000, 1.104)	
Menopausal status			1.000
Pre-menopause	1		
Post-menopause	0.817	(0.098, 6.301)	
Grade			0.816
1	1		
2–3	2.518	(0.135, 143.895)	
Hormone status			0.208
Negative	1		
Positive	7.025	(0.475, 384.119)	

The individual expressions of HER3 and HER4 were not significantly correlated with pCR in the univariate and multivariate logistic models. Furthermore, the ORs of HER3 and HER4 were larger than that of HER1. Therefore, the individual impacts of the expressions of HER3 and HER4 on pCR might be smaller than that of the expression of HER1.

DISCUSSION

One limitation of this study is that the sample size was too small to allow definitive conclusion, this pilot study suggested that the immunohistochemical expression of HER1 might be negative predictor for pCR in HER2-positive breast cancer patients treated with trastuzumab-containing neo-adjuvant chemotherapy.

Previous reports have described the individual frequencies of positive immunohistochemical expressions for HER1, HER2, HER3, and HER4 in breast cancer patients. Table V shows the individual positive expressions of HER1, HER3, and HER4 reportedly ranged from 8% to 51%, 12% to 75%, and 11% to 82%, respectively [10–20]. Although all these studies utilized primary surgical specimens from patients without neoadjuvant chemotherapy and most of the studies included more than 100 patients, the positive frequencies of these biomarkers varied considerably. These variations might have been caused by the sample size, immunohistochemical staining method (including the antibodies that were used), or the cut-off levels for the positive or negative expressions of these biomarkers. Although HER2 staining/quantification, which is associated with clinical significance regarding indication for trastuzumab, is established [9], a standard method of HER1/3/4 immunohistochemical staining/quantification has yet to be established for breast cancer. This lack of standard methodology may explain the variation in the frequencies of positive

TABLE V. HER Family Expression in Breast Cancer: Data From the Literature

Author	N	Patient setting	HER1	HER2 (%)	HER3	HER4
Stassen et al. [10]	214	PBC, primary surgery	15%	22	75%	37%
Abd El-Reim et al. [11]	1584	PBC, primary surgery	20%	32	45%	45%
Bartlette et al. [12]	322	PBC, primary surgery	8%	21	29%	19%
Bianchi et al. [13]	145	PBC, primary surgery	21%	45	50%	56%
Esteva et al. [14]	35	PBC, primary surgery	51%	54	48%	57%
Kaya et al. [15]	59	PBC, primary surgery	7%	12	12%	24%
Suo et al. [16]	100	PBC, primary surgery	36%	27	26%	82%
Tovey et al. [17]	55	PBC, primary surgery	13%	19	20%	11%
Wiseman et al. [18]	242	PBC, primary surgery	13%	14	12%	—
Witton et al. [19]	220	PBC, primary surgery	16%	23	18%	12%
Tzaida et al. [20]	312	PBC, primary surgery	17%	32	—	—
Haas et al. [26]	171	HER2-negative PBC, primary surgery	—	0	39%	19%
Gschwantler-Kaulich et al. [27]	57	HER2-positive MBC	35%	100	—	—
Hudelist et al. [28]	46	HER2-positive MBC, H ± CTX	35%	100	—	—
Giuliani et al. [29]	87	HER2-positive MBC, H ± CTX	22%	100	68%	59%
Smith et al. [30]	77	HER2-positive MBC, H + CTX	56%	100	91%	—
Robinson et al. [21]	153	HER2-positive MBC, H + CTX	—	100	9%	—
Gori et al. [23]	45	HER2-positive MBC, H + CTX	20%	100	—	—
Colleoni et al. [33]	485	PBC, neoadjuvant CTX	14%	14	—	—
Current study	44	HER2-positive PBC, neoadjuvant H + CTX	18%	100	27%	18%

N, number of patients; HER, human epidermal receptor; PBC, primary breast cancer; MBC, metastatic breast cancer; CTX, chemotherapy; H, trastuzumab.

