Table 4 List of genes included in the 95-gene classifier

No.	Probe.set.ID	Gene.symbol	UniGene.ID	zScore[a]	*P* value	Direction
1	219306_at	KIF15	Hs.646856	−5.36	<0.0001	Up
2	218585_s_at	DTL	Hs.656473	−5.14	<0.0001	Up
3	221677_s_at	DONSON	Hs.436341	−5.06	0.0013	Up
4	201088_at	KPNA2	Hs.594238	−4.97	0.0010	Up
5	209034_at	PNRC1	Hs.75969	4.97	0.0008	Down
6	202610_s_at	MED14	Hs.407604	−4.90	0.0007	Up
7	218906_x_at	KLC2	Hs.280792	−4.83	0.0023	Up
8	212723_at	JMJD6	Hs.514505	−4.70	0.0050	Up
9	222231_s_at	LRRC59	Hs.370927	−4.63	0.0062	Up
10	208838_at	CAND1	Hs.546407	−4.63	0.0056	Up
11	218039_at	NUSAP1	Hs.615092	−4.60	0.0055	Up
12	209472_at	CCBL2	Hs.481898	4.60	0.0050	Down
13	212898_at	KIAA0406	Hs.655481	−4.60	0.0052	Up
14	202620_s_at	PLOD2	Hs.477866	−4.58	0.0049	Up
15	201059_at	CTTN	Hs.596164	−4.58	0.0048	Up
16	201841_s_at	HSPB1	Hs.520973	−4.56	0.0048	Up
17	203755_at	BUB1B	Hs.631699	−4.54	0.0047	Up
18	211750_x_at	TUBA1C	Hs.719091	−4.53	0.0044	Up
19	38158_at	ESPL1	Hs.153479	−4.52	0.0042	Up
20	204709_s_at	KIF23	Hs.270845	−4.51	0.0042	Up
21	201589_at	SMC1A	Hs.211602	−4.47	0.0040	Up
22	218460_at	HEATR2	Hs.535896	−4.44	0.0044	Up
23	207430_s_at	MSMB	Hs.255462	−4.43	0.0045	Up
24	212139_at	GCN1L1	Hs.298716	−4.42	0.0045	Up
25	211596_s_at	LRIG1	Hs.518055	4.40	0.0045	Down
26	212160_at	XPOT	Hs.85951	−4.40	0.0045	Up
27	219238_at	PIGV	Hs.259605	4.40	0.0043	Down
28	203432_at	TMPO	Hs.11355	−4.35	0.0047	Up
29	201377_at	UBAP2L	Hs.490551	−4.34	0.0052	Up
30	218875_s_at	FBXO5	Hs.520506	−4.33	0.0052	Up
31	221922_at	GPSM2	Hs.584901	−4.32	0.0050	Up
32	218727_at	SLC38A7	Hs.10499	−4.27	0.0060	Up
33	207469_s_at	PIR	Hs.495728	−4.27	0.0058	Up
34	218483_s_at	C11orf60	Hs.533738	4.26	0.0056	Down
35	204641_at	NEK2	Hs.153704	−4.26	0.0058	Up
36	219502_at	NEIL3	Hs.405467	−4.25	0.0058	Up
37	209054_s_at	WHSC1	Hs.113876	−4.24	0.0061	Up
38	220318_at	EPN3	Hs.670090	−4.24	0.0061	Up
39	210297_s_at	MSMB	Hs.255462	−4.23	0.0061	Up
40	209186_at	ATP2A2	Hs.506759	−4.23	0.0059	Up
41	219787_s_at	ECT2	Hs.518299	−4.18	0.0077	Up
42	45633_at	GINS3	Hs.47125	−4.18	0.0075	Up
43	200848_at	AHCYL1	Hs.705418	4.18	0.0075	Down
44	200822_x_at	TPI1	Hs.524219	−4.18	0.0075	Up
45	211072_x_at	TUBA1B	Hs.719075	−4.16	0.0077	Up
46	200811_at	CIRBP	Hs.634522	4.16	0.0076	Down
47	202864_s_at	SP100	Hs.369056	4.14	0.0083	Down

Table 4 continued

No.	Probe.set.ID	Gene.symbol	UniGene.ID	zScore[a]	*P* value	Direction
48	202154_x_at	TUBB3	Hs.511743	−4.13	0.0083	Up
49	213152_s_at	SFRS2B	Hs.476680	4.11	0.0093	Down
50	209368_at	EPHX2	Hs.212088	4.09	0.0101	Down
51	211058_x_at	TUBA1B	Hs.719075	−4.09	0.0100	Up
52	209251_x_at	TUBA1C	Hs.719091	−4.08	0.0100	Up
53	213646_x_at	TUBA1B	Hs.719075	−4.08	0.0098	Up
54	204540_at	EEF1A2	Hs.433839	−4.07	0.0101	Up
55	202026_at	SDHD	Hs.719164	4.06	0.0101	Down
56	201090_x_at	TUBA1B	Hs.719075	−4.06	0.0101	Up
57	213119_at	SLC36A1	Hs.269004	−4.05	0.0101	Up
58	217840_at	DDX41	Hs.484288	−4.04	0.0102	Up
59	206559_x_at	EEF1A1	–	4.03	0.0106	Down
60	202066_at	PPFIA1	Hs.530749	−4.03	0.0105	Up
61	203108_at	GPRC5A	Hs.631733	−4.02	0.0108	Up
62	218697_at	NCKIPSD	Hs.655006	−4.02	0.0110	Up
63	222039_at	KIF18B	Hs.135094	−3.99	0.0126	Up
64	202069_s_at	IDH3A	Hs.591110	−3.99	0.0124	Up
65	203362_s_at	MAD2L1	Hs.591697	−3.98	0.0124	Up
66	202666_s_at	ACTL6A	Hs.435326	−3.97	0.0126	Up
67	204892_x_at	EEF1A1	Hs.520703	3.96	0.0134	Down
68	205682_x_at	APOM	Hs.534468	3.95	0.0137	Down
69	209714_s_at	CDKN3	Hs.84113	−3.95	0.0136	Up
70	218381_s_at	U2AF2	Hs.528007	−3.94	0.0136	Up
71	201947_s_at	CCT2	Hs.189772	−3.94	0.0135	Up
72	212722_s_at	JMJD6	Hs.514505	−3.94	0.0137	Up
73	204825_at	MELK	Hs.184339	−3.93	0.0136	Up
74	203184_at	FBN2	Hs.519294	−3.93	0.0139	Up
75	201266_at	TXNRD1	Hs.708065	−3.93	0.0137	Up
76	202969_at	DYRK2	Hs.173135	−3.92	0.0140	Up
77	204817_at	ESPL1	Hs.153479	−3.90	0.0150	Up
78	209523_at	TAF2	Hs.122752	−3.90	0.0148	Up
79	218491_s_at	THYN1	Hs.13645	3.90	0.0146	Down
80	217363_x_at	–	–	3.89	0.0149	Down
81	218009_s_at	PRC1	Hs.567385	−3.89	0.0148	Up
82	204026_s_at	ZWINT	Hs.591363	−3.88	0.0153	Up
83	218355_at	KIF4A	Hs.648326	−3.88	0.0151	Up
84	202153_s_at	NUP62	Hs.574492	−3.88	0.0153	Up
85	213011_s_at	TPI1	Hs.524219	−3.88	0.0151	Up
86	217966_s_at	FAM129A	Hs.518662	3.88	0.0149	Down
87	214782_at	CTTN	Hs.596164	−3.87	0.0151	Up
88	217967_s_at	FAM129A	Hs.518662	3.87	0.0150	Down
89	204649_at	TROAP	Hs.524399	−3.86	0.0150	Up
90	35671_at	GTF3C1	Hs.371718	−3.86	0.0150	Up
91	213502_x_at	LOC91316	Hs.148656	3.86	0.0149	Down
92	221285_at	ST8SIA2	Hs.302341	3.85	0.0154	Down
93	221519_at	FBXW4	Hs.500822	3.84	0.0158	Down

Table 4 continued

No.	Probe.set.ID	Gene.symbol	UniGene.ID	zScore[a]	*P* value	Direction
94	202551_s_at	CRIM1	Hs.699247	3.84	0.0158	Down
95	217138_x_at	IGL@	Hs.449585	3.83	0.0168	Down

[a] The value in which each effect size of training sets were combined

Up up-regulated in recurrent cases, *Down* down-regulated in recurrent cases

that as many as 58% of the patients classified into the low-risk group with this classifier could be safely spared adjuvant chemotherapy. Although our current study is based on a relatively large number of patients, i.e., 549 patients in the training set and 105 in the validation set, it still seems to be required for the 95-gene classifier to be validated in other cohorts including a larger number of patients in future studies using desirably both Japanese patients and Caucasian patients before it can be used routinely in clinical practice.

