Table 2 Multivariate analysis for factors associated with time to PIPN

Variables	HR	95% CI		*P* value
Regimen				
triPTX	1			
wPTX	0.66	0.43	1.03	0.070
Age				
<60				
≥60	0.99	0.72	1.37	0.960
Lymph				
<4				
≥4	1.20	0.82	1.77	0.341
Tumor size (cm)				
<5				
≥5	0.98	0.68	1.42	0.917
Radiation				
No				
Yes	0.78	0.51	1.20	0.259
Surgery				
Mastectomy				
Lumpectomy	1.08	0.75	1.56	0.666
Endocrine				
No				
Yes	0.87	0.65	1.18	0.366
Grade				
1				
2 or 3	1.35	0.97	1.87	0.073
Diabetes mellitus				
No				
Yes	1.34	0.81	2.21	0.260

PIPN paclitaxel-induced peripheral neurotoxicity, *triPTX* tri-weekly paclitaxel, *wPTX* weekly paclitaxel, *HR* hazard ratio, *CI* confidence interval

Table 3 Multivariate analysis for factors associated with duration of PIPN

Variables	HR	95% CI		*P* value
Regimen				
triPTX	1			
wPTX	0.48	0.19	1.21	0.119
Age				
<60				
≥60	0.55	0.32	0.94	0.027
Lymph				
<4				
≥4	0.86	0.46	1.59	0.621
Tumor size (cm)				
<5				
≥5	1.03	0.59	1.77	0.927
Radiation				
No				
Yes	1.05	0.52	2.12	0.900
Surgery				
Mastectomy				
Lumpectomy	0.67	0.36	1.26	0.213
Endocrine				
No				
Yes	1.10	0.70	1.73	0.668
Grade				
1				
2 or 3	0.53	0.32	0.88	0.015
Diabetes mellitus				
No				
Yes	0.66	0.28	1.56	0.345

PIPN paclitaxel-induced peripheral neurotoxicity, *triPTX* tri-weekly paclitaxel, *wPTX* weekly paclitaxel, *HR* hazard ratio, *CI* confidence interval

any correlation with grade 2/3 PIPN (Table 4). Based on the results of multivariate analyses, there were no significant associations between diabetes mellitus and time to PIPN onset ($P = 0.260$) or duration of PIPN ($P = 0.345$) or grade 2/3 PIPN ($P = 0.229$).

Duration of PIPN

The median duration of PIPN was 727 days for the total patient group (range 14–2621) (Fig. 2). With weekly administration, the median duration was not reached (range 14–1089); the median duration for patients with tri-weekly administration was 651 days (range 23–2621). One year after initiating PTX treatment, PIPN (all grades included) persisted in 64% of patients; 3 years after treatment initiation, this number had dropped to 41%.

Discussion

This is the first published report to our knowledge that investigates the time to onset and duration of PIPN among breast cancer patients and explores potential risk factors related to severe and/or persistent PIPN. The data from this study confirm that most patients (97%) developed PIPN with a severity of at least grade 1. Peripheral neuropathy persisted in 64% of patients at 1 year and 41% at 3 years after the first administration of PTX. Approximately half of the patients who received PTX and developed PN experienced recovery from PN within 9 months after cessation of PTX treatment. We found correlations between the maximum PIPN severity and both the time to onset of PIPN and the duration of PIPN. In addition, we observed that PN lasted significantly longer in patients >60 years of age.

Table 4 Multivariate analysis for factors associated with grade 2 or 3 PIPN

Variables	Odds ratio	95% CI		*P* value
Regimen				
triPTX	0.57	0.18	1.83	0.345
wPTX				
Age				
<60	1.65	0.81	3.36	0.171
≥60				
Lymph				
<4	0.98	0.40	2.41	0.968
≥4				
Tumor size (cm)				
<5	0.47	0.18	1.24	0.125
≥5				
Radiation				
No	0.98	0.35	2.77	0.975
Yes				
Surgery				
Mastectomy	0.73	0.29	1.82	0.499
Lumpectomy				
Endocrine				
No	0.72	0.36	1.45	0.360
Yes				
Diabetes mellitus				
No	2.05	0.69	6.09	0.197
Yes				
Dose intensity				
<58	1.00	0.50	2.01	1.000
≥58				
Cumulative dose				
<700	0.31	0.08	1.13	0.077
≥700	0.57	0.18	1.83	0.345

PIPN paclitaxel-induced peripheral neurotoxicity, *triPTX* tri-weekly paclitaxel, *wPTX* weekly paclitaxel, *CI* confidence interval

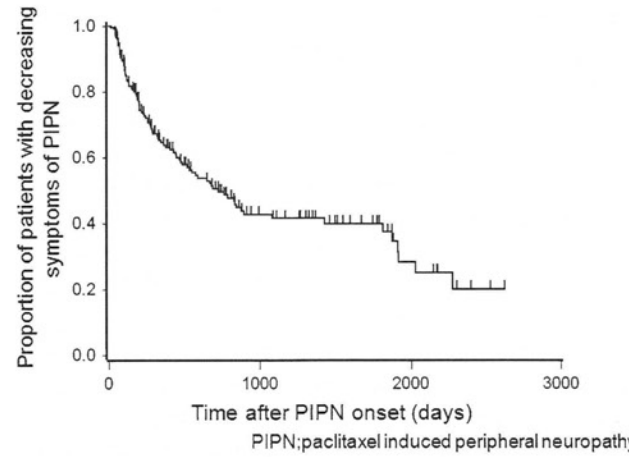

Fig. 2 Time to resolving PIPN from the time of developing paclitaxel-induced peripheral neuropathy