HER1/3/4 expressions recorded in the abovementioned studies. The present study demonstrated that the individual positive frequencies for HER1, HER3, and HER4 were each about 20%. Unlike previous studies, the present study included all the patients with a HER2-positive status who underwent trastuzumab-containing neoadjuvant chemotherapy.

Accumulating data have suggested that the individual positive expressions of HER family receptors might be used as predictors of clinical outcome in patients with breast cancer. Indeed, many studies have reported that HER1, HER2, and HER3 individually are predictors of a poor clinical outcome, such as a short disease-free survival or overall survival period. In contrast to the other HER family receptors, HER4 expression is paradoxically associated with a good clinical outcome [10,12,17–20]. In addition, the co-expressions of HER family receptors (including HER1 and HER2; HER2 and HER3; and HER1, HER2, and HER3) have been frequently reported to have a synergistic influence on a poor clinical outcome [11,16,19,21]. HER4 has an opposite influence, compared with the three other HER family receptors; therefore, uniform results between clinical outcome and the co-expression of HER4 and other HER family receptors have not been reported [15,16,19]. Thus, the statistical significance of the correlation between the co-expression of HER family receptors and clinical outcome has varied in previous reports [11,15,16,18,19,22]. Previous study reported that a HER1-positive status resulted in a large increase in the risk of a poor survival outcome and that a HER4-positive status reduced this risk in HER2-positive breast cancer patients [16]. The formation of dimerized receptor complexes by HER family members has been shown to be a trigger for tumor growth and potential tumor behavior [22]. It induces intrinsic receptor tyrosine kinase activity and subsequent activation of downstream signaling components via mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K-Akt) cascades. Although trastuzumab reduces HER2-mediated signaling through these pathways, it may not reduce signaling mediated by other HER receptors. Therefore, cells containing HER1/HER1 or HER1/HER2 heterodimers may initiate mitogenic PI3K-Akt and MAPK signaling even in the presence of trastuzumab [3]. Structural studies have indicated that trastuzumab binds to a region of the HER2 receptor ectodomain that is not involved in receptor dimerization, and thus trastuzumab is unable to block the formation of ligand-induced HER1/HER2 and HER2/HER3 heterodimers [23,24]. Although the role of each HER family receptor in cell signaling remains fully unknown, the present study supported previous studies suggesting that HER1 positive expression is associated with a poor outcome. A tendency between clinical outcome and the co-expression of HER family members was observed, but little is known about the interactions of the HER family member. Additionally, expression of phosphorylated/activated HER family members may more accurately reflect the signaling and functional activity of the HER family proteins than detection of the actual HER family receptors. Accordingly, recent studies have investigated the association between phosphorylated HER family receptors and clinical outcomes [25,26–29]. Although in the present pilot study we did not employ phosphorylated HER family antibodies, further investigations on the phosphorylation of HER family receptors and downstream signaling proteins in larger cohorts will be needed for a better assessment of predictive biomarkers in the treatment of breast cancer.

The expression of HER1 appears to be implicated in the development and progression of malignancies, and enhanced expression of HER1 has been associated with increased tumor proliferation, angiogenesis, and metastatic potential [30,31]. The present study suggested that the immunohistochemical expression of HER1 might be negatively associated with pCR, rather than the expression of HER3 and HER4, in HER2-positive breast cancer patients treated with trastuzumab-containing neo-adjuvant chemotherapy. In HER2-positive metastatic breast cancer patients treated with trastuzumab-containing chemotherapy, several studies reported that a HER1-positive status was not significantly associated with clinical response or overall survival [21,27,28], while Smith et al. [29] reported HER1 expression significantly correlated with progression-free rate. On the other hand, Robinson et al. reported that a HER3-positive status was a significant predictor of overall survival in HER2-positive metastatic breast cancer patients treated with trastuzumab-containing chemotherapy [32]. In another report, positive HER1 expression was associated with a poor disease-free survival period but was not associated with pCR (16.7% in HER1-positive patients vs. 11.7% in HER1-negative patients, $P = 0.33$) in a neo-adjuvant chemotherapy setting without trastuzumab [33]. Although differences in the definitions of response in neo-adjuvant and metastatic settings (i.e., clinical response and pathologic response) and differences in the patient cohorts (i.e., with or without chemotherapy, with or without trastuzumab therapy) make compar-

isons among these conflicting studies difficult, the present pilot study suggested that positive HER1 expression might play a role in the classification of different prognostic populations in HER2-positive breast cancer patients. At present, definite conclusions regarding the role of HER family co-expression in patients treated with trastuzumab-containing chemotherapy are difficult to make based on the results of the present study and these limited previously reported data sets [21,23,27–29].