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Expression of Estrogen Receptor Beta and Phosphorylation of Estrogen Receptor Alpha Serine 167 Correlate with Progression-Free Survival in Patients with Metastatic Breast Cancer Treated with Aromatase Inhibitors

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Key Words
Aromatase inhibitor · Breast cancer · Estrogen receptor-α · Estrogen receptor-β · Prognostic factor

Abstract
Aromatase inhibitor (AI) is widely used as an endocrine treatment in postmenopausal patients with hormone receptor-positive breast cancer. To identify useful prognostic factors for patients with metastatic breast cancer treated with AI therapy, we investigated the association between several hormone receptor-related factors and prognosis. The expressions of estrogen receptor-α (ERα), ERβ, progesterone receptor, the phosphorylation of ERα serine 118 (Ser118) and ERα Ser167 were examined using immunohistochemical techniques for the primary tumors of 41 patients with metastatic breast cancer who received first-line AI therapy after relapse. To assess the associations of protein expression and phosphorylation levels with progression-free survival (PFS), the levels of each factor were categorized into low and high values at optimal cutoff points. In univariate analysis, high ERα expression and high ERα Ser167 phosphorylation correlated with longer PFS (p = 0.016 and 0.013, respectively). In multivariate analysis, low ERβ expression and high ERα Ser167 phosphorylation correlated with longer PFS (p = 0.031 and 0.004, respectively). Patients with both low ERβ expression and high ERα Ser167 phosphorylation had longer PFS than the others (p = 0.0107). These data suggest that the expression of ERβ and phosphorylation of ERα Ser167 may be useful prognostic factors in patients with metastatic breast cancer who received first-line AI therapy.

Introduction

Aromatase inhibitors (AIs) have been widely used as endocrine treatment in postmenopausal patients with hormone receptor-positive breast cancer. AIs have been effective as adjuvant hormonal therapy and have shown longer disease-free survival than tamoxifen in large clinical trials [1–3].

Estrogen receptor (ER) and progesterone receptor (PgR) are good predictive factors for the efficacy of endocrine therapy. There are 2 known ER isoforms, ERα and ERβ, which are encoded by 2 different genes and differ

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in their ligand specificities and physiological functions [4–6]. The expression level of ERα is well correlated with the response to endocrine therapy and prognosis. On the other hand, the role of ERβ has not been elucidated.

ERα is phosphorylated on multiple amino acid residues [7]. Phosphorylations of ERα (pERα) serine 118 (Ser118) and pERα Ser167 seem to be controlled by different mechanisms [8–13]. It is reported that pERα Ser167 is a predictive biomarker of the response to endocrine therapy [14, 15]; however, the biomarkers for the efficacy of AIs have not been investigated sufficiently.

In this study, we investigated the association between several hormone receptor-related factors and prognosis to identify useful prognostic factors for patients with metastatic breast cancer treated with AI therapy.

Patients and Methods

Patients

Primary breast tumor specimens from 41 postmenopausal patients with metastatic breast cancer who were treated with AI therapy at Osaka Medical Center for Cancer and Cardiovascular Diseases between 2001 and 2007 were included in this study, excluding 31 patients in which the efficacy judgment of first-line AI therapy was unclear (table 1). All patients presented a hormone receptor-positive (ER positive and/or PgR positive) primary tumor at diagnosis. Four patients (10%) received no adjuvant therapy. Of the remaining 37 patients, 15 (37%) received adjuvant endocrine therapy, 3 (7%) received adjuvant chemotherapy and 19 (46%) received combined endocrine and chemotherapy. When the patients relapsed and were diagnosed with metastatic breast cancer, they started endocrine therapy (table 1). Thirty-seven patients (90%) received anastrozole, 3 (7%) received exemestane and 1 (2%) received fadrozole hydrochloride. The median age at start of AI therapy was 57 years (range 42–79).

Patients were assessed monthly for clinical response, which was defined according to the World Health Organization criteria as complete response, partial response, no change or progressive disease. Clinical benefit and overall response were defined as complete response + partial response + no change, and complete response + partial response [16].

All patients provided informed consent, and this study was approved by the Osaka Medical Center for Cancer and Cardiovascular Diseases Institutional Review Board.

Immunohistochemical Analysis

One 4-μm section of each submitted paraffin block was stained with hematoxylin and eosin to confirm the location of the tumor cells. The slides of other serial sections were stained with antibodies against each protein (ERα, ERβ, PgR, as well as pERα Ser118 and pERα Ser167). Primary antibodies included monoclonal mouse antihuman ERα antibody (Clone 6F11; Ventana, Tucson, Ariz., USA) for ERα; monoclonal mouse antihuman PgR antibody (Clone 16; Ventana) for PgR; polyclonal rabbit antiphospho-ERα (Ser118) antibody (sc-12915; Santa Cruz Biotechnology, Santa Cruz, Calif., USA) at 1:100 dilution for pERα Ser118; polyclonal rabbit antiphospho-ERα (Ser167) antibody (No. 11073; Signalway Antibody, Pearland, Tex., USA) at 1:100 dilution for pERα Ser167; monoclonal mouse anti-ERβ antibody (clone PPG5/10, M7292; Dako) at 1:10 dilution for ERβ. The Ventana iView DAB Universal kit (Ventana) and the autostainer (NX-IHC/20; Ventana) were used as a detection system for ERα, PgR, pERα Ser118 and pERα Ser167. The Dako Mouse EnVision+, HRP/DAB+ Labeled Polymer detection kit (K4007; Dako) and the autostainer (Autostainer; Dako) were used as a detection system for ERβ.

Two pathology experts evaluated the staining intensity of the stained cells among cancer cells using the grade of 4 intensity

Table 1. Characteristics of patients

Factor	Patients
Total	41 (100)
Age at surgery	
<50 years	15 (37)
≥50 years	26 (63)
Tumor size	
≤2 cm	14 (34)
>2–5 cm	25 (61)
>5 cm	2 (5)
Histological grade	
1	19 (46)
2	11 (27)
3	11 (27)
Lymph node status	
Negative	18 (44)
Positive	23 (56)
ER/PgR	
Positive/negative	2 (5)
Negative/positive	1 (2)
Positive/positive	38 (93)
HER2	
Negative	38 (93)
Positive	3 (7)
Adjuvant therapy	
None	4 (10)
Endocrine therapy	15 (37)
Tamoxifen	10
Tamoxifen, anastrozole	1
Tamoxifen, exemestane	1
Anastrozole	1
Toremifen	1
Tamoxifen, zoladex	1
Chemotherapy	3 (7)
Combined	19 (46)
Tamoxifen	17
Anastrozole	1
Tamoxifen, exemestane	1
First-line endocrine therapy	
Anastrozole	37 (90)
Exemestane	3 (7)
Fadrozole hydrochloride	1 (2)

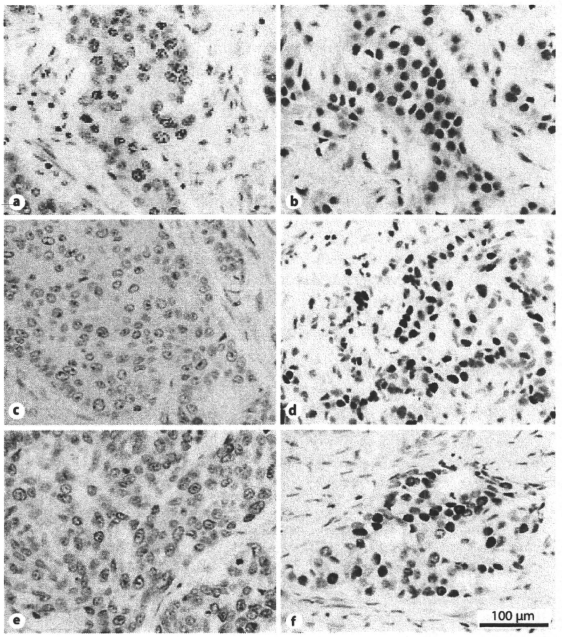

Fig. 1. Representative immunohistochemical staining of ERβ negative (**a**), ERβ positive (**b**), pERα Ser118 negative (**c**), pERα Ser118 positive (**d**), pERα Ser167 negative (**e**) and pERα Ser167 positive (**f**) in human breast cancer sections. ×200.

scores as follows: no staining, score 0; weak staining, score 1+; moderate staining, score 2+; intense staining, score 3+. They also evaluated staining intensity by the positive cell occupancy rate (% positive, by 5%) inside tumor tissues. Each staining intensity score multiplied by the positive occupancy rate and the sum of the products was used as the H score:

$$H\ score = \Sigma(\%\ positive \times intensity\ score)$$

The HER2 protein expression status was assessed using the immunohistochemistry (IHC) assay method, the Herceptest (Dako, Glostrup, Denmark) and the HER2 Pathvysion fluorescence in situ hybridization (FISH) assay (Abbott Laboratories, Abbott Park, Ill., USA), according to the manufacturer's instructions, as summarized elsewhere [17, 18]. HER2 immunostaining was scored as 0, 1+, 2+ and 3+ by 2 pathology experts, with 0 and 1+ considered negative and 3+ considered positive. The gene amplification of HER2 of samples with IHC 2+ was assessed using the FISH assay. IHC 2+ and FISH-negative samples were defined as HER2 negative, and IHC 2+ and FISH-positive samples as HER2 positive.

