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

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

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

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

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

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

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

Conclusions

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

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Conflict of interest statement

None declared.

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

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

Locoregiocal recurrence is an important prognostic factor for patients with invasive ductal carcinoma of the breast,[1] and several studies have been performed to clarify factors that are significantly associated with locoregional recurrence.[2,3] These studies demonstrated that lymph vessel invasion, histological grade, tumor size, hormone receptor status, and HER2 status are very important predictors of locoregional recurrence in patients with invasive ductal carcinoma. We have already reported histological factors that are significantly associated with distant-organ metastasis or the tumor-related death of patients with invasive ductal carcinoma of the breast.[4] Since the publication of our previous study,[4] we have performed additional studies that identified the following new histological factors as predictors of the outcome of patients with invasive ductal carcinoma of the breast[5]: (i) type of invasive ductal carcinoma;[6] (ii) grading system for lymph vessel tumor emboli;[7] (iii) number of apoptotic figures in blood vessel tumor emboli;[8] (iv) number of mitotic figures in metastatic carcinomas to the lymph node;[9] and (v) maximum dimension of metastatic carcinomas to the lymph node.[8] Although our studies clearly demonstrated that the factors we previously reported were very useful for accurately predicting tumor recurrence, distant-organ metastasis or tumor-related death,[4–9] we have not yet investigated whether these factors are significantly associated with the locoregional recurrence of invasive ductal carcinoma of the breast. We are confident that clarification of the recurrent or metastatic patterns of invasive ductal carcinomas based on their histological features will provide clinicians, pathologists, and scientists with very important clues for accurately evaluating the true biological characteristics of invasive ductal carcinomas. Such a result would likely contribute to the establishment of targeted therapies for patients with invasive ductal carcinoma of the breast.

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

Materials and Methods

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

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

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Table 1. Groups and factors

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

Table 1. (continued)

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

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

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

The following antibodies were used for immunohistochemistry: anti-estrogen receptor mouse monoclonal antibody ER88 (BioGenex, Fremont, CA, USA), anti-progesterone receptor mouse monoclonal antibody PR88 (BioGenex), and anti-HER2 mouse monoclonal antibody CB11 (BioGenex). Allred scores for estrogen receptor or progesterone receptor were assessed according to our previously study.[13] We defined an Allred score of 0 or 2 for ER or PR as being negative for ER or PR and Allred scores of 3 or more for ER or PR as being positive for ER or PR. HER2 expression in tumor cells was categorized according to the definition of Wolf.[14] All types 2 and 4 invasive ductal carcinomas were immunohistochemically studied using monoclonal antibodies to keratins (AE1/3) to confirm that the atypical tumor-stromal fibroblasts were not modified invasive tumor cells, and fibroblasts that were negative for keratins were considered as atypical tumor-stromal fibroblasts (Fig. 1b). We also performed immunohistochemical staining for alpha-smooth muscle actin for types 2 and 4 invasive ductal carcinomas to investigate whether atypical tumor-stromal fibroblasts are myofibroblasts (Fig. 1c). Some invasive ductal carcinomas contained large lymph vessel tumor emboli, especially in invasive ductal carcinomas containing a grade 2 or grade 3 lymph vessel tumor emboli, and it was difficult to determine whether they were true lymph vessel tumor emboli or a non-invasive ductal carcinoma component by hematoxylin and eosin staining alone. We therefore performed immunohistochemical staining with D2-40 antibody (monoclonal mouse antibody, Signet, Dedham, MA, USA, 1:200) to confirm that the lymph vessel tumor emboli identified by hematoxylin and eosin staining were true tumor emboli in some invasive ductal carcinomas with grade 2 or grade 3 lymph vessel tumor emboli (Fig. 1e). Histologic grade, nuclear feature of primary invasive tumors, and mitotic activity index in primary invasive tumors were evaluated according to the criteria of Elston and Ellis.[15] Tumor necrosis in primary invasive tumors was evaluated according to the definition of Gilchrist.[16]

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

Results

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

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

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

(1) The presence of atypical tumor-stromal fibroblasts was defined based on the presence of one or more atypical tumor-stromal fibroblasts in the tumor stroma inside and outside of the fibrotic foci in invasive ductal carcinoma. Although atypical tumor-stromal fibroblasts are occasionally distributed at random locations in the tumor stroma, they tend to exist within the cellular area of the tumor-stromal fibroblasts

(2) The number of nuclei in an atypical tumor-stromal fibroblast is one or more. The nuclear size of an atypical tumor-stromal fibroblast is two or more times larger than that of an ordinary tumor-stromal fibroblast. The nuclear features of an atypical tumor-stromal fibroblast include an irregular or convoluted shape, and also include various bizarre shapes

(3) An obvious small to large size nucleolus or nucleoli are seen in the nucleus or nucleoli of atypical tumor-stromal fibroblasts and some atypical tumor-stromal fibroblasts show a coarsely granulated nuclear chromatin pattern

Type	Fibrotic focus	Atypical tumor-stromal fibroblast not forming a fibrotic focus	Atypical tumor-stromal fibroblast forming a fibrotic focus
1	Absent	Absent	Not applicable
2	Absent	Present	Not applicable
3	Present	Not assessed	Absent
4	Present	Not assessed	Present

2 *Grading system for lymph vessel tumor embolus*

Grade 0: Invasive ductal carcinomas with no lymph vessel tumor embolus

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

	No. of mitotic figures	No. of apoptotic figures
Grade 1	0	0
	0	Any
	Any	0
Grade 2	1–4	>0
	>0	1–6
Grade 3	>4	>6

(1) The numbers of tumor cell mitotic figures and apoptotic figures in lymph vessels are counted in 20 high-power fields. In carcinomas containing a small number of lymph vessel tumor emboli, the mitotic figures and apoptotic figures are counted in fewer than 20 high-power fields

(2) A large lymph vessel tumor emboli located far from the stroma-invasive tumor margin is selected and the mitotic figures and apoptotic figures in the lymph vessel tumor emboli or embolus are counted

(3) The numbers of mitotic figures and apoptotic figures in tumor cells composing the lymph vessel tumor embolus or emboli in the high-power field containing the largest number of mitotic figures, and/or the largest number of apoptotic figures are recorded as the number of mitotic figures and apoptotic figure in the lymph vessel tumor emboli or embolus. The cumulative numbers of tumor cell mitotic figures and apoptotic figures in the lymph vessel tumor emboli in all 20 high-power fields are not used