With the appearance of trastuzumab as a molecular target therapy for HER2, trastuzumab resistance has become a critical therapeutic problem. Cross-talk between different HER family receptors is associated with resistance to HER2-targeted therapy [3]. Several molecular target therapy agents for HER family members or for blocking dimerization have been recently developed for the treatment of breast cancer [4]. Clinical trial of lapatinib, an oral-small molecule dual inhibitor of HER1 and HER2, demonstrated no association with either HER1 expression and response or progression-free survival but a favorable response to lapatinib was observed in patients with the co-expression of phosphorylated HER2 and HER3 [34,35]. Although the present study demonstrated a tendency for the co-expression of HER1 and HER2 to be negatively associated with pCR, ongoing randomized clinical trials have suggested that neoadjuvant trastuzumab-containing chemotherapy with or without lapatinib and trasutuzmab plus lapatinib with or without chemotherapy in a neoadjuvant setting might lead to a breakthrough in the treatment of a subset of patients, such as those with HER2-positive breast cancer with the co-expression of HER1 or HER3 [4].

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Original contribution

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☆

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

[a]*Clinical Trials and Practice Support Division, Pathology Consultation Service, 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]*Division of Breast and Medical Oncology, National Cancer Center Hospital, Tokyo 104-0045, Japan*
[d]*Clinical Laboratory Division, National Cancer Center Hospital, Tokyo 104-0045, Japan*
[e]*Cancer Genomics Project, National Cancer Center Research Institute, Tokyo 104-0045, Japan*
[f]*Epidemiology and Prevention Division, Research Center for Cancer Prevention and Screening, National Cancer Center, Tokyo 104-0045, Japan*

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Summary The purpose of this study was to determine whether p53 immunoreactivity in tumor-stromal fibroblasts assessed by the Allred scoring system in biopsy specimens obtained before neoadjuvant therapy and assessed in surgical specimens obtained after neoadjuvant therapy is significantly associated with nodal metastasis by invasive ductal carcinoma and with the outcome of 318 patients with invasive ductal carcinoma who received neoadjuvant therapy, according to UICC pathologic TNM stage, in multivariate analyses with well-known clinicopathologic factors. The Allred scores for p53 in tumor-stromal fibroblasts in the surgical specimens were significantly associated with the presence of nodal metastasis. The Allred scores for p53 in the tumor-stromal fibroblasts of biopsy and surgical specimens were a very important outcome predictive factor for patients who received neoadjuvant therapy, independent of UICC pathologic TNM status, but the outcome predictive power of the Allred scores for p53 in tumor-stromal fibroblasts assessed in the surgical specimens was superior to that of the Allred scores for p53 in tumor-stromal fibroblasts in the biopsy specimens. The results indicated a close association between p53 protein expression in tumor-stromal fibroblasts, especially in surgical

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

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specimens, and both the presence of nodal metastasis and the outcome of invasive ductal carcinoma patients who received neoadjuvant therapy.

1. Introduction

It has recently been reported that the gene expression profile and protein expression profile of the tumor stroma play a very important role in tumor progression in carcinoma [1-3] and that the interaction between tumor cells and stromal cells also plays a very important role in tumor progression by carcinoma [4,5]. We have already reported that the proliferative activity of tumor-stromal fibroblasts plays a very important role in nodal metastasis and distant organ metastasis by invasive ductal carcinoma (IDC) of the breast [6,7]. Recently, a high frequency of p53 mutations in tumor cells and the surrounding stroma has also been reported [8], and p53 mutations in breast cancer stromal cells have been reported to be closely associated with nodal metastasis [9]. These findings strongly suggest a significant role of the tumor stroma in tumor progression by IDC, and the p53 status of tumor-stromal fibroblasts may play a very important role in tumor progression by IDC.