Previous studies have reported that the incidence of PIPN is related to several risk factors, including treatment schedule, doses per course, patient age, diabetes mellitus, and cumulative dose [6–11]. We found no association between the severity of PIPN and the PTX administration schedule including single dose, dose intensity, diabetes mellitus, or interval of administration. In our study, the mean cumulative dose at the onset of grade 1 or higher PN was 175 mg/m^2 for patients treated with PTX every 3 weeks and 320 mg/m^2 for weekly PTX patients. In contrast to an earlier study [14], our clinical outcomes indicated that tri-weekly administration of PTX was associated with more severe PIPN than weekly administration. However, this result may be attributed to frequent hospital visits and/or the relatively small number of patients treated by weekly PTX.

Previous reports suggest there are several risk factors for PIPN, including concurrent administration of cisplatin [19] and various genetic predispositions for neuropathy, such as *Wlds* (slow Wallerian degeneration gene) and *CYP3A* genotype [20, 21], but we did not examine any of those risk factors in this study.

Axonal microtubules are composed largely of β-tubulin. Neurotoxicity is caused by disruption of the microtubule structure, impairing axoplasmic transport and leading to dying-back neuropathy [22]. The most widely accepted mechanism of taxane neurotoxicity is a dying-back process that starts from distal nerve endings and progresses to affect Schwann cells, neuron bodies, or axons, resulting in transport changes that disturb cytoplasmic flow in the affected neurons [23]. Another possible cause of PIPN is that sensory nerves may be particularly vulnerable to the inhibition of tubulin assembly, as sensory nerves have long axons. However, motor neurons and C-neurons are not as sensitive to taxanes as are sensory nerves, despite the fact that these neurons are as long as sensory nerves. Some reports suggest that induction of Ca$\alpha 2\delta$-1 expression by PTX in the spinal root may be important, but further investigation is necessary to understand the mechanisms of PIPN [24].

There are no medications that prevent or relieve PIPN. Likewise, there are no laboratory tests that can predict the severity of PN. Management of PIPN is now based on early detection during chemotherapy to prevent its progression to grade 3 or 4. Clinical assessment, including a physical examination, is currently the most reliable method of assessing PIPN because we lack more reliable objective methods, and the symptoms of PIPN, such as numbness, sensory pain, fatigue, and weakness, are complicated [12, 25]. If grade 2 PN is diagnosed, it may be prudent to

withhold PTX until PN improves to at least grade 1; PTX administration can then be resumed at a reduced dose.

There were several limitations to our study. We used physician-based assessments, which relies on patients' report and examiners' interpretation and could have resulted in underestimation and under-reporting of the frequency and severity of PN [26]. In addition, physicians were more prone to quit following symptoms periodically once patients recovered from maximum PIPN. In fact, there were many censored cases in this study (Fig. 2). Therefore, features of PIPN such as location, presence of accompanying symptoms, and triggers for increase or decrease in severity were unclear. This study was retrospective, with censored data; the neurotoxicity corresponding to each grade of PIPN was unclear. In fact, time to onset of PIPN was faster for grades 2 and 3 than grade 1. In order to properly evaluate the correlation between severity and duration of PIPN, we will need further studies to determine whether or not the duration of PIPN is longer when the maximum severity increases from grade 1 to grade 2.

In conclusion, we analyzed the incidence and duration of PIPN and identified correlations between these and several risk factors. We found that the median time to onset of PIPN was 21 days, and the median duration of PIPN was 727 days. Patient age and PIPN severity were the independent risk factors significantly associated with longer PIPN duration. Urgent needs currently include identification of specific risk factors for PIPN, establishment of subjective methods for evaluating PIPN, and development of effective strategies for prevention and treatment of PIPN. To meet these ends, further investigation of the biological mechanisms leading to PIPN is warranted.

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Conflict of interest The authors have declared no conflicts of interest.

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Original article

The differences in the histological types of breast cancer and the response to neoadjuvant chemotherapy: The relationship between the outcome and the clinicopathological characteristics

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ABSTRACT

Although effective regimens have been established for invasive ductal carcinoma-not otherwise specified (IDC), the efficacy and prognosis of other minor types of breast cancer are unknown because of their rareness. The clinicopathological features and prognosis of other minor types concerning the response to neoadjuvant chemotherapy (NAC) were evaluated in this study.

A total of 562 patients were classified according to the Japanese and the World Health Organization (WHO) classifications, and the number of IDC and other special types (SP) was 500 and 62. The SP patients had a significantly poorer clinicopathological response to NAC and less breast-conservative therapy than those with IDC. According to the WHO classification, mucinous carcinoma, metaplastic carcinomas and apocrine carcinoma also responded poorly, and patients with metaplastic carcinomas and invasive lobular carcinoma had a significantly poorer prognosis. Despite the poor response to chemotherapy, patients with mucinous carcinoma and apocrine carcinoma had a good prognosis.

The response to NAC and the prognosis vary for each histological type. For some types, the prognosis was not related to the clinicopathological response to NAC.

Background: In the treatment of breast cancer, neoadjuvant chemotherapy (NAC) has become the standard treatment modality for downstaging purposes. Although effective regimens have been established for the treatment of invasive ductal carcinoma-not otherwise specified (IDC), the data about the efficacy and prognosis for patients with other minor types of breast cancer are insufficient because of the rareness of these tumors. Defining the relationship between each histological type and the clinicopathological response to NAC is essential to optimizing individualized treatment.