Statistical Analyses

We examined the progression-free survival (PFS) and the objective tumor response to AI therapy after relapse. PFS was calculated as the period from the start of first-line AI therapy until disease progression.

To assess the association of the protein expression and phosphorylation levels (H score) with PFS, the expression and phosphorylation levels of each protein were categorized into low and high values at optimal cutoff points. The maximal χ^2 method [19–21] was used to determine which protein expression (optimal cutoff point) best segregated patients into poor- and good-outcome subgroups (in terms of the likelihood of response and survival). Cox's proportional hazards model was used for univariate and multivariate analyses of PFS. PFS was estimated using the Kaplan-Meier method, and differences between survival curves were assessed with the log-rank test.

To assess the associations of protein expression and phosphorylation levels (H score) with the tumor response, the levels of each protein were categorized into low and high values at optimal cutoff points. The optimal cutoff point for protein expression and

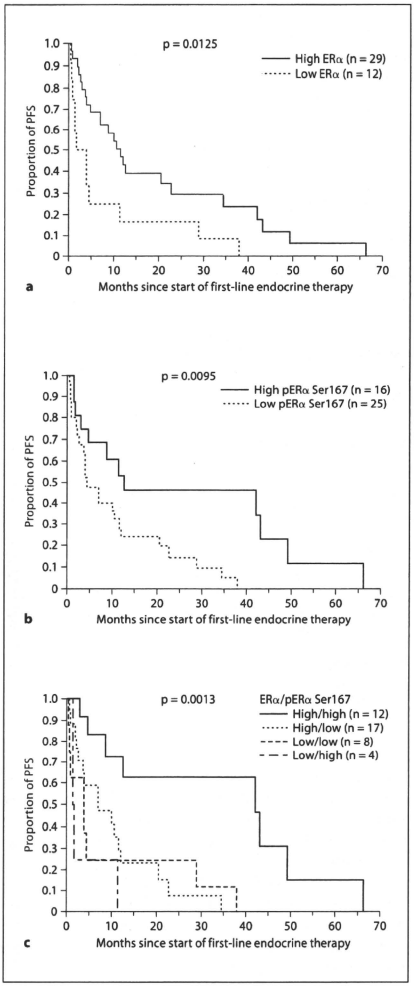

Fig. 2. Kaplan-Meier plot of PFS according to ERα expression levels (**a**), pERα Ser167 levels (**b**) and a combination of both factors (**c**). p values were calculated by the log-rank test.

phosphorylation levels was determined by the receiver operating characteristic curve. Fisher's exact test was used to analyze the association between protein expression and phosphorylation levels and tumor response.

All reported p values are 2-sided, and the level of statistical significance was set at $p < 0.05$. Variables for multivariate analysis were selected by the stepwise method, using a significance level of <0.05 for entering or remaining in the model. All analyses were performed using statistical software, JMP 7.0.1, and the SAS statistical package, version 9.1.3 (SAS Institute Inc., Cary, N.C., USA).

Results

Immunohistochemical Staining of Breast Tumors

Cancer cell nuclei of breast tumors were positively stained with specific antibodies for ERα, PgR, ERβ (fig. 1b), pERα Ser118 (fig. 1d) and pERα Ser167 (fig. 1f).

Correlation between H Scores for Hormone Receptors and PFS in Patients Treated with First-Line AI Therapy

We analyzed whether the expression and phosphorylation levels of hormone receptors in the primary breast tumors affected the PFS. In univariate analysis, a high expression of ERα (H score >70) and high pERα Ser167 (H score >75) significantly correlated with longer PFS (p = 0.016 and 0.013, respectively) (table 2). In multivariate analysis, a low expression of ERβ (H score ≤180) and high pERα Ser167 (H score >75) significantly correlated with longer PFS (p = 0.031 and 0.004, respectively) (table 2). Patients with both a high expression of ERα Ser167 (H score >75) and a high expression of ERα (H score >70) or a low expression of ERβ (H score ≤180) had longer PFS than the others (p = 0.0013 and 0.0107) (fig. 2c, 3). On the other hand, pERα Ser118 and expression of PgR did not affect the PFS (table 2).

Correlation between H Scores for Hormone Receptors and Response to First-Line AI Therapy in Breast Cancer

We analyzed whether the expression and phosphorylation levels of hormone receptors in primary breast tumors affected the tumor response. Patients with a high expression of ERα (H score ≥85) significantly had a higher response rate of clinical benefit than those with a low expression (high 72.4% vs. low 25.0%; p = 0.013) (table 3). Patients with a high expression of PgR (H score ≥125) significantly had a higher rate of overall response than those with a low expression (high 45.5% vs. low 10.0%; p = 0.021) (table 3). Although other factors did not sig-

Table 2. Cox regression univariate and multivariate analysis of PFS

Factor	Cutoff point	Patients	Median months	Univariate analysis		Multivariate analysis	
				hazard ratio	p	hazard ratio	p
ERα	≤70	12	4.0	0.41 (0.20–0.85)	0.016*		
	>70	29	11.6				
PgR	≤130	33	7.2	0.61 (0.26–1.43)	0.254		
	>130	8	22.8				
ERβ	≤180	27	11.6	1.69 (0.80–3.58)	0.168	2.42 (1.08–5.40)	0.031*
	>180	14	4.9				
pERα Ser118	≤125	32	7.0	0.57 (0.23–1.39)	0.216		
	>125	9	11.4				
pERα Ser167	≤75	25	4.6	0.36 (0.16–0.80)	0.013*	0.29 (0.12–0.67)	0.004**
	>75	16	12.8				

Figures in parentheses are 95% CIs. The optimum cutoff point for the protein expression level was determined by the maximal χ^2 method. The stepwise method was used to select factors for multivariate analysis. * $p < 0.05$; ** $p < 0.01$.

Table 3. Protein expression levels and tumor response in patients with breast cancer according to first-line endocrine therapy

Factor	Patients	Clinical benefit				Overall response			
		cutoff point (H score)	response rate in low group, %	response rate in high group, %	p[1]	cutoff point (H score)	response rate in low group, %	response rate in high group, %	p[1]
ERα	41	85	25.0 (3/12)	72.4 (21/29)	0.013*	135	9.5 (2/21)	30.0 (6/20)	0.130
PgR	41	45	46.7 (7/15)	65.4 (17/26)	0.328	125	10.0 (3/30)	45.5 (5/11)	0.021*
ERβ	41	165	69.6 (16/23)	44.4 (8/18)	0.125	165	26.1 (6/23)	11.1 (2/18)	0.429
pERα Ser118	41	135	50.0 (16/32)	88.9 (8/9)	0.056	65	11.8 (2/17)	25.0 (6/24)	0.433
pERα Ser167	41	80	48.0 (12/25)	75.0 (12/16)	0.113	80	12.0 (3/25)	31.3 (5/16)	0.225

The optimum cutoff point for the protein expression level was determined by the receiver operating characteristic curve analysis. * $p < 0.05$. [1] Fisher's exact test.

nificantly affect the response rate, patients with high pERα Ser167 or pERα Ser118, or a low expression of ERβ had a high response rate (table 3). The overall response to first-line AI therapy correlated with longer PFS ($p = 0.0222$).

Discussion

In this report, we have demonstrated that patients in whom tumors showed high pERα Ser167 and a low expression of ERβ had better PFS. pERα Ser167 has been reported to be predictive of the response to tamoxifen therapy [14, 15]. It has been suggested that pERα Ser167 may represent the strength of the ER dependency of breast tumor because it is mainly caused by ER signaling. Our results of AI therapy are consistent with their results; therefore, pERα Ser167 may be a common predictive factor of endocrine therapy including AI or tamoxifen.