The purpose of the present study was to determine whether p53 protein expression in tumor-stromal fibroblasts assessed in biopsy specimens obtained before neoadjuvant therapy and surgical specimens obtained after neoadjuvant therapy is significantly associated with the presence of nodal metastasis by IDC, and significantly associated with the outcome of IDC patients who received neoadjuvant therapy, according to the UICC (International Union Against Cancer) pathologic TNM (pTNM) stage. The results indicated that p53 protein expressions in tumor-stromal fibroblasts in both the biopsy specimens and the surgical specimens were closely associated with the presence of nodal metastasis and the outcome of IDC patients who received neoadjuvant therapy.

2. Materials and methods

2.1. Cases

The subjects of this study were 318 consecutive patients with IDC of the breast and who received neoadjuvant therapy before surgery at the National Cancer Center Hospital between January 2000 and December 2005. The IDCs were 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 26 to 75 years (median, 54 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 patients with IDC.

Of the 318 patients, 37 (12%) achieved a pathologic complete response (34, no residual tumor; 3, only residual ductal carcinoma in situ; they have no nodal metastasis) to neoadjuvant therapy.

The neoadjuvant therapy consisted of chemotherapy in 235 patients, endocrine therapy in 43 patients, and chemoendocrine therapy in 3 patients; and 214 of 281 patients 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 UICC pTNM classification.

For the pathologic 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 histologic 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.

2.2. Histologic 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 9 histologic 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) histologic grade (1, 2, 3) [10], (3) tumor necrosis (absent, present) [11], (4) fibrotic focus (FF) (biopsy specimen: absent, present; surgical specimen: absent; FF diameter ≤8 mm, FF diameter >8 mm) [12,13], (5) lymph vessel invasion (absent, present), (6) blood vessel invasion (absent, present), (7) adipose tissue invasion (absent, present), (8) skin invasion (absent, present), and (9) 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].

2.3. Immunohistochemistry

Immunohistochemical staining for estrogen receptors (ERs), progesterone receptors (PRs), p53, and HER2 products was performed with autoimmunostainer (Optimax Plus; BioGenex, San Ramon, CA). Antigen retrieval device for Optimax Plus was 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 mouse anti-ER monoclonal antibody (mAb), ER88 (BioGenex), mouse anti-PR mAb, PR88 (BioGenex), and mouse anti-HER2 mAb, CB11 (BioGnex) and mouse p53 mAb, DO7 (Dako, Glostrup, Denmark). ER88, PR88, and CB11 were already diluted and DO7 was applied at 1:100 dilution. After immunostaining, the sections were counterstained with hematoxylin. Sections of IDCs positive for ER, PR, HER2, and p53 were used each time as positive control. As a negative control, the primary antibody was replaced with normal mouse immunoglobulin.