3 *Grade of stromal fibrosis in metastatic carcinomas to the lymph node*

None: Metastatic carcinoma with no tumor-stromal fibrosis

Mild: Metastatic carcinoma occupied by ≤30% tumor-stromal fibrosis

Moderate: Metastatic carcinoma occupied by >30 to ≤80% tumor-stromal fibrosis

Severe: Metastatic carcinoma occupied by >80% tumor-stromal fibrosis

4 *Extranodal blood vessel tumor embolus or emboli*

Tumor embolus or emboli in blood vessel or vessels with a smooth muscle-supported endothelial lining in perinodal adipose tissues was/were assessed as extranodal blood vessel tumor embolus or emboli

5 *Mitotic figures in metastatic carcinomas to the lymph node*

(1) A random search for mitotic figures in metastatic mammary carcinoma to the lymph nodes is performed using high-power magnification fields (×10 or ×20) of the tumor area

(2) Next, one high-power magnification field (×40) of the tumor area containing the highest number of mitotic figures is selected to determine the largest number of metastatic mammary carcinoma to the lymph nodes exhibiting mitotic figures

modified radical mastectomy had been performed in 584. The surgical margins of all the partial mastectomy materials were histologically examined to confirm whether tumor cells were absent or present at the surgical margins of the materials; we confirmed that all the materials had been completely resected because the outermost edges of the tumors were 5 mm or further from the surgical margin of the materials. A Level I and II axillary lymph node dissection had been performed in all the patients, and a Level III axillary lymph node dissection had been performed in some of the patients. Of the 1042 patients, 873 received adjuvant therapy, consisting of chemotherapy in 217 patients, endocrine therapy in 281 patients, and chemoendocrine therapy in 375 patients. The chemotherapy regimens used were anthracycline-based with or without taxane and

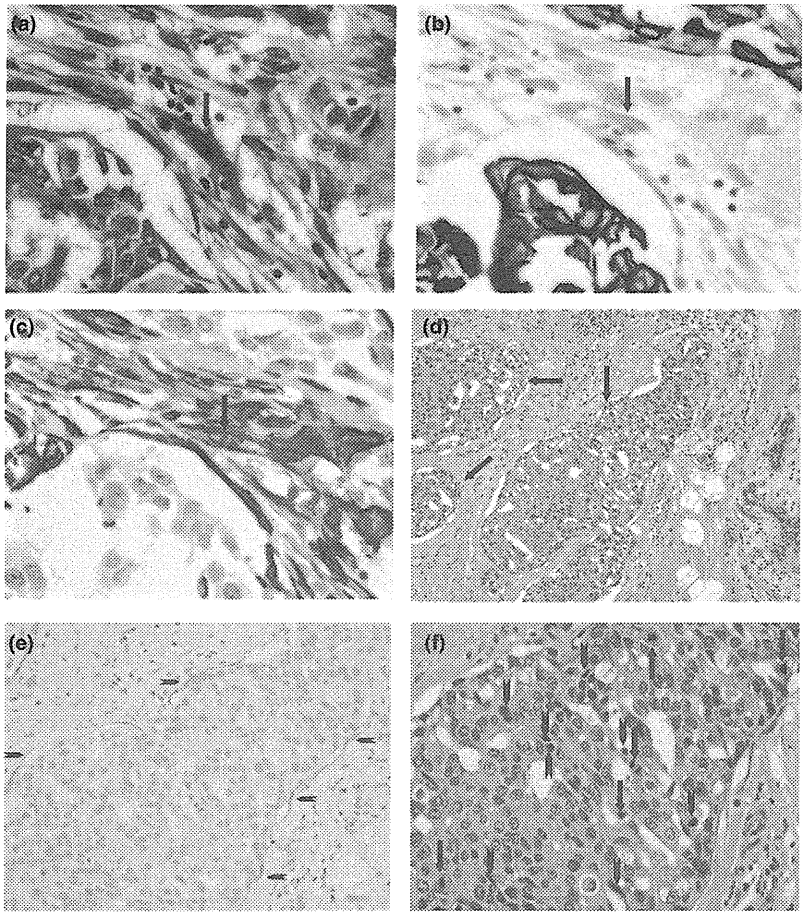

Fig. 1. (a–c) Type 2 invasive ductal carcinoma. One atypical tumor-stromal fibroblast with a large spindle nucleus is visible in the tumor stroma (arrow). The fibroblast was stained negative for AE1/3 (arrow, b) and positive for smooth muscle actin (arrow, c). The invasive tumor cells were stained positive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

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

Univariate analyses for first locoregional recurrence. Overall, age ($P = 0.026$), the Allred score for estrogen receptors in the tumor cells ($P = 0.017$), the histologic grade ($P = 0.009$), the invasive tumor size ($P < 0.001$), nuclear features of the primary-invasive tumor cells ($P < 0.001$), the number of mitotic figures in the primary-invasive tumor cells ($P = 0.002$), tumor necrosis ($P = 0.019$), the type of invasive ductal carcinoma, the grading system for lymph vessel tumor emboli, the UICC pN category, the grade of stromal fibrosis in metastatic carcinomas to the lymph node, the maximum dimension of metastatic carcinomas to the lymph node, the number of extranodal blood vessel tumor emboli, and the number of mitotic figures in metastatic carcinomas to the lymph node were significantly associated with first locoregional recurrence in the univariate analyses (Table 3). The fibrotic focus dimension (Table 3), the number of apoptotic figures in blood vessel tumor emboli (Table 3), adjuvant therapy, adjuvant radiotherapy, the Allred score for progesterone receptors in the tumor cells, the HER2 category, and the skin invasion were not significantly associated with first locoregional recurrence in the univariate analyses (data not shown). Atypical tumor-stromal fibroblast was observed in 69 (7%) cases (type 2 and 4 invasive ductal carcinoma cases) among 1042 cases (Table 3). The presence of atypical tumor-stromal fibroblasts stained positive for alpha-smooth muscle actin was observed in 60 (87%) out of 69 types 2 and 4 invasive ductal carcinomas (type 2: 35/40 cases, 88%; type 4: 25/29 cases, 86%).[6]

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

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

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

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

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

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

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

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

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

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

–, not significant; NA, not available.

pTNM stages I and II patients (Tables 5 and 8), the patients who had received endocrine therapy (Tables 6 and 8), the patients who had received chemotherapy (Tables 6 and 8), the patients who had not received adjuvant radiotherapy (Tables 7 and 8) and the patients who had received adjuvant radiotherapy (Tables 7 and 8). Lymph vessel tumor embolus grade 2 was significantly associated with first locoregional recurrence among the overall patients who had received adjuvant therapy ($P < 0.001$), the UICC pN0 patients (Tables 4 and 8), the UICC pTNM stages I and II patients (Tables 5 and 8) and the patients who had received chemoendocrine therapy (Tables 6 and 8). Twenty or more mitotic figures in primary invasive tumors, the presence of tumor necrosis, and the presence of skin invasion were significantly associated with first locoregional recurrence among the UICC pN0 patients (Tables 4 and 8), among the UICC pTNM stages I and II patients .(Tables 5 and 8) and among the patients who had

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

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

–, not significant; NA, not available.