On the other hand, the role of ERβ, unlike ERα, in the responsiveness to endocrine therapy and prognosis has not been fully elucidated. Several investigators have reported that increased expression of ERβ predicts a more favorable response to tamoxifen therapy [22–31] but other investigators have reported that ERβ expression predicts a poor outcome in patients treated with tamoxifen [32, 33]. In regard to AI therapy, one study has reported no correlation between the expression of ERβ and the re-

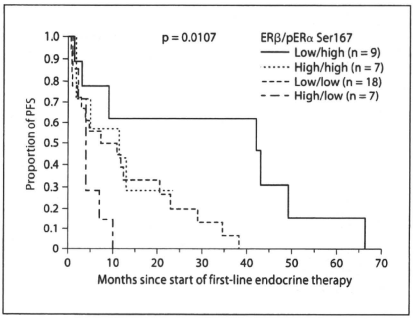

Fig. 3. Kaplan-Meier plot of PFS according to the combination of ERβ expression levels and pERα Ser167 levels. The p value was calculated by the log-rank test.

sponse to exemestane as primary endocrine therapy in ERα-positive breast cancer [34]. In this study, we demonstrated that low ERβ expression is associated with a better response to AI therapy (table 2; fig. 3).

ERβ is reported to exhibit an inhibitory action on ERα-mediated gene expression and in many instances to oppose the actions of ERα [35]. Moreover, it has been reported that AI and tamoxifen had a different effect on the expression of ERα and ERβ in breast cancer cells [36]. Tamoxifen increased ERα expression and left ERβ unchanged. In contrast, AI upregulated ERβ; that is, tamoxifen increased the ERα/ERβ ratio, and AI decreased the ratio. Therefore, AI may have an activity of decreasing the ERα/ERβ ratio, and patients with low ERβ expression levels may have greater sensitivity to the change of the ratio. In this study, the combination of ERβ and pERα Ser167 was a more powerful predictive factor of the outcome of AI therapy than ERβ only. These results might suggest that the ERα/ERβ balance is a good predictive biomarker of AI therapy in patients with breast cancer. Further investigation of the molecular basis is required in the future.

Although ERα Ser167 and ERβ did not significantly affect the response rate, patients with high pERα Ser167 or low expression of ERβ had a high response rate (table 3). This study was a retrospective study; therefore, a further large-scale prospective study is required to verify the effect of protein expression of ERβ and pERα Ser167 on the efficacy of AI therapy.

In conclusion, the present data suggest that ERβ and pERα Ser167 may be useful prognostic factors in patients with metastatic breast cancer who received first-line AI therapy.

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Phase II Trial in Japan of Sequential Administration of Weekly Paclitaxel Followed by FEC as Neoadjuvant Chemotherapy for Locally Advanced Breast Cancer [KBCSG0206 Trial: Kinki Breast Cancer Study Group (KBCSG)]

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Key Words
Paclitaxel · Epirubicin · 5-Fluorouracil · Cyclophosphamide · Breast cancer · Neoadjuvant chemotherapy

Abstract
Objective: We conducted a phase II trial in Japan to evaluate the efficacy and tolerability of weekly paclitaxel followed by fluorouracil, epirubicin, and cyclophosphamide (FEC) as neoadjuvant chemotherapy (NAC) for locally advanced breast cancer (LABC). ***Methods:*** Patients with clinical stage IIIA–IIIB breast cancer received NAC consisting of 12 once-a-week cycles of paclitaxel followed by 4 once-every-third-week cycles of FEC. ***Results:*** Fifty patients with LABC were enrolled, 47 of whom were administered paclitaxel followed by FEC as NAC. The clinical response rate for all chemotherapies was 85.1%, and the pathological complete response rate was 27.7%. Regarding toxicity, grade 3–4 neutropenia was observed in 10% of patients. No serious toxicities requiring the discontinuation of treatment were encountered. The rate of breast conservation surgery was 31.9%, median survival had not been reached at the time of conclusion of this study, and the 3-year survival rate was 85.1%. Median disease-free survival was 40.2 months, and the 3-year disease-free survival rate was 62.1%. ***Conclusions:*** Weekly paclitaxel followed by FEC demonstrated efficacy and tolerable toxicity in a neoadjuvant setting for LABC.

Introduction

Breast cancer is the most common form of cancer in Japanese women and its incidence is increasing, with about 40,000 new cases diagnosed each year [1]. The introduction of drugs such as anthracyclines and taxanes

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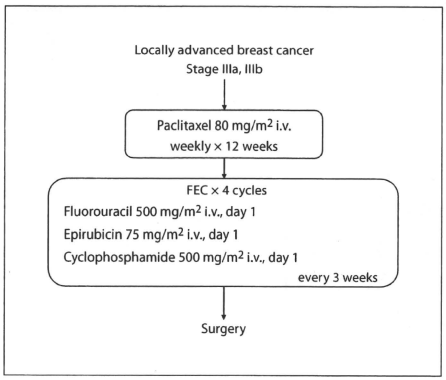

Fig. 1. Treatment schema.

has resulted in progressive improvements in outcomes, and prognosis appears to be particularly influenced by perioperative therapy [2]. Neoadjuvant chemotherapy (NAC) is a beneficial perioperative treatment modality that improves the real-time tumor response as well as the breast conservation rate. As such, NAC may provide a greater chance for breast conservation surgery (BCS) in stage III breast cancer, which usually requires mastectomy.

The type of response to chemotherapy also appears to be important, with many researchers reporting a better prognosis in patients achieving a pathological complete response (pCR) than in those with a pathological non-complete response [3, 4]. These reports have encouraged investigation into more effective regimens that may improve the pCR rate [5, 6].

Conventional NAC consists of the sequential administration of an anthracycline followed by a taxane. Of the few reports investigating the reverse, namely a taxane followed by an anthracycline, the 2005 study of Green et al. [7] of NAC with paclitaxel followed by fluorouracil, doxorubicin, and cyclophosphamide (FAC) showed that the pCR rate with weekly administration of paclitaxel was superior to that of the conventional once-every-3-weeks regimen, and that tolerability in the node-negative group which received weekly paclitaxel 80 mg/m^2 was better than in the node-positive group which received a higher (175 or 150 mg/m^2) weekly dose. Further, overall weekly dosing was better tolerated than 3-weekly dosing. Green et al. [7] also provided an elegant explanation supporting the superiority of taxane-leading regimens. The 2005 study of Gianni et al. [8] of NAC with doxorubicin and paclitaxel followed by cyclophosphamide, methotrexate, and fluorouracil similarly reported favorable feasibility and tolerability. Hence, despite regimens involving the sequential administration of anthracyclines followed by taxanes being in the majority, we focused on the studies of Green et al. [7] and Gianni et al. [8] as they indicated better results for taxane-leading regimens [7, 8]. We therefore designed a phase II trial in Japan of a regimen leading with 12 consecutive weeks of paclitaxel followed by 4 cycles of fluorouracil, epirubicin, and cyclophosphamide (FEC) in patients with stage III breast cancer.

Patients and Methods

Eligibility Criteria

Women with a histological diagnosis of clinical stage IIIA or IIIB primary breast cancer were eligible for enrolment. The main inclusion criteria were: ECOG Performance Status (PS) 0–2, normal cardiac (absence of serious arrhythmias or serious ischemic changes on ECG), renal (serum creatinine level ≤1.5 mg/dl), hepatic (AST and ALT twice or less than twice the normal limits), and hematologic (white blood cell count ≥3,000/mm^3, neutrophil count ≥1,500/mm^3, platelet count ≥75,000/mm^3, and hemoglobin ≥8.0 g/dl) functions confirmed by a prestudy examination, and no prior chemotherapy or surgery for breast cancer. Written informed consent was obtained from all patients and the study was approved by each institutional review board.

Treatment

The treatment schema is shown in figure 1. Paclitaxel at 80 mg/m^2 was administered by intravenous (i.v.) infusion for 1 h once a week for 12 consecutive weeks. Premedication 30 min prior to paclitaxel administration consisted of i.v. dexamethasone 20 mg (decreasing to 10 mg from the second week on), oral diphenhydramine 50 mg, and i.v. ranitidine 50 mg. The FEC regimen was started within 1–3 weeks after the last paclitaxel administration and consisted of 4 cycles of i.v. cyclophosphamide 500 mg/m^2, epirubicin 75 mg/m^2, and fluorouracil 500 mg/m^2 administered on day 1 of a 21-day cycle.

The entry criteria for each paclitaxel administration were: (i) neutrophil count ≥1,500/mm^3, (ii) platelet count ≥75,000/mm^3, (iii) body temperature <38°C, (iv) PS ≤2, and (v) nonhematological toxicity less than grade 3 (except nausea, vomiting, alopecia, and fatigue). The entry criteria for each FEC cycle were the same as for paclitaxel, except a required platelet count of ≥100,000/mm^3.

Surgery was performed within 3–5 weeks after the completion of NAC and the nature of the surgery was decided in consultation with the patient. Adjuvant therapy was not prescribed to any of the patients; this includes trastuzumab for HER2-positive patients as trastuzumab had not been approved under the Japanese health insurance scheme for adjuvant therapy at the time of this trial.