Methods: We retrospectively evaluated the clinicopathological features and classification of the histological types based on the Japanese and the World Health Organization (WHO) classifications before and after NAC in 562 patients with primary breast cancer who underwent curative treatment after NAC between 1998 and 2008. The prognosis was estimated for each histological type.

Results: Of the 562 patients, the number of cases of IDC and other special types (SP) was 500 and 62. In the SP group, the clinicopathological response to NAC was significantly poorer, and the patients underwent breast-conservative therapy less frequently than did the IDC patients. According to the WHO classification, mucinous carcinoma, metaplastic carcinomas and apocrine carcinoma responded poorly to NAC. The disease-free survival and overall survival were significantly worse for patients with metaplastic carcinomas ($p < 0.001$ and $p < 0.001$) and with invasive lobular carcinoma ($p = 0.03$ and $p < 0.001$) than other cancers. Despite their poor response to treatment, patients with mucinous carcinoma and apocrine carcinoma had a good prognosis.

Conclusions: The response to standardized NAC and prognosis varies for each histological type. For some types, the prognosis was not associated with the clinicopathological response to NAC. Innovative regimens should therefore be investigated for each histological type to achieve the best response.

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Introduction

In the treatment of breast cancer, neoadjuvant chemotherapy (NAC) has become the standard treatment modality for down-staging purposes. With the introduction of NAC, many patients have been able to be treated with breast-conserving therapy (BCT) as a result of the tumor reduction prior to surgery. Especially for patients with invasive ductal carcinoma-not otherwise specified (IDC), NAC had been confirmed to be efficient and beneficial, and is now widely applied for treatment. At present, invasive breast carcinoma is treated with a standardized regimen of NAC, regardless of the pathological type. However, because of their rareness, the efficiency and outcomes of NAC for the other minor types of breast carcinoma have not been fully elucidated.

In this study, we made a comparison between the patients with IDC and other types of breast cancer about clinicopathological features with regard to NAC. The histological types were classified using the Japanese classification[1,2] and the World Health Organization (WHO) classification.[3] We have correlated these histological types with the overall survival (OS) and disease-free survival (DFS) of the patients, and assessed the association between the tumor response to standardized NAC and the outcome for each histological type.

Material and methods

Patients

This study was a retrospective analysis of 562 breast cancer patients who underwent NAC during the period from 1998 to 2008 at the National Cancer Center Hospital, Tokyo, Japan. NAC was indicated for clinical stage II tumors that were larger than 3 cm in diameter, and for all stage III tumors. Axillary lymph node metastasis was diagnosed by cytology or imaging studies. Prior to NAC, all the patients underwent a core needle biopsy (CNB) for histological examination and were staged according the International Union Against Cancer (UICC) TNM classification.

Neoadjuvant chemotherapy regimens

NAC regimens were introduced based on current reviews at the time. Anthracycline-based chemotherapy included four cycles of CEF (cyclophosphamide 500 mg/m^2, epirubicin 100 mg/m^2, and fluorouracil 500 mg/m^2) every 3 weeks, or four cycles of AC (doxorubicin 60 mg/m^2, and cyclophosphamide 600 mg/m^2). Taxane chemotherapy included 12 cycles of weekly paclitaxel (wPTX, 80 mg/m^2).[4,5] Concurrent anthracycline and taxane chemotherapy included four cycles every 3 weeks of doxorubicin and docetaxel (AT, 50 and 60 mg/m^2).[6] Sequential anthracycline and taxane chemotherapy included AT (two cycles) followed by wPTX, AC followed by wPTX, and CEF followed by wPTX. Trastuzumab (first cycle; 4 mg/kg: after second cycle; 2 mg/kg) combined with anthracycline and taxane chemotherapy was administered to the patients with overexpression of the human epidermal growth factor receptor 2 (HER2).[7]

Histological diagnosis and evaluation

Prior to NAC, CNB specimens were examined for the histological sub-type and histological grade (HG) by hematoxylin and eosin (HE) staining. After NAC, the surgical specimen was examined for the histological sub-type, HG, and presence or absence of lymphatic or vascular space invasion. The histological sub-types were defined based on the General Rules for Clinical and Pathological Recording of Breast Cancer that were proposed by The Japanese Breast Cancer Society (JBCS classification)[1,2] and the WHO classification.[3] As the feature of the Japanese histological classification, all breast carcinomas are first classified according to the existence of invasion while, in addition, invasive carcinoma is classified as invasive ductal carcinoma, or other types called 'special types (SP)', and the SP category includes invasive lobular carcinoma (ILC) and other minor histological types.

The HG was assessed using the Scaff-Bloom-Richardson classification.[8] Immunohistochemistry was used to examine the tissue samples for the expression of the estrogen receptor (ER), progesterone receptor (PgR), and HER2. The cutoff values for the ER and PgR were 10% positive cells. HER2 status was defined based on immunohistochemical staining (IHC). The specimens that were HER2 2 + by IHC were then subjected to fluorescence *in situ* hybridization (FISH). HER2 positive samples were defined as those that were HER2 3+ in IHC or HER2 2+ in IHC and had an amplification ratio in FISH of >2.0. The degree of lymphatic invasion (ly) was classified by HE staining as follows: absent, no lymphatic invasion; ly1+, minimal lymphatic invasion; ly2+, moderate lymphatic invasion; and ly3+, marked lymphatic invasion. These diagnoses and evaluations were performed separately by two qualified pathologists, and the final diagnosis and evaluations were decided as a result of conferences between the pathologists.