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

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

–, not significant; NA, not available.

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

Discussion

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

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

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

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

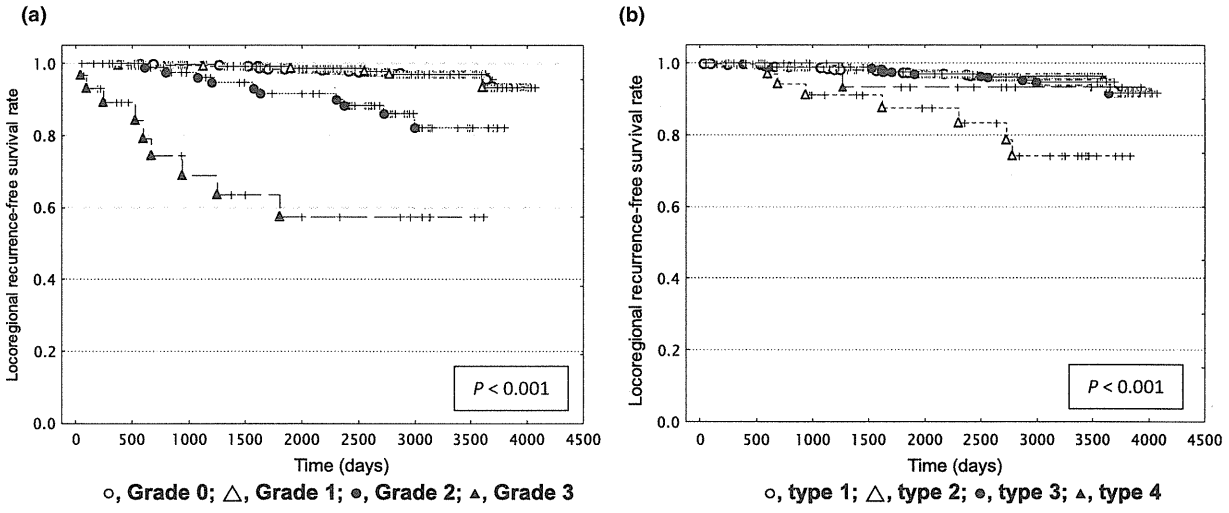

Fig. 2. First locoregional recurrence-free survival curves for overall patients with invasive ductal carcinoma (a and b). (a) Patients with grade 3 lymph vessel tumor emboli had the shortest locoregional recurrence-free survival curve. Patients with grade 2 lymph vessel tumor emboli also had a significantly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

rately predicting the locoregional recurrence of invasive ductal carcinoma,[19,20] we confirmed that the grading system for lymph vessel tumor emboli is superior to the presence or absence of lymph vessel invasion, the number of invaded lymph vessels or the location of lymph vessels invaded for accurately predicting first locoregional recurrence in this study. Thus, we can conclude that the lymph vessel tumor embolus grade is the only lymph vessel assessment parameter that can accurately divide patients with lymph vessel invasion into a good prognosis group and a poor prognosis group. However, the locoregional predictive power of the lymph vessel tumor embolus grade was inferior to type 2 invasive ductal carcinoma or >5 mitotic figures in metastatic carcinomas to the lymph node in patients who had received adjuvant radiotherapy; this finding strongly suggests that adjuvant radiotherapy prevents locoregional recurrence in patients with lymph vessel tumor embolus grades 3 or 2.[21] Since the lymph vessel tumor embolus grade is assessed based on the numbers of mitotic figures and apoptotic figures in tumor cells in the lymph vessel,[7] adjuvant radiotherapy probably inhibits the acceleration of the cell cycle in tumor cells in the lymph vessel. Thus, adjuvant radiotherapy may contribute to improving the outcome of patients with lymph vessel tumor embolus grade 3 or those with lymph vessel tumor embolus grade 2. From these, we can conclude that the lymph vessel tumor embolus grade in the tumor embolus-related group was the best grade for accurately predicting first locoregional recurrence among patients with invasive ductal carcinoma of a low-risk, intermediate-risk or high-risk

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

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

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

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Disclosure Statement

The authors have no conflict of interest.

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RESEARCH ARTICLE Open Access

A simple immunohistochemical panel comprising 2 conventional markers, Ki67 and p53, is a powerful tool for predicting patient outcome in luminal-type breast cancer

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Abstract

Background: Ki67 is widely used in order to distinguish the "A" and "B" subtypes of luminal-type breast cancer. This study aimed to validate the prognostic value of adding p53 to Ki67 for characterizing luminal-type breast cancer.

Methods: Immunostaining for Ki67, p53, and the molecular markers HER2, CK5/6, CK14, EGFR, FOXA1, GATA3, and P-cadherin was examined hormone receptor (HR)-positive cancer tissues from 150 patients. The prognostic value of an immunohistochemical panel comprising Ki67 and p53 was compared with that of the single Ki67 labeling index (LI), and uni- and multivariate analyses were performed.

Results: Division of the patients based on the immunohistochemistry results into favorable- (low Ki67 LI, p53-negative) and unfavorable- (high Ki67 LI and/or p53-positive) phenotype groups yielded distinctly different Kaplan-Meier's curves of both disease-free ($P<0.0001$) and overall survival ($P=0.0007$). These differences were much more distinct than those between the corresponding low Ki67 LI vs. high Ki67LI curves. While the prognostic values of the other molecular markers were not significant, combined Ki67-p53 status was an independent prognostic factor by multivariate analysis.

Conclusion: These data indicate that an immunohistochemical panel comprising Ki67 and p53 is a practical tool for management of patients with HR-positive breast cancer.