Assessment of Response to Therapy

The primary endpoint was pCR and secondary endpoints included clinical overall response (OR), overall survival (OS), disease-free survival (DFS), rate of BCS, and toxicity. The evaluation of pathological response was performed in accordance with the General Rules for Clinical and Pathological Recording of Breast Cancer of the Japanese Breast Cancer Society [9] and pathological response was classified as: grade 0: 'no response' (almost no change in cancer cells after treatment); grade 1a: 'mild response' (mild changes in cancer cells regardless of the area, or marked changes in less than one third of the cancer cells); grade 1b: 'moderate response' (marked changes in one third or more, but less than two thirds of the tumor cells); grade 2: 'marked response' (marked changes in two thirds or more of the tumor cells), or grade 3: 'complete response' (necrosis or disappearance of all tumor cells; replacement of all cancer cells by either granulomatous or fibrous tissue or both). In the case of complete disappearance of cancer cells, pretreatment pathological evidence of the prior presence of cancer was necessary. The entire tumor bed and axillary lymph nodes were submitted for histopathological analysis, and pCR was defined as no histopathological evidence of any residual cancer cells in the breast, namely a grade 3 pathological response. The tumor response was classified in accordance with response evaluation criteria in solid tumors [10], and toxicity was assessed according to the second version of the National Cancer Institute Common Toxicity Criteria (NCI-CTC, v2).

Statistical Design

Based on the study by Green et al. [7], we established an expected pCR rate of 30.4% at a weekly paclitaxel dose of 80 mg/m^2, and a threshold efficacy rate of 13.3%, which was the lowest pCR rate for paclitaxel administration once every 3 weeks. Defining $\alpha = 0.05$ and $\beta = 0.2$, a total of 42 patients was required.

Table 1. Patient characteristics

Patients, n		47
Median age (range)		54 (30–74)
PS	0	46
	1	1
Clinical tumor status	T0	1
	T1	3
	T2	8
	T3	20
	T4	15
Nodal status	N0	6
	N1	24
	N2	16
	N3	1
Clinical stage	IIIa	28
	IIIb	19
Menopausal status	pre	20
	post	26
	unknown	1
Estrogen (and/or progesterone) receptor	positive	26
	negative	18
	unknown	3
HER2/neu	positive (IHC 3+ or FISH+)	13
	negative	30
	unknown	4

Results

From February 2003 to January 2005, 50 patients were enrolled, 47 of whom were eligible. Two patients were ineligible due to staging error (stage II and IV), and 1 patient dropped out as she underwent surgery before the dosing schedule was complete. The characteristics of the eligible patients included: a median age of 54 years (range 30–74); 28 patients (59.6%) with stage IIIa, and 19 patients (40.4%) with stage IIIb disease (table 1), and 14 patients (29.8%) with tumor size ≤3 cm, 32 patients (68.1%) with tumor size >3 cm, and 1 patient (2.2%) with an unknown tumor size.

OR at the end of paclitaxel treatment was 9 patients with complete response (CR), 22 with partial response (PR), 9 with stable disease, 3 with progressive disease, and 4 patients with an unknown response, giving a response rate of 66.0% (31 of 47 patients) [95% confidence interval (CI) 52.4–79.5%]. OR at the end of FEC was 18 patients with CR, 22 with PR, 3 with stable disease, and 4 with progressive disease, giving a response rate of 85.1% (40 of 47 patients) (95% CI 74.9–95.3%) (table 2).

Among the 47 patients included in this study, 44 (93.6%) underwent surgery, including mastectomy in 29 (67.1%) and BCS in 15 (31.9%), while 3 (6.4%) had no surgery. Based on guidelines in Japan, patients with tumor size ≤3 cm are eligible for BCS [11]. Of the 32 patients with tumor size >3 cm at baseline, 28 (85%) exhibited a reduction to ≤3 cm after the NAC regimen.

Regarding the histopathological response of the primary breast tumor among the 47 patients, 2 patients (4.3%) had grade 0, 13 (27.7%) had grade 1a, 3 (6.4%) had grade 1b, 13 (27.7%) had grade 2, and 13 (27.7%) had grade 3 response (table 3). A pCR in the breast was achieved in 27.7% of patients (13 of 47) (95% CI 14.9–40.4%), and a pCR in the breast plus lymph nodes was achieved in 21.3% of patients (10 of 47) (95% CI 9.6–33.0%). With regard to tumor size, pCR was achieved in 35.7% of patients (5 of 14) with tumor size ≤3 cm, and in 25.0% of patients (8 of 32) with a tumor >3 cm (table 4). The pCR rates stratified by HER2 and hormone receptor (HR, either estrogen receptor or progesterone receptor or both) status were 50% (1 of 2 patients) in HER2-positive/HR-positive, 25% (1 of 4

Table 2. Clinical response to therapy and surgical management

	Patients	
	n	%
Clinical response after paclitaxel (includes US/MMG)		
CR	9	19.1
PR	22	46.8
SD	9	19.1
PD	3	6.4
Not assessable	4	8.5
Clinical response after all NAC (includes US/MMG)		
CR	18	38.3
PR	22	46.8
SD	3	6.4
PD	4	8.5
Local therapy/type of surgery		
Mastectomy	29	61.7
BCS	15	31.9
None	3	6.4

US = Ultrasound; MMG = mammogram.

Table 3. Pathological response rate (n = 47)

	Grade				
	0	1a	1b	2	3
Patients, n (%)	2 (4.3)	13 (27.7)	3 (6.4)	13 (27.7)	13 (27.7)

Three patients did not undergo surgery. pCR: breast tumor 27.7% (95% CI 14.9–40.4%), and breast tumor and lymph nodes 21.3% (95% CI 9.6–33.0%).

Table 4. pCR and breast conservation rate according to breast tumor size

Tumor size	n	pCR	BCS	Mastectomy
≤3 cm	14	5 (35.7%)	9 (64.3%)	5 (35.7%)
>3 cm	32	8 (25.0%)	6 (18.8%)	24 (75.0%)

patients) in HER2-positive/HR-negative, 12.5% (2 of 16 patients) in HER2-negative/HR-positive, and 33.3% (7 of 21 patients) in HER2-negative/HR-negative cancers.

Major toxicities greater than grade 3 with paclitaxel administration included 3 patients with leukocytopenia (6.4%), 5 with neutropenia (10.6%), 1 with anemia (2.1%), 2 with neuropathy (4.3%), and 2 with a rash (4.3%). Major toxicities greater than grade 3 in the 44 patients receiving FEC included 14 patients with leukocytopenia (31.8%), 17 with neutropenia (38.6%), 1 with anemia (2.3%), and 1 with fever (2.3%). There were no serious toxicities that required the discontinuation of treatment (table 5).

The median follow-up period was 27.3 months. Median survival had not been reached at the time of conclusion of this study, and the 3-year OS rate was 85.1% (fig. 2). Median DFS was 40.2 months, and the 3-year DFS rate was 62.1% (fig. 3).

Discussion

In randomized controlled trials on breast cancer, the National Surgical and Adjuvant Breast and Bowel Project (NSABP) B-18 and EORTC studies confirmed no difference in the DFS and OS periods between neoadjuvant and adjuvant chemotherapy [3, 4]. With regard to correlating response and prognosis, improved prognosis was only observed in patients with a pCR, and pCR was an appropriate surrogate marker for predicting prognosis with NAC [5]. In this context, Green et al. [7] conducted a taxane-leading study involving NAC with paclitaxel followed by FAC, and found that paclitaxel achieved a higher pCR rate with good tolerability with weekly administration when compared to conventional administration once every third week.

The phase II and phase III studies of either an anthracycline or a taxane or both as NAC are shown in table 6. We obtained an OR rate of 85.1%, and pCR rates of 27.7 and 21.3% in the breast alone and in the breast plus lymph nodes, respectively. Following the paclitaxel regimen, the CR rate was 19.1% and the PR rate was 46.8%; after the FEC regimen, the CR rate rose to 38.3% and the PR rate to 46.8%. We therefore showed that in a NAC setting, the addition of an anthracycline regimen (FEC therapy) after a paclitaxel regimen leads to an increased response rate. Although comparing the pCR rates between the present and previous studies is impossible as the criteria for pCR differed, a comparison of anthracycline and taxane NAC regimens indicates that protocols leading with a taxane have sufficient efficacy compared to the more common sequence [5]. We were unable to demonstrate a significant relationship between pCR and receptor (HER2 and HR) status due to small sample size.

Recently, Chen et al. [17] reported the use of 4 cycles of a nonanthracycline-containing, weekly, paclitaxel-plus-carboplatin regimen in neoadjuvant treatment for stage III breast cancer. pCR rate was 19.4%, the incidence of grade

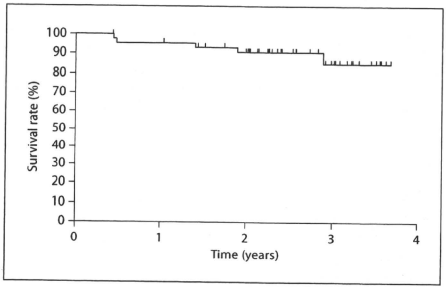

Fig. 2. Overall survival (n = 47).