Evaluation of the response to NAC

Prior to and after NAC, all of the patients and tumors were evaluated by physical examinations and radiographic imaging. The tumor diameter was evaluated using calipers and by ultrasonography. The clinical response was assessed using the Response Evaluation Criteria in Solid Tumor (RECIST) guidelines.[9] The tumor was judged to be 'progressive disease (PD)' when the tumor size increased by 20% or more. At that time, chemotherapy was discontinued and surgery was performed. The pathological response was evaluated from surgical specimens. The histopathological response was assessed using the General Rules for Clinical and Pathological Recording of Breast Cancer.[10] Response grade 0 was no response, and was defined by almost no change in the cancer cells after treatment. Grade 3 was a complete response, and was defined as necrosis or the disappearance of all tumor cells. The definition of a pathological complete response (pCR) was 'necrosis and the disappearance of all invasive cells' of the primary tumor. Cases with only intraductal carcinoma remaining were included in the pCR category.

Table 1
The Japanese histological classification of breast tumors (extraction) and the number of patients with each histological type ($n = 562$).

Histological type	No. of patients	%
B. Malignant (Carcinoma)		
a. Invasive carcinoma	500	89.0
a1. Papillotubular carcinoma	126	22.4
a2. Solid-tubular carcinoma	202	35.9
a3. Scirrhous carcinoma	172	30.6
b. Special types	62	11.0
b1. Mucinous carcinoma	12	2.1
b2. Medullary carcinoma	0	0
b3. Invasive lobular carcinoma	29	5.2
b4. Adenoid cystic carcinoma	0	0
b5. Squamous cell carcinoma	5	0.9
b6. Spindle cell carcinoma	4	0.7
b7. Apocrine carcinoma	5	0.9
b8. Carcinoma with cartilaginous and/or osseous metaplasia	1	0.2
b9. Tubular carcinoma	0	0
b10. Secretory carcinoma	1	0.2
b11. Invasive micropapillary carcinoma	1	0.2
b12. Matrix-producing carcinoma	4	0.7
b13. Others	0	0

Table 2
The administered NAC regimens ($n = 562$).

	No. of patients	%
AT	150	26.7
AT followed by wPTX	25	4.4
AT followed by wPTX/Trastuzumab	2	0.4
AC followed by wPTX	142	25.3
AC followed by wPTX/Trastuzumab	17	3.0
CEF followed by wPTX	181	32.2
CEF followed by wPTX/Trastuzumab	26	4.6
wPTX	12	2.1
wPTX/Trastuzumab	7	1.2

AT, doxorubicin and docetaxel; wPTX, weekly paclitaxel; AC, doxorubicin and cyclophosphamide; CEF, cyclophosphamide, epirubicin and fluorouracil.

Surgery and post-operative treatment

The breast surgery was either a lumpectomy or a total mastectomy. When the patient who underwent a lumpectomy was detected to have cancer in the pathological margin, additional excision was performed until the specimen became pathologically margin free. All of the patients underwent axillary lymph node dissection (level II). Adjuvant therapy was given in some cases based on the most current recommendations from the St. Gallen's Consensus Meeting at the time.[11–15] Tamoxifen (20 mg/day) or anastrozole (1 mg/day) was administered for five years when CNB specimens or surgical postchemotherapy specimens were positive for the ER or PgR. Radiotherapy was performed for the patients who underwent BCT for the residual breast or the patients with tumors >5 cm and/or with massive metastatic lymph nodes (≥4 nodes) for the chest wall, axilla, and supraclavicular area.

Follow-up and statistical analysis

The number of follow-up months was recorded from the first day of NAC to the most recent medical visit on record.

OS and DFS were calculated using the Kaplan–Meier methods and compared using the log-rank test. For comparisons of categorical variables, the chi-square test was used. Odds ratios (OR) and associated 95% confidence intervals (95% CI) were calculated as estimates of the relative risk. Values of $p < 0.05$ were considered to be statistically significant. All data were analyzed using the SPSS software program (SPSS Inc., Chicago, IL).

Results

Patient characteristics and clinical features

Table 1 presents the Japanese histological classification and the number of each histological type. The total number of IDC and SP

Table 3
The results of the analysis of the patient and tumor characteristics by histological groups (JBCS).