2.4. Assessment of ER, PR, p53, and HER2 expression

Sections of biopsy specimens and surgical specimens immunostained for ER, PR, and p53 in tumor cells were scored by the Allred system as described previously [16-19]. In brief, each entire slide was evaluated by light microscopy as follows. First, one of the following proportion scores was assigned according to the estimated proportion of tumor cells that stained positive: 0, 0/100 (0%); 1, <1/100 (<1%); 2, 1/100 to 1/10 (1% to 10%); 3, >1/10 to 1/3 (>10% to 33%); 4, >1/3 to 2/3 (>33% to 67%); 5, >2/3 (>67%). Next, one of the following intensity scores was assigned according to the average intensity of staining by the positive tumor cells: 0, no staining; 1, weak; 2, intermediate; 3, strong. The proportion score and intensity score were then added to obtain a total score, with possible total scores ranging from 0 and 2 to 8. However, the number of tumor-stromal fibroblasts that express p53 in tumors is relatively small, and examination of the distribution of tumor-stromal fibroblasts expressing p53 shows that they are scattered even in IDCs with tumor-stromal fibroblasts having Allred scores of 4 to 8. We therefore modified the Allred scoring system to assess nuclear expression of p53 in tumor-stromal fibroblasts by identifying one field with the highest of both proportion score and intensity score for p53 nuclear expression in the whole tumor area by scanning the tumor section stained for p53 at medium-power field (×20 objective and ×10 ocular). The highest intensity score, not the average intensity score, for nuclear expression of p53 was assigned to the tumor-stromal fibroblast staining, and the highest p53 nuclear expression proportion score and intensity score were then evaluated in one high power field (×40 objective and ×10 ocular) (Fig. 1). The HER2 status of the tumor cells was semiquantitatively scored on a scale of 0 to 3 according to the level of HER2 protein expression [20]. Immunohistochemistry was used to score 290 of the 318 IDCs for ER, PR, HER2, and p53 expression in biopsy specimens. In surgical specimens, immunohistochemistry was used to score 273 of the 318 IDCs for ER, PR, and p53 expression and to score 271 of them for HER2 expression.

One author (T. H.) assessed all of the immunohistochemical parameters, and 1 of 3 other authors (H. T., T. S., or Y. S.) identified the immunohistochemical parameters to confirm the IDC immunohistochemical characteristics recorded by TH. Discordant results were reevaluated jointly to reach a consensus. 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 52 months (range, 18-102 months) until June 2008. At that time, 199 of the 281 patients were alive and well, 82 had developed tumor recurrence, and 24 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.

The correlation analyses were performed using Pearson correlation coefficients. The univariate and multivariate analyses for pathologic complete response were performed by using the logistic regression model for all patients. 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 9 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 univariate analyses were then entered together into the multivariate analyses according to UICC pTNM stage. Because the 9 factors were examined using both biopsy specimens obtained before neoadjuvant therapy and surgical specimens obtained