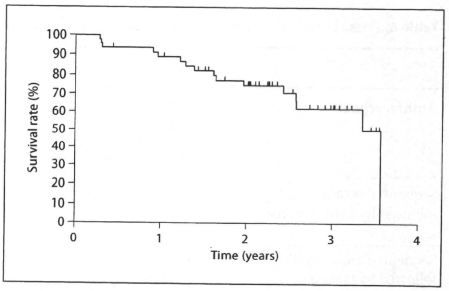

Fig. 3. Disease-free survival (n = 47).

Table 5. Adverse events

	Paclitaxel (n = 47)				FEC (n = 44)			
Grade:	1	2	3	4	1	2	3	4
Leukocytopenia	18	7	3 (6.4%)	0	6	16	12 (27.3%)	2 (4.5%)
Neutropenia	8	6	5 (10.6%)	0	5	13	9 (20.5%)	8 (18.2%)
Thrombocytopenia	0	0	0	0	0	0	0	0
Anemia	13	6	0	1 (2.1%)	14	7	0	1 (2.3%)
Fever	7	0	0	0	3	2	1 (2.3%)	0
Fatigue	12	1	0	0	12	2	0	0
Anorexia	2	2	0	0	15	2	0	0
Nausea/vomiting	7	2	0	0	19	5	0	0
Mucositis	0	1	0	0	2	1	0	0
Dysgeusia	1	0	0	0	4	0	0	0
Constipation	6	2	0	0	7	3	0	0
Diarrhea	0	1	0	0	1	0	0	0
Edema	4	0	0	0	2	0	0	0
Nail changes	3	0	0	0	0	0	0	0
Neuropathy	32	2	2 (4.3%)	0	3	1	0	0
Hypersensitivity reaction	1	0	0	0	0	0	0	0
Rash	6	1	2 (4.3%)	0	0	1	0	0
Alopecia	4	28	–	–	0	6	–	–
Myalgia/arthralgia	5	0	0	0	0	0	0	0
Cardiac toxicity	2	1	0	0	1	0	0	0

Adverse events were assessed according to NCI-CTC, version 2.

3–4 neutropenia was 40.2%, and only 1 patient was reported with febrile neutropenia. Weekly paclitaxel in combination with carboplatin showed marked activity and was tolerable as NAC. Further, Sparano et al. [18] reported in the ECOG 1199 trial a significant lengthening of OS (odds ratio 1.32; p = 0.01) and DFS (odds ratio 1.27; p = 0.006) for a weekly compared to once-every-third-week paclitaxel regimen. With the group with paclitaxel once every third week as the control, comparison of the groups with weekly paclitaxel, with docetaxel once every third week, and with weekly docetaxel showed that the group with weekly paclitaxel had the most favorable improvement in overall

Table 6. Phase II and III studies of anthracycline and/or taxane as neoadjuvant chemotherapy

	Study	Treatment regimen	pCR rate, %
Anthracycline	NSABP B-18 [3]	AC ×4	13
	EORTC 10902 [4]	CEF ×4	4.2
	multicenter trial [12]	AC ×4	10
	NSABP B-27 [13]	AC ×4	14
Sequential taxane followed by anthracycline	MDACC [7]	paclitaxel ×4 → FAC ×4	14
	MDACC [7]	paclitaxel ×12 weeks → FAC ×4	29
	ECTO [8]	AT ×4 → CMF ×4	23
Sequential anthracycline followed by taxane	AGO [14]	ddE ×3 → ddT ×3	18
	NSABP B-27 [12]	AC ×4 → docetaxel ×4	26
	GEPAR-DUO [15]	AC ×4 → docetaxel ×4	22
	JBCRG001 [16]	AC ×4 → docetaxel ×4	7.9
Concurrent anthracycline + taxane	multicenter trial [12]	AT ×4	16
	AGO [14]	ET ×4	10
	GEPAR-DUO [15]	ddAD ×4	11

AC = Doxorubicin + cyclophosphamide; CEF = cyclophosphamide + epirubicin + 5-fluorouracil; AT = doxorubicin + paclitaxel; CMF = cyclophosphamide + methotrexate + 5-fluorouracil; ddE = dose-dense epirubicin; ddT = dose-dense paclitaxel; ET = epirubicin + doxorubicin; ddAD = dose-dense doxorubicin + dose-dense docetaxel.

survival and DFS. Regarding toxicity, 28% of patients receiving paclitaxel weekly had grade 3–4 toxic effects, compared with 30% of those receiving paclitaxel every 3 weeks ($p = 0.32$), 71% of those receiving docetaxel every 3 weeks ($p < 0.001$), and 45% of those receiving docetaxel weekly ($p < 0.001$). Weekly administration of paclitaxel was associated with better treatment efficacy and tolerability than administration every 3 weeks and docetaxel.

Toxicities resulting from NAC influence subsequent treatment options and in this study we showed that a regimen leading with a weekly dosing of paclitaxel was well tolerated with acceptable levels of toxicity. We also investigated weekly paclitaxel therapy for metastatic breast cancer in a previous study and found similarly high levels of tolerability with a high completion rate [19].

Regarding OS and DFS, median survival had not been reached at the time of conclusion of this study. The 3-year OS rate was 85.1%, median DFS was 40.2 months, and the 3-year DFS rate was 62.1%. De Laurentiis et al. [20] conducted a meta-analysis of randomized trials that evaluated the efficacy of incorporating taxanes into anthracycline-based regimens for early breast cancer. The addition of a taxane to an anthracycline-based regimen improved the DFS and OS of high-risk early breast cancer patients. In a meta-analysis, the 3-year OS rate was 91% and the 3-year DFS rate was 81%. Our DFS rate is slightly below that of the meta-analysis data, but our OS rate is similar.

The 31.9% breast conservation rate demonstrated in this study is relatively modest, and this is in part due to patient preference as a determinant in the decision to undertake BCS. In Japan, patients with tumor size ≤3 cm are eligible for BCS, taking into consideration the minimization of local recurrence and satisfactory postsurgical esthetics [11]. In this study, tumors in 28 of 32 patients (84.8%) with tumor size >3 cm before NAC showed a reduction in tumor size to ≤3 cm after NAC. Thus, if all patients demonstrating a reduction in tumor size to ≤3 cm had gone on to have BCS, the breast conservation rate would have been considerably higher.

In 2005, Taghian et al. [21] conducted a randomized trial in patients with breast cancer for the sequential administration of paclitaxel and doxorubicin in the neoadjuvant setting, with 1 group leading with paclitaxel and the other with doxorubicin. They found that interstitial fluid pressure at the tumor sites decreased significantly after the administration of paclitaxel, but not after doxorubicin. They speculated that if the leading drug causes a decrease in interstitial fluid pressure at the tumor site, the tumor uptake of the following drug will be accelerated and improve the toxicity of oxygen-dependent drugs (e.g. doxorubicin and cyclophosphamide), possibly leading to an improvement in OR. This physiological consideration supports the argument of starting the sequential regimen with paclitaxel.

In summary, we found that weekly paclitaxel followed by FEC is an effective regimen with good tolerability in the neoadjuvant setting for locally advanced breast cancer. To confirm the clinical benefit of NAC involving the sequential administration of weekly paclitaxel followed by FEC, phase III trials comparing FEC followed by weekly paclitaxel may be required in the future.