	Univariate			Multivariate	
	IDC ($n = 500$)	SP ($n = 62$)	p value	OR (95% CI)	p value
Age, mean ± SD	50.7 ± 10.4	50.6 ± 11.7	0.932		
Age (years)			0.335		
<41	74 (14.8)	13 (21.0)			
41–50	147 (29.4)	15 (24.2)			
51–60	178 (0.356)	18 (29.0)			
≥61	101 (0.202)	16 (25.8)			
Tumor size (cm), mean ± SD					
Prior NAC	5.7 ± 1.7	5.5 ± 2.5	0.075		
After NAC	2.1 ± 1.9	3.5 ± 2.7	<0.001	1.318 (1.063–1.632)	0.012
Stage			0.841		
II	320 (64.0)	39 (62.9)			
III	180 (36.0)	23 (37.1)			
Hormone receptors					
ER positive (%)	223 (44.6)	27(43.5)	0.892		
PgR positive (%)	198 (39.6)	21 (33.9)	0.408		
HER2 positive (%)	105 (21.0)	4 (6.5)	0.006	0.275 (0.080–0.948)	0.041
Histological grade			<0.001	0.674 (0.403–1.125)	0.131
G1 (%)	32 (6.4)	14 (22.6)			
G2 (%)	216 (43.2)	27 (43.5)			
G3 (%)	252 (50.4)	21 (33.9)			
Clinical response					
Responded (CR + PR) (%)	425 (85.0)	42 (67.7)	0.002	0.841 (0.341–2.076)	0.707
CR	165 (33.0)	6 (9.6)	<0.001	0.938 (0.633–1.390)	0.750
PD	13 (2.6)	7 (11.3)	0.003	5.279 (1.715–16.249)	0.004
BCT cases (%)	208 (53.0)	16 (25.8)	0.019	0.386 (0.082–1.247)	0.240
Pathological response					
pCR	113 (22.6)	5 (8.1)	0.080		
Pathological response grade					
G0 (%)	15 (3.0)	6 (9.7)	0.021	2.911 (0.777–10.909)	0.113
G3 (%)	65 (13.0)	5 (8.1)	0.314		
G0/1 (%)	312 (62.4)	42 (67.7)	0.086		
G2/3 (%)	188 (37.6)	20 (32.3)			
Cases of LN metastasis (%)	265 (53.0)	38 (61.3)	0.227		
No. of LN metastasis, mean ± SD	2.8 ± 5.1	3.7 ± 6.6	0.215		
Lymphatic invasion					
present	133 (26.6)	12 (19.4)	0.042	0.385 (0.174–0.851)	0.018
ly(1+)	85 (17.0)	10 (16.1)	0.302		
ly(2+/3+)	48 (9.6)	2 (3.2)	0.018	0.324 (0.073–1.448)	0.140
Vascular invasion, present	17 (3.4)	2 (3.2)	1.000		

IDC, invasive ductal carcinoma-not otherwise specified; SP, special types; OR, odds ratio; CI, confidence interval; SD, standard deviation; NAC, neoadjuvant chemotherapy; ER, estrogen receptor; PgR, progesterone receptor; HER2, human epidermal growth factor receptor 2; G, grade; CR, complete response; PR, partial response; PD, progressive disease; BCT, breast-conserving therapy; pCR, pathological complete response; LN, lymph node.

Table 4
The results of the univariate analysis of the patient and tumor characteristics for each histological type (WHO).

	IDC ($n = 500$)	ILC($n = 29$)		Metaplastic ($n = 14$)		Mucinous ($n = 12$)		Apocrine ($n = 5$)	
			p value		p value		p value		p value
Tumor size (cm), mean + SD									
Prior NAC	5.0 + 1.7	4.7 + 1.2	0.169	5.9 + 2.9	0.254	7.4 + 3.4	0.036	5.3 + 1.4	0.709
After NAC	2.1 + 1.9	2.4 + 1.7	0.400	5.9 + 3.9	0.003	4.1 + 1.7	0.001	3.0 + 0.1	<0.001
Hormone receptors									
ER positive (%)	223 (44.6)	14 (50.0)	0.698	1 (7.1)	0.005	9 (75.0)	0.039	0 (0)	0.061
PgR positive (%)	198 (39.6)	13 (46.4)	0.556	1 (7.1)	0.011	6 (50.0)	0.348	1 (25.0)	0.163
HER2 positive (%)	105 (21.0)	2 (7.1)	0.091	0 (0)	0.085	1 (8.3)	0.252	1 (20.0)	0.999
Histological grade			0.069		0.016		<0.001		0.372
G1	32 (6.4)	4 (14.3)		0 (0)		8 (66.7)		1 (20.0)	
G2	216 (43.2)	17 (60.7)		1 (7.1)		3 (25.0)		4 (80.0)	
G3	252 (50.4)	7 (25.0)		13 (92.9)		1 (8.3)		0 (0)	
Clinical response									
Responded (CR + PR)	425 (85.0)	21 (75.0)	0.211	5 (35.7)	0.003	9 (75.0)	0.271	5 (100)	0.446
CR	165 (33.0)	5 (17.9)	0.067	0 (0)	0.007	0 (0)	0.009	0 (0)	0.137
PD	13 (2.6)	0 (0)	0.428	7 (50.0)	<0.001	0 (0)	0.603	0 (0)	0.737
Pathological response									
pCR	113 (22.6)	2 (7.1)	0.032	0 (0)	0.047	0 (0)	0.045	0 (0)	0.272
Pathological response grade			0.357		0.094		0.116		0.372
G0/1	312 (62.4)	19 (67.9)		12 (85.7)		10 (83.3)		2 (40.0)	
G2/3	188 (37.6)	9 (32.1)		2 (14.3)		2 (16.7)		3 (60.0)	
Cases of LN metastasis	265 (53.0)	19 (67.9)	0.089	7 (50.0)	0.968	9 (75.0)	0.111	1 (20.0)	0.194
No. of LN metastasis, mean + SD	2.8 + 5.1	4.4 + 6.8	0.223	5.2 + 9.5	0.341	1.7 + 2.0	0.453	0.2 + 0.4	0.260
Lymphatic invasion, present	133 (26.6)	4 (14.2)	0.307	3 (21.4)	0.385	4 (33.3)	0.771	0 (0)	0.161
Vascular invasion, present	17 (3.4)		0.357	0 (0)	1.000	0 (0)	1.000	0 (0)	1.000

IDC, invasive ductal carcinoma-not otherwise specified; ILC, invasive lobular carcinoma; SD, standard deviation; NAC, neoadjuvant chemotherapy; ER, estrogen receptor; PgR, progesterone receptor; HER2, human epidermal growth factor receptor 2; G, grade; CR, complete response; PR, partial response; PD, progressive disease; pCR, pathological complete response; LN, lymph node.