Keywords: Ki67 labeling index, p53, IHC panel, Luminal-type breast cancer

Background

Approximately 70% of breast cancers express a hormone receptor (HR). HR status is a powerful predictor of response to therapies that inhibit estrogen synthesis or block the action of its receptor [1]. Endocrine therapies are established in the adjuvant setting [2-4]. For example, women with node-negative, HR-positive breast cancer who are treated with tamoxifen alone after surgery have an average 10-year recurrence rate of only 15% [5]. If all of these patients were offered chemotherapy, 85% would be over-treated [6]. It is therefore important to distinguish patients with HR-positive tumors at high risk for recurrence who need additional chemotherapy from those for whom adjuvant hormonal therapy alone may suffice [7].

Multi-gene assays are strong candidate tools for predicting the risk of recurrence in HR-positive patients. For example, the Oncotype DX™ assay analyzes the expression levels of 21 genes (including 5 reference genes) in formalin-fixed paraffin embedded tissues and produces a Recurrence Score (RS) that predicts the likelihood of distant recurrence [6] and the benefit of chemotherapy in women with early HR-positive breast cancer [8]. Although Oncotype DX™ is a potentially powerful tool for stratification of HR-positive patients, it is too expensive to use in routine clinical practice. Many

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oncologists are eager for an alternative assay that is inexpensive as well as easy to use; one possible approach would be an immunohistochemical (IHC) assay.

Sorlie et al. reported that breast cancers could be divided, based on their gene expression profiles, into at least 4 groups: luminal-type, HER2-type, normal-like-type, and basal-type. Luminal-type cancers are characterized by an activated estrogen receptor (ER) signaling pathway and are divided into 2 subtypes, luminal subtypes "A" and "B. In general, luminal-subtype-A tumors express higher levels of ER and carry a better prognosis than do luminal-subtype-B tumors [9]. Recent studies have shown that tumors of luminal subtype A have a lower rate of *p53* mutation [9-12] and are less proliferative [13,14] than those of luminal subtype B, suggesting that the combination of p53 status and proliferation markers could be useful to distinguish between luminal subtypes A and B.

The tumor suppressor gene *p53* plays a most important role in regulating normal cell fate in response to various stresses, and disruption of p53 function is often involved in tumor progression. Since the co-authors first reported in 1991 that distinct immunoreaction with p53 in the nuclei of breast cancer cells is an independent prognostic indicator [15], more than 1000 articles about the correlation between p53 status and breast cancer prognosis have been published. Recent studies have shown that abnormalities of the *p53* gene [16] and accumulation of p53 protein in the nuclei [17,18] are also robust prognostic indicators in HR-positive patients.

Ki67 is the marker most often used to evaluate tumor proliferation status. The Ki67 protein is a large (395 kD) nuclear protein that is present during all active phases of the cell cycle except for the G0 phase. Because proliferation status is closely correlated with tumor aggressiveness, the Ki67 labeling index (LI) is considered an established prognostic marker for various tumor types, including breast cancer [19,20]. Previous clinical studies have revealed Ki67 LI to be a good prognostic indicator for HR-positive breast cancer patients [7,21].

Although Ki67 is a strong prognostic indicator for HR-positive breast cancer patients, adding Ki67 to the commonly used indices in daily practice is controversial [19]. Furthermore, its predictive value is weaker than that of multi-gene expression assays such as Oncotype DX™. In this study, we attempted to validate the classification of HR-positive breast cancer patients by combined analysis of Ki67 LI and p53 status. We performed immunohistochemical examination of Ki67 and p53 expression in 150 samples of surgically resected HR-positive invasive breast cancers and analyzed the relationships between combined Ki67-p53 status and clinicopathological factors, including prognosis.

Methods

Patients and Samples

Of the 247 patients who had undergone mastectomy or breast-conserving surgery for invasive ductal carcinoma of the breast at the National Defense Medical College (NDMC) Hospital between 1995 and 1999, 150 patients with ER-positive and/or progesterone-receptor (PgR)-positive localized breast carcinomas were selected based on immunohistochemical reevaluation of ER and PgR expression. Tissue microarray (TMA) blocks of the tumors from these 150 patients were constructed as previously described [22]. Briefly, double tissue cores 2 mm in diameter were taken from each donor block, and these core specimens were transferred to a recipient block using a Tissue Microarrayer (Beecher Instruments, Silver Spring, MD, USA). The use of the tissue blocks was internally reviewed and approved by the NDMC Ethics Committee.

The 150 patients had been followed up for a median of 82 months (range, 1–151 months), during which time there were 30 relapses and 15 deaths. In most cases, the patients were prescribed adjuvant endocrine therapy (for example, tamoxifen, toremifene, fadrozole, or LHRH analogues). Forty-nine patients with large tumors and/or 4 or more lymph node metastases had received adjuvant chemotherapy (cyclophosphamide-epirubicin-5-fluorouracil (CEF), cyclophosphamide-adriamycin-5-fluorouracil (CAF), cyclophosphamide-methotrexate-5-fluorouracil (CMF), or oral fluoropyrimidines), and 12 patients with locally advanced breast cancer had received preoperative chemotherapy (for example, CAF or CEF). One hundred forty-eight patients were females and 2 were males. The clinical stage of the patients was determined based on the TNM classification according to general rules of the Japanese Breast Cancer Society [23]. Clinicopathological data were obtained from the medical records and pathology reports, but ER, PgR and HER2 status were examined in our previous study [22].

Immunohistochemistry

Immunohistochemistry was performed on a TMA composed of 150 breast cancer tissue specimens. The antibodies used were mouse monoclonal anti-p53 antibody (DO-7, Dako, Glostrup, Denmark), mouse monoclonal anti-Ki67 antibody (MIB-1, Dako), mouse monoclonal anti-FOXA1 antibody (2D7, Abnova, Taipei, Taiwan), mouse monoclonal anti-GATA3 antibody (HG3-31, Santa Cruz, Santa Cruz, CA, USA) mouse monoclonal anti-CK5/6 antibody (D5/16 B4, Dako), mouse monoclonal anti-CK14 antibody (LL002, NeoMarkers, Fremont, CA, USA), mouse monoclonal anti-P-cadherin antibody (56C1, Novocastra, Newcastle, UK), and a mouse monoclonal anti-EGFR antibody included in an EGFR pharmDX kit (Dako).