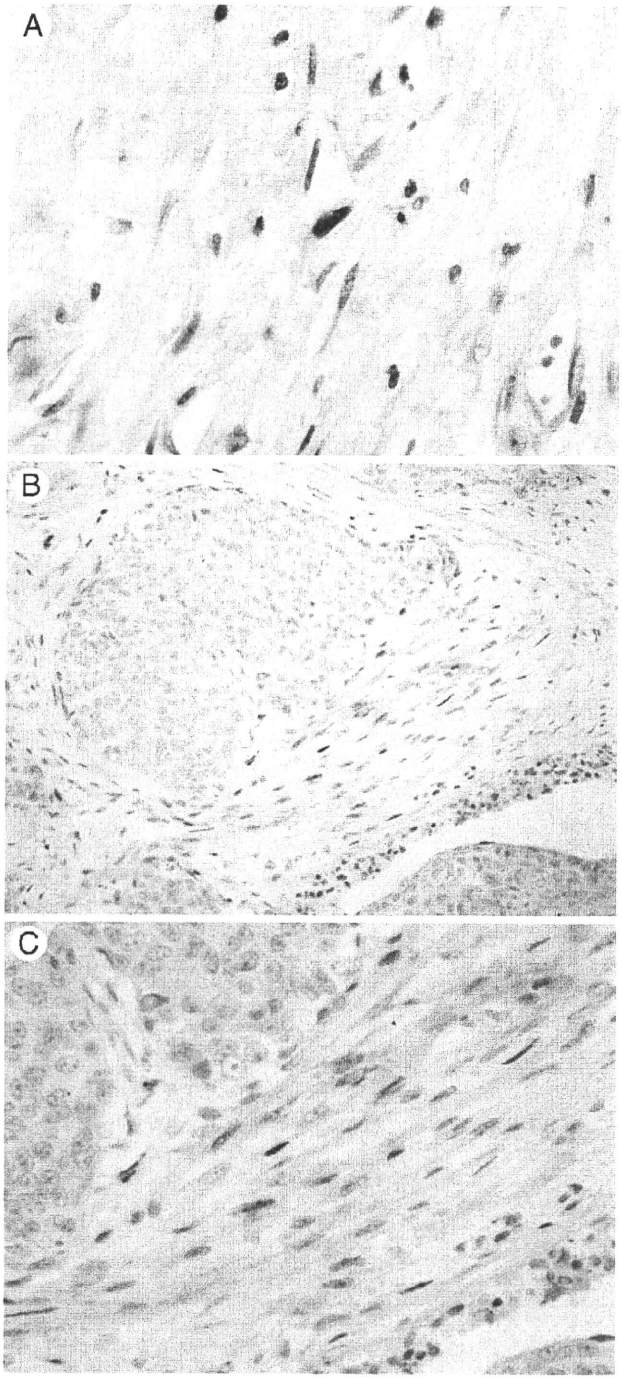

Fig. 1 p53 expression in tumor-stromal fibroblasts of IDCs. (A) Allred score of 3 for p53 in tumor-stromal fibroblasts. One tumor-stromal fibroblast shows moderately intense nuclear staining for p53 in the high-power field (magnification ×40). (B and C) Allred score of 7 for p53 in tumor-stromal fibroblasts. Several tumor-stromal fibroblasts show moderately intense nuclear staining for p53 at the medium-power field (B) and in the high-power field (C). None of the nuclei of the tumor cell have stained positive for p53 (B, magnification ×20; C, magnification ×40).

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 of the remaining factors were significant at a P value less than .05. Because there were fewer than 10 tumor deaths among the patients with UICC pTNM stage 0 and I disease and the patients with UICC pTNM stage II disease, we were unable to perform multivariate analyses for tumor death in these groups. Survival curves were drawn by the Kaplan-Meier method. All analyses were performed with Statistica/Windows software (StatSoft, Tulsa, OK).

3. Results

3.1. Correlations between Allred scores for ER, PR, and p53, and HER2 category assessed in the biopsy specimens and assessed in the surgical specimens

The Allred scores for ER, PR, and p53 in tumor cells and HER2 category in the biopsy specimens were significantly correlated with the Allred scores for ER, PR, and p53 in tumor cells and HER2 category in tumor cells in the surgical specimens (ER: $r = 0.730$, $P < .001$; PR: $r = 0.407$, $P < .001$; p53: $r = 0.576$, $P < .001$; HER2, $r = 0.550$, $P < .001$). There were marginally significant correlations between the Allred scores for p53 in tumor-stromal fibroblasts assessed in the biopsy specimens and the Allred scores for p53 in tumor-stromal fibroblasts assessed in the surgical specimens ($r = 0.109$, $P = .088$) (Table 1, Fig. 1).

3.2. Analysis for nodal metastasis

Although the Allred scores for p53 in tumor-stromal fibroblasts in the biopsy specimens were not significantly associated with the presence of nodal metastasis, the Allred scores for p53 in tumor-stromal fibroblasts in the surgical specimens were significantly associated with the presence of nodal metastasis (Table 1).

3.3. Factors significantly associated with pathologic complete response

In the multivariate analysis, UICC pTNM-pathologic node (pN) category significantly decreased the trend values for the relative risk for pathologic complete response, and

Table 1 Nodal metastasis, tumor recurrence, and tumor-related deaths according to Allred scores for p53 in tumor-stromal fibroblasts in all patients with IDC who received neoadjuvant therapy