cases was 500 and 62. Table 2 shows the NAC regimens that were administered. Prior to NAC, the average age and tumor size were not significantly different for the different groups. The HG was significantly higher in the IDC group ($p < 0.001$). The immunohistochemical findings and ER and PgR status were not significantly different in the two groups, however, the HER2 status was more frequently positive in the IDC group ($p = 0.006$). After NAC, the SP group was significantly less likely to achieve a clinical response ($p = 0.002$) and had tumors that were larger in size ($p < 0.001$). There were 20 patients who discontinued NAC because of PD. This was 11.3% of the cases in the SP group, which was significantly higher ($p = 0.003$) than that in the IDC group. BCT was performed significantly more often for IDC patients than SP patients (53.0% vs. 25.8%, $p = 0.019$). Axillary lymph node metastasis was present in 53.0% of patients in the IDC group and 61.3% of those in the SP group, which was not significantly different. The average number of metastatic lymph nodes was not significantly different between the groups. With regard to the pathological response, the pCR rate was 22.6% in the IDC group and 8.1% in the SP group, which was not significantly different. The rate of pathological response grade 3 was also not significantly different between the groups. However, 9.7% of SP patients had no pathological response, and this was a significantly higher rate than that in IDC patients ($p = 0.021$). The IDC group had larger tumors ($p = 0.042$), and more severe ($p = 0.018$) lymphatic invasion. The frequency of vascular invasion was not significantly different between the groups. According to a multivariate analysis, the significantly different characteristics in the SP group were a larger tumor size after NAC, more frequent HER2-negative status, more PD and a lower severity of lymphatic invasion (Table 3).

Histological classification and clinicopathological response to NAC

According to the WHO classification, squamous cell carcinoma, spindle cell carcinoma, carcinoma with cartilaginous and/or osseous metaplasia, and matrix-producing carcinoma were included in the category of metaplastic carcinomas (MPC). The total number of MPC was 14 cases. The tumor size of mucinous carcinomas, MPC and apocrine carcinomas was only minimally reduced, and this was significantly different from IDC ($p = 0.001$, $p = 0.003$ and $p < 0.001$). The clinical response of MPC was significantly poorer than that of IDC ($p = 0.003$) and a half of MPC cases

Table 5
The results of the multivariate analysis of the patient and tumor characteristics of patients with metaplastic carcinomas and mucinous carcinoma (WHO).

	Metaplastic		Mucinous	
	OR (95% CI)	p value	OR (95% CI)	p value
Tumor size, Prior NAC			1.416 (0.983–2.041)	0.062
Tumor size, After NAC	1.443 (1.065–1.956)	0.018	1.226 (0.765–1.964)	0.398
ER positive	0.122 (0.012–1.265)	0.079	1.746 (0.350–8.703)	0.496
PgR positive	0.389 (0.042–3.603)	0.406		
Histological grade	5.935 (0.709–49.680)	0.100	0.077 (0.021–0.280)	<0.001
Clinical response, (CR + PR)	0.545 (0.125–2.367)	0.418		
Clinical response, CR	0.117 (0.001–35.290)	0.830	0.071 (0.001–20.076)	0.861
Clinical response, PD	36.409 (3.408–289.011)	0.003		
Pathological response, pCR	0.028 (0.001–27.724)	0.835	0.003 (0.001–17.390)	0.898

OR, odds ratio; CI, confidence interval; NAC, neoadjuvant chemotherapy; ER, estrogen receptor; PgR, progesterone receptor; CR, complete response; PR, partial response; PD, progressive disease; pCR, pathological complete response.

developed PD, which was significantly higher than the rate of IDC ($p < 0.001$). The HG was lower in mucinous carcinomas ($p < 0.001$) and higher in cases of MPC ($p = 0.016$) than in IDC (Table 4). A multivariate analysis indicated that mucinous carcinoma had a lower HG and that MPC had a larger tumor size after NAC and more frequently developed PD than did patients with IDC (Table 5).

Prognosis after treatment and histological features

The patient survival was evaluated using a median follow-up period of 49 months (range, 1–136 months). The 10 year DFS rate was 28% in the SP group and 62% in the IDC group ($p < 0.001$). The OS was significantly worse in the SP group than the IDC group ($p < 0.001$). The incidence of recurrence or death was also significantly higher in the SP group (OR. 2.359; 95% CI, 1.443–3.856; $p < 0.001$ and OR, 4.825; 95% CI, 2.473–9.412; $p < 0.001$, respectively). The independent risk of recurrence or death was analyzed using a Cox multivariate analysis (Table 6). The independent risk factors for recurrence were a younger age, a high HG and the presence of lymphatic invasion. The pathological response grade was a significant factor associated with OS. However, PD was not a significant factor for predicting the DFS or OS.

According to the WHO classification, the DFS and OS of MPC and ILC were significantly worse than those of IDC. However, there were no cases of recurrence or death in the patients with apocrine carcinoma (Fig. 1). The incidence of recurrence or death was significantly higher in the MPC group (OR, 3.076; 95% CI, 1.057–8.951; $p = 0.031$ and OR, 7.053; 95% CI, 2.347–21.197; $p < 0.001$, respectively). The other three types were not significantly different with regard to the incidence of recurrence or death. Because there was only a small number of cases of each histological type, no significant independent risk factor for recurrence or death were identified in the multivariate analysis of each histological type.