Sections (4-um-thick) were cut from the formalin-fixed, paraffin-embedded TMA blocks. Antigens were retrieved by microwave heating for 30 min in 10 mM sodium citrate (pH 6.0) for CK5/6 and GATA3 or by autoclaving for 20 min in 10 mM Tris–HCl (pH 9.0) for Ki67, p53, CK14, FOXA1, and P-cadherin. To block endogenous peroxidase activity, the sections were treated for 5 min with 100% methanol containing 3% H_2O_2. Non-specific binding was blocked by incubation in 1% normal swine serum (Dako) in phosphate-buffered saline. The slides were incubated with primary antibodies at 4°C overnight and then reacted with a dextran polymer reagent combined with secondary antibodies and peroxidase (Envision Plus; Dako) for 30 min at room temperature. Specific antigen-antibody reactions were visualized with 0.2% diaminobenzidine tetrahydrochloride and hydrogen peroxide. Immunostaining for EGFR was performed in accordance with the package inserts of the EGFR pharmDX Kit. The sections were counterstained with Mayer's hematoxylin.

Evaluation of immunohistochemistry

Although there is no universal cut-off value for Ki67 LI, Cheang et al. showed that, using the cases which were subtyped by gene expression profile, the best Ki67 LI cut-off value to distinguish luminal B from luminal A was 13% [7]. Furthermore, similar to the 10% cut-off value was used in several reports [21,24-28]. So, in this study, Ki67 LI greater than 10% was classified as high. The Ki67 LI was calculated as the percentage of positive tumor nuclei divided by the total number of tumor cells examined on the basis of a manual count of 500 or more cells under high power (400×).

For p53, FOXA1, and GATA3, cells with immunostaining in the nucleus were defined as positive, while for CK5/6, CK14, and P-cadherin, cells with immunostaining along the cellular periphery and/or in the cytoplasm were defined as positive. For p53, positive staining of fewer than 10% of the tumor cells was defined as negative tumor expression and staining of 10% or more of the tumor cells as positive tumor expression [15]. For P-cadherin, membrane staining of fewer than 50% of the tumor cells was defined as negative tumor expression and staining of 50% or more of the tumor cells as positive tumor expression. P-cadherin positive tumors were further divided into "weakly" and "strongly" expressing tumors based on staining intensity. Finally, negative and weakly P-cadherin-staining tumors were classified as

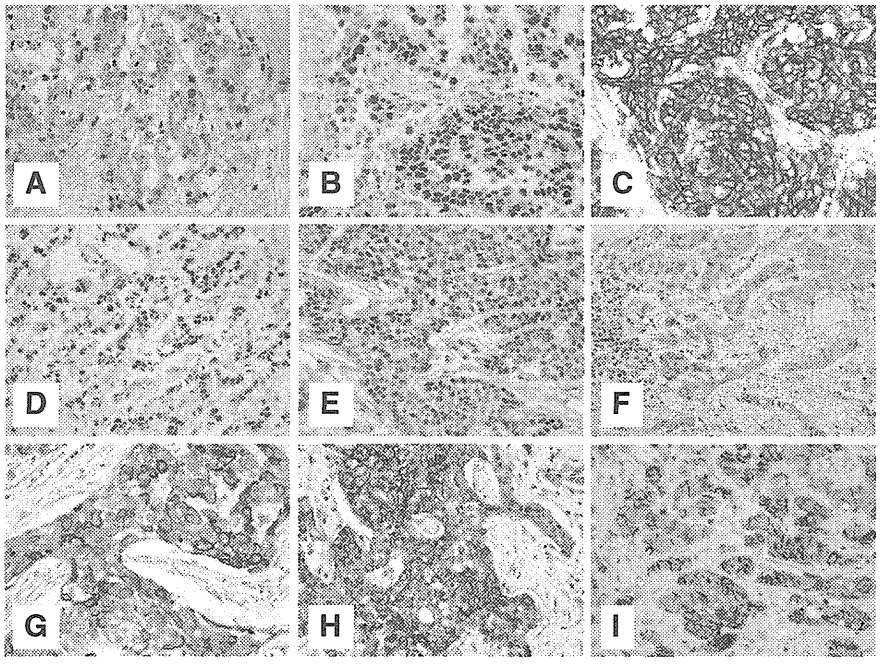

Figure 1 Representative images of immunostaining for 9 molecular markers. A, Positive nuclear Ki67 staining. The Ki67 labeling index of this specimen is 12%. **B**, p53 staining in the nucleus. This tumor was scored as p53-positive. **C**, Positive HER2 membrane staining. The HER2 expression of this tumor was scored as 3+. **D**, Positive nuclear FOXA1 staining. This tumor was classified as FOXA1-positive. **E**, Positive nuclear GATA3 staining. This tumor was classified as GATA3-positive. **F**, Positive CK5/6 membrane or sub-membrane staining of tumor cells. This tumor was classified as CK5/6-positive. **G**, Positive CK14 membrane or sub-membrane staining of tumor cells. This tumor was classified as CK14-positive. **H**, Positive EGFR membrane staining of tumor cells. The EGFR expression of this tumor was scored as 3+. **I**, Positive P-cadherin membrane or sub-membrane immunoreactivity of tumor cells. This tumor was classified as P-cadherin-high. The magnification of all figures is ×400.

Table 1 Clinicopathological implication of Ki67-p53 combination status in surgically resected hormone receptor-positive breast cancers

Variables	Cases			
	Ki67-p53 combination status			
	Total (n=150)	Low Ki67 LIand Negative p53 (n=88)	High Ki67 LI and/or Positive p53 (n=62)	*P* - value
Age				
≦50	71	40	31	
>50	79	48	31	0.58
Tumor size				
<5.0 cm	128	78	50	
≧5.0 cm	19	8	11	0.12
Unknown	3	2	1	
Lymph node metastasis				
(−)	84	52	32	
(+)	63	34	29	0.33
Unknown	3	2	1	
Stage				
I or II	129	78	51	
III	17	7	10	0.13
Unknown	4	3	1	
Nuclear grade				
1, 2	115	76	39	
3	35	12	23	0.0008
HER2 status				
Negative	142	88	54	
Positive	8	0	8	0.0006
Basal phenotype marker (CK5/6, CK14, EGFR)				
Negative	140	87	53	
Positive	10	1	9	0.0016
FOXA1				
Negative	20	14	6	
Positive	127	72	55	0.38
NE	3	2	1	
GATA3				
Negative	30	18	12	
Positive	120	70	50	0.99
P-cadherin				
Low	94	62	32	
High	56	26	30	0.0019
Chemotherapy				
No	98	59	39	0.59
Yes	52	29	23	

Abbreviation: *LI* labeling index, *NE* not evaluable.