	Cases (%)	Number of patients (%)					
		Nodal metastasis		Tumor recurrence		Tumor-related death	
		Present	Absent	Present	Absent	Yes	No
Model 1			0.680		<0.001		0.035
Allred score	290	166 (57)	124 (43)	75 (26)	215 (74)	21 (7)	269 (93)
0	63 (22)	33 (52)	30 (48)	3 (5)	60 (95)	0	63 (100)
2	24 (8)	14 (58)	10 (42)	4 (17)	20 (83)	0	24 (100)
3	65 (22)	38 (58)	27 (42)	17 (26)	48 (74)	7 (11)	58 (89)
4	64 (22)	40 (63)	24 (37)	23 (36)	41 (64)	8 (13)	56 (87)
5	29 (10)	18 (63)	11 (37)	8 (28)	21 (72)	1 (3)	28 (97)
6	27 (9)	12 (44)	15 (56)	12 (44)	15 (56)	5 (19)	22 (81)
7	16 (6)	10 (63)	6 (37)	7 (44)	9 (56)	0	16 (100)
8	2 (1)	1 (50)	1 (50)	1 (50)	1 (50)	0	2 (100)
Model 2			<0.001		<0.001		<0.001
Allred score	273	176 (65)	97 (35)	80 (29)	193 (71)	24 (9)	249 (91)
0	142 (52)	77 (54)	65 (46)	19 (14)	123 (86)	4 (3)	138 (97)
2	4 (1)	3 (75)	1 (25)	0	4 (100)	0	4 (100)
3	39 (14)	27 (69)	12 (31)	13 (33)	26 (67)	1 (3)	38 (97)
4	44 (16)	37 (84)	7 (16)	23 (52)	21 (48)	7 (16)	37 (84)
5	33 (12)	26 (79)	7 (21)	19 (58)	14 (42)	9 (27)	24 (73)
6	9 (3)	5 (56)	4 (44)	5 (56)	4 (44)	2 (22)	7 (78)
7	1 (1)	0	1 (100)	1 (100)	0	1 (100)	0
8	1 (1)	1 (100)	0	0	1 (100)	0	1 (100)

NOTE. Model 1: Allred scores for p53 in tumor-stromal fibroblasts based on biopsy specimens obtained before neoadjuvant therapy. Model 2: Allred scores for p53 in tumor-stromal fibroblasts based on surgical specimens obtained after neoadjuvant therapy.

HER2 category in tumor cells significantly increased the trend values for relative risk for pathologic complete response (Table 2).

3.4. Factors significantly associated with tumor recurrence and tumor death

The univariate analyses of all of the cases as a whole showed that the Allred scores for p53 in tumor-stromal fibroblasts in the biopsy specimens and the surgical specimens were significantly associated with tumor recurrence and tumor-related death (Table 1, Fig. 2). In the multivariate analyses using model 1, UICC pTNM-pN category and the presence of lymph vessel invasion significantly increased the trend values for the hazard rates (HRs) for tumor recurrence and tumor-related death (data not shown). The Allred scores for p53 in tumor-stromal fibroblasts and the presence of an FF significantly increased the trend values for the HRs for tumor recurrence, and the Allred scores for ER in tumor cells significantly increased the trend value for the HR for tumor-related death in the multivariate analyses (data not shown). When model 2 was used, the Allred scores for p53 in tumor-stromal fibroblasts, the Allred scores for ER in tumor cells, UICC pTNM-pN category, and histologic grade significantly increased the trend values for the HRs for tumor recurrence and tumor-related death, and the Allred scores for p53 in tumor cells and residual tumor size significantly increased the trend values for the HRs for tumor-related death in the multivariate analyses (data not shown).

Table 2 Multivariate analysis for pathologic complete response in all patients with IDC who received neoadjuvant therapy

	Pathologic complete response	
	Trend RR (trend 95% CI)	*P* for trend
UICC pTNM-pN category (N0, N1, N2, N3)	0.07 (0.02-0.27)	<.001
HER2 category in tumor cells (0, 1, 2, 3)	1.81 (1.19-2.76)	.005

Abbreviations: RR, relative risk; N0, no nodal metastasis; N1, 1 to 3 nodal metastases; N2, 4 to 9 nodal metastases; N3, 10 or more nodal metastases.
NOTE. Pathologic complete responses were adjusted for UICC pTNM-pN category assessed in surgical specimens obtained after neoadjuvant therapy, and HER2 category in tumor cells, Allred scores for ERs, Allred scores for PRs, histologic grade, and tumor necrosis assessed in biopsy specimens obtained before neoadjuvant therapy.