Discussion

For breast cancer patients, NAC has been standardized for the purpose of reducing the tumor or for downstaging the tumor. For IDC, standardized NAC regimens have been established, and the effects of treatment have been widely shown.[16,17] However, because of their rareness, the therapeutic effect and outcome after NAC for other types (excluding IDC) were unclear, and standardized regimens for each histological type have not been established. In Japan, standardized NAC was started in 1998, and has been administered for all types of invasive breast carcinoma. We have demonstrated that there are differences in the clinicopathological effects and outcomes after NAC for different types of invasive breast carcinoma, and that these differences are especially pronounced between IDC and other minor types based on the Japanese and the WHO classifications.

Although the SP group had a significantly poorer outcome with regard to tumor reduction and the pathological response, there were actually two sub-types of tumors; those that were effectively reduced by NAC (mucinous carcinoma, ILC and apocrine carcinoma) and those that increased in size despite treatment (squamous carcinoma and spindle cell carcinoma). Under the WHO classification, these increased types were included among the MPC group.

Overall, the SP group had a significantly poorer prognosis than the IDC group. However, according to the WHO classification, the SP group could be sub-classified into better and worse prognostic types, irrespective of the poor response to NAC. ILC and MPC had significantly poorer outcomes than IDC, but mucinous carcinoma and apocrine carcinoma did not have significant differences in their DFS and OS compared to IDC patients. These results suggest that the SP group in the JBCS classification includes different biological and clinical types.

The behavior and a better prognosis of mucinous carcinoma and apocrine carcinoma were reported.[18–21] Because of their better prognosis regardless of the little effect of NAC, the role of NAC for these carcinomas was limited and NAC might not be needed.

MPC was characterized that the biological and clinical malignancies,[22,23] and the subgroups of MPC included carcinoma with cartilaginous and/or osseous metaplasia and matrix-producing carcinoma were previously reported by Wargotz et al.[24–28] Because of its sarcomatous lesion, MPC has only a minimal response to NAC using the conventional regimens[29] and the effeteness of anti-sarcoma regimens including ifosfamide and etoposide was reported.[30] In our study, the clinicopathological characteristics and response to NAC were similar to other reports,[31–34] but the prognosis was poorer and different. From 1990

Table 6
The hazard ratio of the disease free interval and overall survival in patient with special types based on the multivariate Cox regression analysis.

	DFS			OS		
	HR	95% CI	*p* value	HR	95% CI	*p* value
Age	0.898	0.832–0.969	0.005	0.979	0.885–1.082	0.673
Tumor size						
Prior NAC	0.874	0.537–1.424	0.589	1.273	0.884–2.415	0.084
After NAC	1.166	0.696–1.956	0.559	1.604	0.948–2.713	0.078
Stage	0.815	0.154–4.305	0.810	0.914	0.241–5.214	0.897
Hormone receptors						
ER positive	1.416	0.197–10.187	0.730	0.383	0.040–3.687	0.406
PgR positive	3.540	0.449–27.927	0.230	0.547	0.018–17.071	0.731
HER2 positive	0.007	0.001–3.142	0.974	0.071	0.001–4.682	0.991
Histological grade	6.022	1.458–24.864	0.013	3.195	0.312–31.992	0.330
Clinical response						
Responded (CR + PR)	0.480	0.029–7.985	0.609	0.555	0.072–40,281	0.572
CR	0.004	0.001–6.486	0.991	0.013	0.001–9.246	0.995
PD	4.628	0.353–60.629	0.243	4.560	0.221–92.262	0.326
Pathological response, pCR	0.871	0.001–17.512	1.000	0.653	0.032–12.486	0.998
Pathological response grade	0.754	0.314–1.811	0.528	0.339	0.117–0.983	0.046
Cases of LN metastasis	1.084	0.091–12.867	0.949	1.898	0.032–23.623	0.868
No. of LN metastasis	1.111	0.949–1.301	0.188	5.856	0.031–52.465	0.889
Lymphatic invasion, present	6.384	1.329–30.666	0.021	2.243	0.225–22.394	0.491
Vascular invasion, present	12.136	0.001–144.730	0.964	4.467	0.001–35.241	0.994

DFS, disease-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; NAC, neoadjuvant chemotherapy; ER, estrogen receptor; PgR, progesterone receptor; HER2, human epidermal growth factor receptor 2; CR, complete response; PR, partial response; PD, progressive disease; pCR, pathological complete response; LN, lymph node.