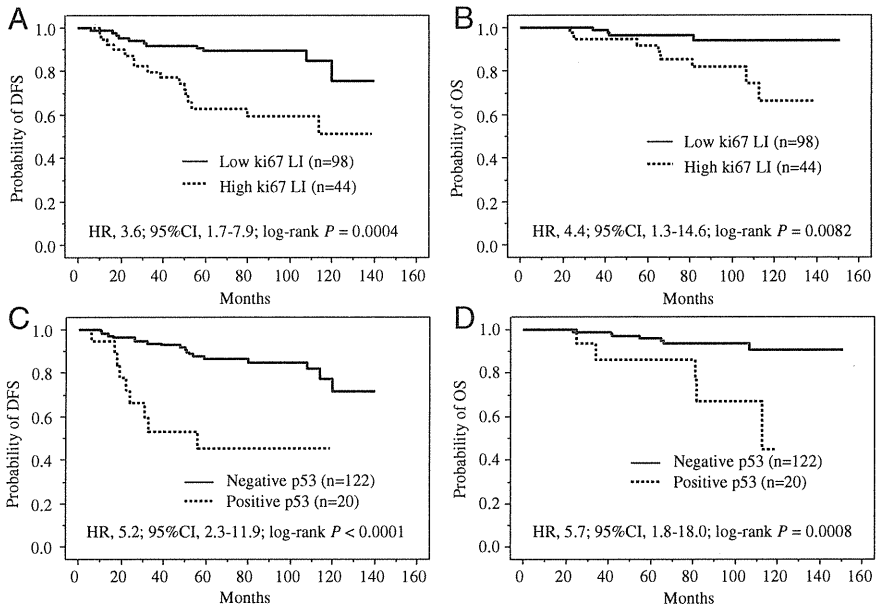

Figure 2 Prognostic impact of Ki67 labeling index (LI) and p53 status in patients with hormone receptor-positive and HER2-negative breast cancer (n=142). A, Disease free survival curves for 98 patients with Ki67 LI-low tumors and 44 patients with Ki67 LI-high tumors. The 2 curves differ significantly (HR, 3.6; 95% CI, 1.7–7.9; log-rank $P = 0.0004$). **B,** Overall survival curves for 98 patients with Ki67 LI-low tumors and 44 patients with Ki67 LI-high tumors. The 2 curves differ significantly (HR, 4.4; 95% CI, 1.3–14.6; log-rank $P = 0.0082$). **C**, Disease-free survival curves for 122 patients with p53-negative tumors and 20 patients with p53-positive tumors. The 2 curves differ significantly (HR, 5.2; 95% CI, 2.3–11.9; log-rank $P < 0.0001$). **D,** Overall survival curves for 122 patients with p53-negative tumors and 20 patients with p53-positive tumors. The 2 curves differ significantly (HR, 5.7; 95% CI, 1.8–18.0; log-rank $P = 0.0008$).

"low" and strongly P-cadherin-staining tumors as "high". A tumor expression cutoff point of 10% of cells stained was used for GATA3, CK5/6, and CK14 and a cutoff of 70% of cells stained for FOXA1, regardless of staining intensity. An EGFR score of 0–3+ was assigned according to the manufacturer's package insert, and scores of 1–3+ were classified as positive. CK5/6, CK14, and EGFR were considered basal phenotype markers.

ER and PgR were examined immunohistochemically as described in the previous study [22], using mouse monoclonal anti-human ER (clone 1D5, Dako) and mouse anti-human PgR (clone PgR636, DAKO). ER and PgR were defined as positive if the nuclear staining was seen in 10% or more of tumor cells. Hormone receptor positive was defined as at least one of ER or PgR positive, and hormone receptor negative was defined as ER and PgR negative. HER2 was evaluated by IHC using rabbit polyclonal anti-HER2 antibody (HercepTest kit, Dako) and FISH (in case of IHC 2+) using Path Vysion kit (Abbott Park, IL, USA). HER2 was defined as positive if the IHC score was "3+" according to the standard procedure, or gene amplification (HER2:CEP17 ratio > 2.0) was detected by FISH [29].

The immunohistochemistry results were evaluated independently by 2 observers (TK and KI), and cases with disparate scores were re-evaluated and discussed until a consensus was reached. Ki67-positive cells were counted and the labeling index calculated by TK alone.

Statistical analysis

Comparisons between groups were evaluated with the chi-squared test or Fisher's exact test. Patient survival curves were drawn using the Kaplan-Meier method and analyzed by the log-rank test. The hazard ratios and corresponding 95% confidence intervals (CIs) were calculated with Cox's proportional hazards model. Univariate and multivariate Cox's proportional hazards models were used to explore the associations of variables with disease-free and overall survival. For all tests, differences at $P < 0.05$ were considered statistically significant. All analyses were performed using the software JMP 6.0 for Windows (SAS Institute Inc., Cary, NC, USA).

Results

Expression of markers (Ki67, p53, HER2, FOXA1, GATA3, CK5/6, CK14, EGFR, and P-cadherin) in HR-positive tumors (n=150)

Representative images of immunostaining for the markers examined in this study are shown in Figure 1. Among 150 HR-positive tumors, there were 51 (34%) Ki67 LI-high tumors, 22 (15%) p53-positive tumors, 127 (85%) FOXA1-positive tumors, 120 (80%) GATA3-

Table 2 Univariate and multivariate analyses of immunohistochemical parameters (disease-free survival and overall survival)

			Univariate			Multivariate		
		Total (n=142)	Hazard ratio	(95% CI)	*P*-value	Hazard ratio	(95% CI)	*P*-value
Disease-free survival								
Ki67 LI	Low	98	1			1		
	High	44	3.6	1.7-7.9	0.0010	3.2	1.4-7.6	0.0073
p53	Negative	122	1			1		
	Positive	20	5.2	2.3-11.9	<0.0001	3.9	1.6-9.4	0.0025
FOXA1	Low	20	1			1		
	High	119	1.4	0.51-3.6	0.54	1.7	0.56-5.2	0.34
GATA3	Negative	30	1			1		
	Positive	112	1.5	0.65-3.5	0.34	1.2	0.46-3.4	0.66
Basal phenotype marker	Negative	133	1			1		
(CK5/6, CK14, EGFR)	Positive	9	1.0	0.24-4.3	0.98	0.47	0.10-2.2	0.34
P-cadherin	Low	90	1			1		
	High	52	1.3	0.59-2.8	0.52	0.87	0.37-2.1	0.75
Overall survival								
Ki67 LI	Low	98	1			1		
	High	44	4.4	1.3-14.6	0.016	3.2	0.91-11.9	0.070
p53	Negative	122	1			1		
	Positive	20	5.7	1.8-18.0	0.0029	3.8	1.1-13.0	0.030
FOXA1	Low	20	1			1		
	High	119	0.49	0.06-3.9	0.50	0.75	0.07-7.5	0.81
GATA3	Negative	30	1			1		
	Positive	112	1.1	0.31-4.3	0.84	1.3	0.29-6.1	0.70
Basal phenotype marker	Negative	133	1			1		
(CK5/6, CK14, EGFR)	Positive	9	1.2	0.15-9.3	0.86	0.60	0.07-5.4	0.64
P-cadherin	Low	90	1			1		
	High	52	1.8	0.57-5.5	0.32	1.2	0.36-4.2	0.73