Acknowledgements

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Bortezomib potentially inhibits cellular growth of vascular endothelial cells through suppression of G2/M transition

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Bortezomib, a selective 26S proteasome inhibitor, has shown clinical benefits against refractory multiple myeloma. The indirect anti-angiogenic activity of bortezomib has been widely recognized; however, the growth-inhibitory mechanism of bortezomib on vascular endothelial cells remains unclear, especially on the cell cycle. Here, we showed that bortezomib (2 nM of the IC_{50} value) potently inhibited the cellular growth of human umbilical vascular endothelial cells (HUVECs) via a vascular endothelial growth factor receptor (VEGFR)-independent mechanism resulting in the induction of apoptosis. Bortezomib significantly increased the vascular permeability of HUVECs, whereas a VEGFR-2 tyrosine kinase inhibitor decreased it. Interestingly, a cell cycle analysis using flow cytometry, the immunostaining of phospho-histone H3, and Giemsa staining revealed that bortezomib suppressed the G2/M transition of HUVECs, whereas the mitotic inhibitor paclitaxel induced M-phase accumulation. A further analysis of cell cycle-related proteins revealed that bortezomib increased the expression levels of cyclin B1, the cdc2/cyclin B complex, and the phosphorylation of all T14, Y15, and T161 residues on cdc2. Bortezomib also increased the ubiquitination of cyclin B1 and wee1, but inhibited the kinase activity of the cdc2/cyclin B complex. These protein modifications support the concept that bortezomib suppresses the G2/M transition, rather than causing M-phase arrest. In conclusion, we demonstrated that bortezomib potently inhibits cell growth by suppressing the G2/M transition, modifying G2/M-phase-related cycle regulators, and increasing the vascular permeability of vascular endothelial cells. Our findings reveal a cell cycle-related mode of action and strongly suggest that bortezomib exerts an additional unique vascular disrupting effect as a vascular targeting drug. (*Cancer Sci* 2010; 101: 1403–1408)

The proteasome is an essential enzyme complex for nonlysosomal and ATP-dependent proteolytic pathways. The ubiquitin–proteasome pathway plays an important role in the intracellular degradation of damaged, oxidized, or misfolded proteins[1–4] as well as in the cell cycle progression. Such damaged, oxidized, or misfolded proteins have been identified as substrates for the ubiquitin/proteasome system.[1,5–7] In addition, this system has been implicated in the regulation of cell proliferation, differentiation, survival, apoptosis, and angiogenesis.[8,9] Because of these unique effects of the proteasome/ubiquitin system on cellular regulation, the proteasome is a novel and promising target for cancer therapy.[10–12]

Bortezomib (Velcade, PS-341), a selective 26S proteasome inhibitor, demonstrates potent antitumor activity against several human cancers and has been clinically used mainly in patients with refractory multiple myeloma.[13–15] The main mechanism of action of this drug was initially thought to be the inhibition of nuclear factor-κB (NF-κB), which acts as a transcription factor for anti-apoptotic proteins, such as Bcl-2, c-IAP2, and survivin. Accumulating data indicates that bortezomib disrupts the cell cycle by modifying cyclins and inhibits the up-regulation of interleukin-6 (IL-6), which plays an important role in the proliferation of myeloma cells, by inhibiting NF-κB and stabilizing p53, p21, and p27, resulting in its anticancer activity.[1,16–18]

Bortezomib exerts an anti-angiogenic effect by decreasing the secretion of vascular endothelial growth factor (VEGF) from myeloma cells.[19,20] This anti-angiogenic effect of bortezomib is considered an indirect effect on vascular endothelial cells resulting from ligand depletion. Meanwhile, direct negative proliferative effects of bortezomib on vascular endothelial cells have emerged which play an important role in its anti-angiogenic activity. Roccaro *et al.*[9] reported that bortezomib induces inhibition of angiogenesis in functional assays of angiogenesis, including chemotaxis, adhesion to fibronectin, capillary formation on Matrigel, and chick embryo chorioallantoic membrane assay using multiple myeloma patient-derived endothelial cells and human umbilical vein endothelial cells (HUVECs). Podar *et al.*[21] reported that Caveolin-1 is a molecular target of bortezomib in multiple myeloma cells and HUVECs and this is required for VEGF-triggered multiple myeloma. However the underlying mechanism responsible for the direct negative proliferative effect of bortezomib on vascular endothelial cells remains unclear, especially with regard to its effect on the cell cycle.

To gain insight into the direct anti-angiogenic effects of bortezomib on HUVECs, we examined cellular proliferation, tube formation, VEGF receptor-2 (VEGFR-2) signaling, the apoptotic pathway, vascular permeability, cell cycle analysis, and effects of drugs on cell cycle-related proteins.

Materials and Methods

Anticancer agents. Bortezomib was provided by Millennium Pharmaceuticals (Cambridge, MA, USA). The VEGFR-2 tyrosine kinase inhibitor (VEGFR-2-TKI) Ki8751 (IC_{50} value for VEGFR-2 kinase inhibition = 0.90 nM) was purchased from Sigma (St. Louis, MO, USA). Paclitaxel was purchased from Wako Pure Chemical Industries (Osaka, Japan). Each chemical agent was dissolved in dimethylsulfoxide for use in the *in vitro* experiments.

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Cell cultures. HUVECs were maintained in Humedia-EG2 (Kurabo, Tokyo, Japan) with 2% fetal bovine serum and 0.1% gentamicin-amphotericinB with the addition of 10 ng/mL of epidermal growth factor, 5 ng/mL of fibroblast growth factor, and 2 ng/mL of VEGF (R&D Systems, Minneapolis, MN, USA). All the cell lines were incubated at 37°C with humidified 5% CO_2.

***In vitro* growth inhibition assay.** Growth inhibition was evaluated using the MTT assay, as described previously.[22] The experiment was performed in triplicate.

Western blotting. The antibodies used for western blotting were anti-phospho-VEGFR-2 (Tyr1175), anti-VEGFR-2, anti-MAPK, anti-phospho-MAPK, anti-β-actin, anti-cleaved or non-cleaved-caspase3, anti-cleaved or non-cleaved-poly ADP-ribose polymerase (PARP), anti-cyclin B1, anti-phospho-cdc2, anti-cdc2, anti-phospho-wee1, anti-wee1, anti-phospho-cdc25C, anti-cdc25C, anti-phospho-chk1 and -2, and anti-chk1 and -2 (Cell Signaling, Beverly, MA, USA). HUVECs were cultured overnight in serum-starved medium and then exposed to the indicated concentrations of bortezomib or Ki8751 for 3 h before the addition of 10 ng/mL of VEGF for 5 min. The western blot analysis was performed as described previously.[23] The experiment was performed in duplicate.

Immunoprecipitation. Total cell lysates were immunoprecipitated with anti-wee1, cdc2 antibodies (Cell Signaling), or anti-cyclin B1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. The protein complex was incubated with protein G-agarose (Invitrogen, San Diego, CA, USA) for 1 h at 4°C and washed three times with lysis buffer. After sequential centrifugation and washing, the pellets were resuspended in 1.5 × sample loading buffer and subjected to immunoblot analyses.

Cell cycle analysis. Cells were treated with the indicated concentrations of bortezomib for 24 h. The cells were then harvested, washed with PBS, fixed with 70% ethanol at −20°C overnight, washed again with PBS, and then stained with 5 μg/mL of propidium iodide containing 0.1% Triton X-100, 0.1 mM EDTA, and RNase I (BD Bioscience, San Jose, CA, USA). The stained cells were then analyzed for DNA content using a FACS Calibur flow cytometer (BD Biosciences) and the cell cycle distributions were calculated using ModFit LT software. The experiment was performed in triplicate.

Giemsa staining. Morphological changes in mitotic cells were evaluated using Giemsa staining. HUVECs treated with bortezomib (1 μM) or paclitaxel (1 μM) for 24 h were fixed with 10% neutral-buffered formaldehyde before staining and were stained for 30 min, then washed with tap water for 5 min. The morphological changes were evaluated using a light microscope (×40).

Immunofluorescence staining of phospho-histone H3. HUVECs were treated with 1 μM of bortezomib or paclitaxel for 24 h and were then fixed and permeabilized with 4% formaldehyde/PBS for 15 min. The cells were blocked with 5% normal goat serum in PBS for 60 min. After washing, anti-phospho-histone H3 antibody (Cell Signaling) was diluted 1:200 in PBS/Triton and incubated for 1 h at room temperature, followed by detection using Alexa Fluor 594 goat antirabbit IgG antibody (Invitrogen) for 1 h. After washing, the cells were counterstained with 1 μg/mL of 4′,6-diamidino-2-phenylindole (DAPI) in PBS for 5 min. Images were obtained using fluorescence microscopy (IX71; Olympus, Tokyo, Japan). The mitotic index was calculated by dividing the number of p-Histone H3-positive cells by the total number of treated cells (DAPI-positive cells). At least 100 cells were scored per low-power field, and the cells were counted over three fields. The experiment was performed in triplicate.

cdc2/cyclinB1 kinase assay. The cdc2/cyclinB1 kinase activity in the cells was quantified using a Cyclex Cdc2-CylinB Kinase Assay Kit (Cyclex, Nagano, Japan) according to the manufacturer's instructions. The experiment was performed in triplicate.

***In vitro* permeability assay.** Transwell permeability assays were performed using monolayers of HUVECs and an *in vitro* vascular permeability assay kit (Chemicon, Temecula, CA, USA). Briefly, HUVECs seeded onto collagen-coated inserts were pretreated with or without bortezomib (1, 0.1 μM) or VEGFR-2-TKI (1 μM) for 6 h, and VEGF (20 ng/mL) was added, except in the control sample, 4 h thereafter. Two hours after the addition of VEGF, fluorescein isothiocyanate dextran (FITC dextran) was added on the top of the cells and the extent of FITC dextran permeation was determined by measuring the fluorescence of the plate well solution, according to the supplier's instructions. The experiment was performed in triplicate.