Abbreviation: *95% CI* 95% confidence interval, *LI* labeling index.

positive tumors, and 6 (4%) CK5/6-positive, 3 (2%) CK14-positive, 3 (2%) EGFR-positive, and 56 (37%) P-cadherin-high tumors. Ten (7%) tumors showed positive staining for at least 1 of the basal phenotype markers CK5/6, CK14, and EGFR. Eight tumors were determined as HER2-positive, which were composed of 6 tumors with IHC 3+ and 2 tumors with IHC 2+ and FISH +.

Correlations of clinicopathological factors (tumor size, lymph-node status, nuclear grade, and molecular markers) with Ki67 LI status and p53 immunoreactivity in HR-positive tumors (n=150)

The tumors with high Ki67 LIs showed significantly higher frequencies of high nuclear grade, HER2 positivity, basal phenotype marker positivity, and P-cadherin (P = 0.013, P = 0.0010, and P = 0.0015, and P = 0.013, respectively). The tumors with positive p53 staining showed significantly higher frequencies of large tumor size and high nuclear grade (P = 0.0013 and P = 0.035, respectively).

Correlation between clinicopathological factors and combined Ki67-p53 status in HR-positive tumors (n=150)

There were 88 (59%), 11 (7%), 40 (27%), and 11 (7%) tumors with the Ki67 LI-low and p53-negative, Ki67 LI-low and p53-positive, Ki67 LI-high and p53-negative, and Ki67 LI-high and p53-positive phenotypes, respectively. The tumors with the "favorable" Ki67 LI-low and p53-negative phenotype (n = 88) showed lower frequencies of high nuclear grade, HER2 positivity, basal phenotype marker positivity, and high P-cadherin expression (P = 0.0008, P = 0.0006, P = 0.0016 and P = 0.0019, respectively; Table 1) than did those with "unfavorable" Ki67 LI-low and p53-positive (n = 11), Ki67 LI-high and

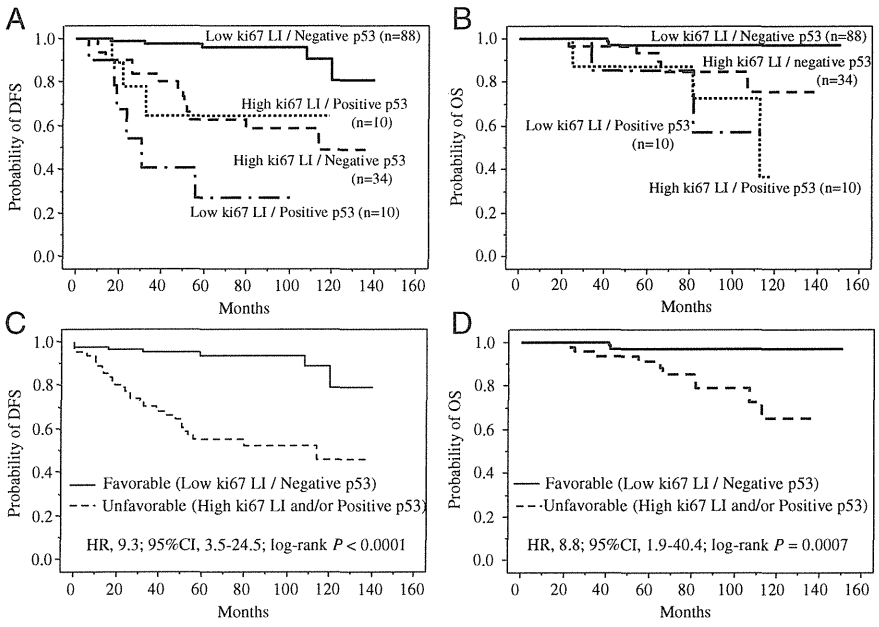

Figure 3 Prognostic impact of combined Ki67-p53 status in patients with hormone receptor-positive and HER2-negative breast cancer (n=142). A, Disease-free survival curves for 88 patients with Ki67 LI-low and p53-negative tumors, 34 patients with Ki67 LI-high and p53-negative tumors, 10 patients with Ki67 LI-low and p53-positive tumors, and 10 patients with Ki67 LI-high and p53-positive tumors. Patients with Ki67 LI-low and p53-negative tumors had significantly longer disease-free survival than those with Ki67 LI-low and p53-positive tumors, those with Ki67 LI-high and p53-negative tumors, or those with Ki67 LI-high and p53-positive tumors ($P < 0.0001$, $P < 0.0001$, and $P = 0.0005$, respectively). **B,** Overall survival curves for 88 patients with Ki67 LI-low and p53-negative tumors, 34 patients with Ki67 LI-high and p53-negative tumors, 10 patients with Ki67 LI-low and p53-positive tumors, and 10 patients with Ki67 LI-high and p53-positive tumors. Patients with Ki67 LI-low and p53-negative tumors had significantly longer disease-free survival than those with Ki67 LI-low and p53-positive tumors, those with Ki67 LI-high and p53-negative tumors, or those with Ki67 LI-high and p53-positive tumors ($P = 0.0010$, $P = 0.011$, and $P < 0.0001$, respectively). **C,** Disease-free survival curves for patients with favorable-phenotype tumors (88 patients with Ki67 LI-low and p53-negative tumors) and unfavorable-phenotype tumors (54 patients with Ki67 LI-high and/or p53-positive tumors). The disease-free survival time was significantly longer in the favorable-phenotype group than in the unfavorable-phenotype group (HR, 9.3; 95% CI, 3.5–24.5; $P < 0.0001$). **D,** Overall survival curves for patients with favorable-phenotype tumors (88 patients with Ki67 LI-low and p53-negative tumors) and those with unfavorable-phenotype tumors (54 patients with Ki67 LI-high and/or p53-positive tumors). The disease-free survival was significantly longer in the favorable-phenotype group than in the unfavorable-phenotype group (HR, 8.8; 95% CI, 1.9–40.4; $P = 0.0007$).

p53-negative (n = 40), and Ki67 LI-high and p53-positive (n = 11) phenotypes (n = 62 total). Interestingly, all HER2-positive tumors were shown to be unfavorable phenotype tumors. This study found no correlations between the combined Ki67-p53 status and the clinical factors tumor size, nodal status.

Prognostic implications of combined Ki67-p53 status in HR-positive and HER2-negative tumors (n=142)

The patients with HER2-positive tumors could be received anti-HER2 treatments which have tremendous effect in both adjuvant and metastatic setting [30,31]. So, we next conducted the further analyses using the cases with HR-positive and HER2-negative tumors in order to evaluate more definitely the clinical implication of Ki67 and p53.

Both the disease-free survival (DFS) and overall survival (OS) curves differed significantly between the patients with Ki67 LI-low tumors and those with Ki67-LI-high tumors (DFS: HR, 3.6; 95% CI, 1.7–7.9; log-rank $P = 0.0004$; Figure 2A, and OS: HR, 4.4; 95% CI, 1.3–14.6; log-rank $P = 0.0082$; Figure 2B). Both curves also differed significantly between the patients with p53-negative tumors and those with p53-positive tumors (DFS: HR, 5.2; 95% CI, 2.3–11.9; log-rank $P < 0.0001$; Figure 2C, and OS: HR, 5.7; 95% CI, 1.8–18.0; log-rank $P = 0.0008$; Figure 2D). Furthermore, a multivariate analysis using Cox's proportional hazard model and including immunostaining for the markers Ki67, p53, HER2, FOXA1, GATA3, the basal phenotype markers, and P-cadherin selected Ki67 and p53 as significant prognostic factors for DFS ($P = 0.0073$ and $P = 0.0025$, respectively; Table 2) and p53 for OS ($P = 0.030$, Table 2).

Figures 3A and 3B show the DFS and OS curves for the 4 combined Ki67-p53 status groups. Patients with Ki67 LI-low and p53-negative tumors survived longer than those in the other 3 groups (the patients with Ki67 LI-low and p53-positive tumors, Ki67-high and p53-

Table 3 Univariate and multivariate analyses of disease-free survival in patients with hormone receptor-positive/HER2-negative primary breast cancer

			Univariate			Multivariate		
		Total (n=142)	Hazard ratio	(95% CI)	*P*-value	Hazard ratio	(95% CI)	*P*-value
Ki67-p53	Low Ki67 LI and Negative p53	88	1			1		
	High Ki67 LI and/or Positive p53	54	9.3	3.5-24.5	< 0.0001	11.6	4.2-32.3	< 0.0001
FOXA1	Low	20	1					
	High	119	1.4	0.51-3.6	0.54			
GATA3	Negative	30	1					
	Positive	112	1.5	0.65-3.5	0.34			
Basal phenotype marker	Negative	9	1					
(CK5/6, CK14, EGFR)	Positive	133	1.0	0.24-4.3	0.98			
P-cadherin	Low	90	1					
	High	52	1.3	0.59-2.8	0.52			
Tumor size	<5.0 cm	17	1			1		
	≧5.0 cm	122	4.9	2.1-11.4	0.0003	5.7	2.3-14.1	0.0002
Lymph-node metastasis	(–)	80	1					
	(+)	59	3.8	1.6-8.8	0.0020			
Nuclear grade	1, 2	111	1					
	3	31	4.2	1.9-9.0	0.0002			
Chemotherapy	No	93	1			1		
	Yes	49	2.6	1.2-5.7	0.014	3.5	1.5-7.9	0.0028

Abbreviation: *95%CI* 95% confidence interval, *LI* labeling index.

negative tumors, and Ki67-high and p53-positive tumors) in both the DFS ($P < 0.0001$, $P < 0.0001$, and $P = 0.0005$, respectively; Figure 3A) and OS ($P = 0.0010$, $P = 0.011$, and $P < 0.0001$, respectively; Figure 3B) analyses. The DFS and OS curves therefore differed significantly between the patients with favorable-phenotype tumors and those with unfavorable-phenotype tumors (DFS: HR, 9.3; 95% CI, 3.5–24.5; log-rank $P < 0.0001$; Figure 3C, and OS: HR, 8.8; 95% CI, 1.9–40.4; log-rank $P = 0.0007$; Figure 3D). This difference was much more distinct than that between the low- and high-Ki67-LI curves (DFS: HR, 3.6; 95% CI, 1.7–7.9, and OS: HR, 4.4; 95% CI, 1.3–14.6). The 5- and 10-year OS rates were 97% and 97%, respectively, for the patients with favorable-phenotype tumors but only 91% and 65%, respectively, for the patients with unfavorable-phenotype tumors.

We next conducted subgroup analysis of HR-positive and HER2-negative breast cancer patients who had and had not received pre- or post-operative chemotherapy. Among the 49 patients who had received chemotherapy, those with favorable-phenotype tumors had significantly longer DFS than those with unfavorable-phenotype tumors ($P < 0.0001$). And then, among the 93 patients who had not received chemotherapy, those with favorable-phenotype tumors had significantly or almost significantly longer DFS than those with unfavorable-phenotype tumors ($P = 0.0002$).

Finally, we performed multivariate analyses of survival using Cox's model of the proportional hazards regression including immunohistochemical parameters (combined Ki67-p53 status, FOXA1, GATA3, basal phenotype marker and P-cadherin) and the established clinicopathological factors (tumor size, lymph-node metastasis, nuclear grade and chemotherapy).

In those analyses, combined Ki67-p53 status, tumor size and chemotherapy was a significant prognostic indicators of DFS ($P < 0.0001$, $P = 0.0001$ and $P = 0.0028$, respectively; Table 3) and combined Ki67-p53 status was an only significant prognostic indicator of OS ($P = 0.0081$, Table 4).

Discussion

The international expert panel at the 2009 St. Gallen Consensus meeting referred to the importance of proliferation markers in deciding whether to include adjuvant chemotherapy in the treatment of patients with HR-positive HER-2-negative breast cancers. Several large-scale studies have evaluated the clinical significance of the Ki67 LI among patients with HR-positive breast cancer [7,18,21]. In this study, we showed that an IHC panel comprising p53 status and Ki67 LI is more accurate than Ki67 LI alone at predicting the prognosis for patients with HR-positive and HER2-negative breast cancer.

The results of our IHC panel divided the patients with HR-positive and HER2-negative invasive breast cancers into