Results

Bortezomib potentially inhibited the cellular growth of HUVECs independent of VEGF signaling. To evaluate the growth inhibitory activity of bortezomib *in vitro*, we performed MTT assays on HUVECs under the 20 ng/mL of VEGF or without it. Bortezomib exhibited a potent growth inhibitory activity on HUVECs with an IC_{50} of 2 nM; however, VEGF stimulation did not influence the growth inhibitory activity of bortezomib (Fig. 1a).

To address the question whether the growth inhibitory activity of bortezomib involves VEGFR-2 signaling, we compared the inhibitory effects of bortezomib with that of a VEGFR-2-TKI, Ki8751, on the phosphorylation levels of VEGFR and MAPK. Bortezomib did not inhibit the phosphorylation level of VEGFR-2, whereas Ki8751 (0.01–1 μM) completely inhibited VEGFR-2 phosphorylation (Fig. 1b). Similar results were observed for MAPK phosphorylation. These results indicate that the growth inhibitory activity of bortezomib is induced via a VEGFR-2 signaling-independent mechanism.

Bortezomib increases vascular permeability *in vitro*. Generally, the characteristics of vascular disrupting agents include a potent anti-proliferative effect. Microtubule-binding drugs (MBD) are widely used in cancer chemotherapy and also have clinically relevant vascular-disrupting properties. The disruption of adherens junctions contributes to the rounding of endothelial cells, leading to a direct increase in vasculature permeability.[24] Therefore, we examined the effect of bortezomib on vasculature permeability to gain an insight into its vascular-disrupting properties. As expected, Ki8751 significantly decreased vasculature permeability during VEGF stimulation, in contrast to the situation in untreated controls. On the other hand, bortezomib significantly increased the vasculature permeability of vasculature endothelial cells in a dose-dependent manner (Fig. 1c). This result supports the hypothesis that bortezomib has vascular-disrupting properties in HUVECs in addition to its potent growth inhibitory effect.

Bortezomib induces apoptosis of HUVECs. We speculated that the potent growth inhibitory activity of bortezomib was based on the induction of apoptosis; thus, we evaluated the expression levels of cleaved caspase 3, cleaved PARP, and ubiquitinated protein from whole cell lysates. The expression levels of cleaved caspase 3 and PARP showed that bortezomib induced the activation of caspase 3 at a dose of 0.1 μM and subsequent PARP cleavage in HUVECs in a dose- and time-dependent manner (Fig. 2). The accumulation of ubiquitinated proteins, which represents a direct effect of bortezomib, was observed at 0.01 μM in a time-dependent manner. These findings indicate that bortezomib is capable of inducing the apoptosis of HUVECs at a relatively low concentration.

Bortezomib inhibits G2/M transition. An analysis of the cell cycle distribution of HUVECs revealed that bortezomib significantly increased the population of cells in the G2/M phase

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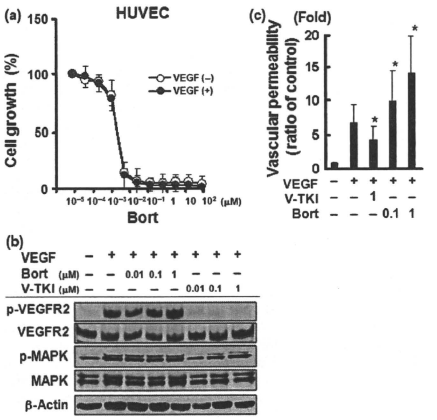

Fig. 1. Bortezomib potently inhibited the cellular growth and increased the vascular permeability of HUVECs. (a) *In vitro* growth-inhibitory effect of bortezomib on HUVECs using an MTT assay with 10 ng/mL vascular endothelial growth factor (VEGF) or without it. The data shown represents the average ± SD of three independent experiments. (b) Effects of bortezomib on VEGF signaling in HUVECs. Western blot analysis was performed for the expression and phosphorylation levels of VEGF receptor-2 (VEGFR-2) and MAPK. HUVECs were cultured under serum-starved conditions and exposed to bortezomib or Ki8751 at the indicated concentrations for 3 h. After 10 ng/mL VEGF stimulation for 5 min, the cells were analyzed. (c) Effect of bortezomib on vascular permeability *in vitro*. HUVECs were seeded onto collagen-coated inserts and were pretreated with or without bortezomib (0.1 and 1 μM) or VEGFR-2 tyrosine kinase inhibitor (VEGFR-2-TKI) (1 μM) for 6 h. After 20 ng/mL of VEGF stimulation for 2 h, fluorescein isothiocyanate dextran (FITC dextran) was added on the top of the inserts and the extent of FITC dextran permeation was determined by measuring the fluorescence of the plate well solution. The relative vascular permeability was calculated using the ratio to the permeability in the control cells (untreated). The data shown represents the average ± SD of three independent experiments. *$P < 0.05$. Bort, bortezomib; V-TKI, VEGFR-2-TKI.

(Fig. 3a). This effect was observed when the cells were exposed to 0.01 μM of bortezomib. Generally, morphological changes, including the disappearance of the nuclear membrane, chromosomal condensation, and cytoplasmic round formation, are observed in mitotic cells. Therefore, we evaluated whether bortezomib induced morphological changes in HUVECs specific to mitotic cells. Paclitaxel, a well-known tubulin binder and mitotic inhibitor, was used as a control. Paclitaxel clearly induced these morphological changes specific to mitotic cells; however, bortezomib did not induce these changes with Giemsa staining (Fig. 3b). Further analysis using phospho-histone H3 immunostaining, an M-phase-specific marker, demonstrated that bortezomib significantly decreased the number of mitotic cells while paclitaxel markedly increased it (Fig. 3c,d). Together, these results indicated that both bortezomib and paclitaxel induced cell cycle arrest at the G2/M phase; however, bortezomib did not increase the number of mitotic cells unlike paclitaxel. These results suggest that bortezomib inhibits the G2/M transition in HUVECs.

Bortezomib decreases cdc2/cyclinB kinase activity. Cell cycle progression at the G2/M transition is regulated by cdc2/cyclin B complex activity, and the activation of this complex is controlled as a consecutive process as follows: (i) the levels of cyclin B protein are increased during late S and G2 phases; (ii) cyclin B binds to unphosphorylated cdc2 and forms an inactive cdc2/cyclinB complex; (iii) cdc2 is phosphorylated at its T14, Y15, and T161 residues during the G2 phase; and (iv) the dephosphorylation of T14 and Y15 on cdc2 by phosphatase cdc25 activates the cdc2/cyclin B complex and introduces the cells to mitosis.

Bortezomib increased the expression of cyclin B1 in a dose- and time-dependent manner, and an immunoprecipitation analysis showed that bortezomib also increased the production of cdc2/cyclin B complexes (Fig. 4a). Bortezomib markedly increased the phosphorylation status of the T14, Y15, and T161 residues on cdc2 in a dose- and time-dependent manner, suggesting that bortezomib promoted the presence of the inactive form of the cdc2/cyclin B complex (Fig. 4b). These results showed that bortezomib inhibits the G2/M transition. In addition, we examined the effects on a competing kinase, wee1, and the phosphatase cdc25C. Increased expression and phosphorylation levels of wee1 were observed after bortezomib treatment, whereas no remarkable changes in cdc25C expression or phosphorylation were observed (Fig. 4b). Regarding the effects of bortezomib on the proteasome–ubiquitin pathway, we found that the ubiquitination of wee1 and cyclin B protein was increased by bortezomib in a dose-dependent manner, suggesting that the increase in the ubiquitination of wee1 and cyclin B may be at least partially involved in the suppression of the G2/M transition and the mode of action of this drug (Fig. 5a). Finally, a kinase assay of the cdc2/cyclin B complex showed that bortezomib (0.01 μM) significantly inhibited the kinase activity of the complex, indicating that the inhibition of kinase activity might suppress the G2/M transition (Fig. 5b).

Together, these results revealed that bortezomib increases the expression levels of cyclin B1, the formation of the cdc2/cyclin

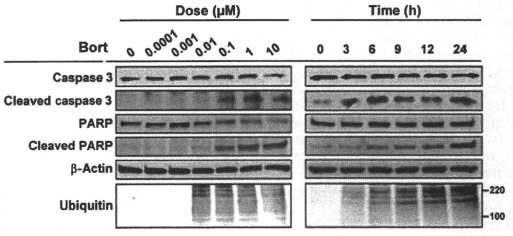

Fig. 2. Bortezomib induces apoptosis of HUVECs. Western blot analysis was performed for the cleaved form and the expression levels of caspase 3, poly ADP-ribose polymerase (PARP), and whole ubiquitinated-protein. HUVECs were treated with bortezomib at the indicated concentrations for 24 h and analyzed (left panel), or they were treated with bortezomib at 0.1 μM for the indicated hours (right panel). Protein size markers are shown at 100 and 220 kDa.