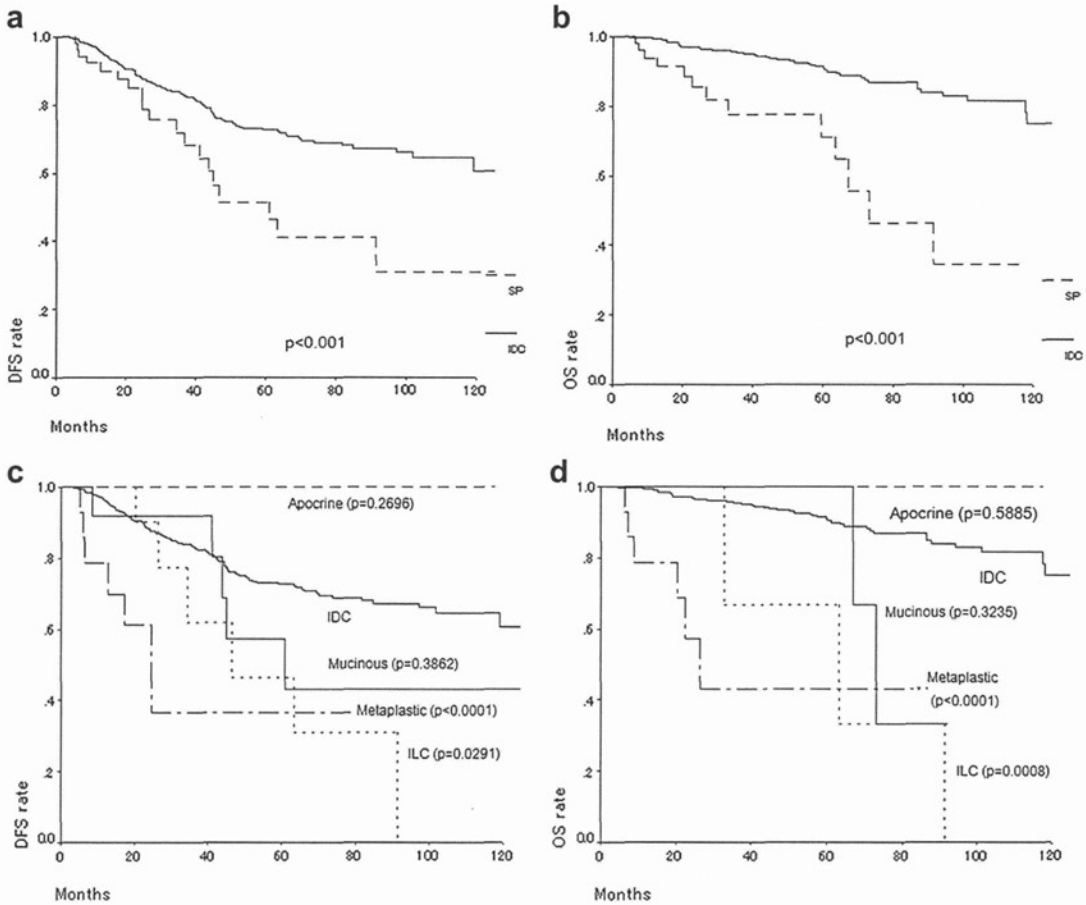

Fig. 1. The disease-free survival (DFS) curves and overall survival (OS) curves. (a) The DFS of IDC and SP patients based on the JBCS classification, (b) The OS of the IDC and SP patients, (c) The DFS for each histological type based on the WHO classification, (d) The OS for each histological type.

to 2009 at our institute, the 10-year survival rate of IDC and ILC patients were 81.8% and 76.5%, which was not significantly different. The reason for the relatively poor prognosis in our study is unclear, but it is possible that the chemosensitivity of ILC may differ in different races as a result of genetic differences.

Currently, breast cancer has been shown to be classifiable into molecular sub-types by gene profiling, and these sub-types related to different prognoses.[35,36] The use of adjuvant or neoadjuvant therapies has been shifting from an emphasis on the histological type to being based on the specific molecular sub-types. With regard to the molecular sub-types, positivity for the ER and/or PgR was not associated with any significant difference between the IDC and SP groups, but there were significantly more HER2-negative cases in the SP group. Several authors have reported that HER2-positive tumors were predicted to have an improved response to chemotherapy and to achieve a much higher pCR rate.[37,38] The HER2-negative status may be one reason why the SP group had a poorer overall response to NAC.

The relationship between chemosensitivity and the molecular sub-types has already clarified that ER-negative tumors have a good response to chemotherapy.[38,39] The molecular sub-types, prognosis and epidemiology of each rare histological type were reviewed by Yerushalmi et al.[40] From the analysis of the histological type in our study, the ER status was found to be positive in cases of mucinous carcinoma and negative in cases of MPC. ER positive status is considered to be the reason for the poor response of mucinous carcinoma. However, MPC had poor response to NAC regardless of the ER status, so the reason for the poorer prognosis is still unclear. MPC is considered to be a basal-like tumor because it is 'triple-negative', and this type has poor chemosensitivity and a poor prognosis.[41] In fact, all of the PD cases in our SP group were MPC. Because of their poor response, NAC is generally omitted for these patients, and surgical resection is performed as the primary therapy for mucinous carcinoma and MPC.

Besides molecular sub-types, other classifications, such as that using the 21-gene expression profile assay and 70-gene assay, have been used for predicting the response to neoadjuvant and adjuvant therapy.[42,43] Although a review concerning the relationship between neoadjuvant endocrine therapy and the 21-gene expression profile assay was reported from Japan,[44] this was a pilot study, and the scoring tools are not yet widespread because of the high price of employing this method. New therapeutic regimens based on the further analysis of the relationship between the immunohistological features or gene expression profiles and therapeutic sensitivity are thus needed.

Some of the limitations associated with this study are the fact that it was a retrospective analysis, and the study population was small due to the rareness of patients with each histological type in the SP group. Trastuzumab therapy was performed in only 52 cases, although there were 109 cases with HER2-positive tumors. The reason for this difference is the date of approval of trastuzumab in Japan. Chemotherapy regimens have been changed during the period of the study, and a uniform evaluation of the effects of therapy cannot be performed. Additionally, treatment for breast cancer has been changed dramatically in the past few years.[45] Because the basis of treatment has been changed from histopathological characteristics of tumor or the presence or absence of lymph node metastasis to intrinsic sub-type of tumor, the role of

chemotherapy has been getting smaller. Therefore the treatment criterion in this review may be different.

In summary, the other minor types of invasive breast carcinoma were different from IDC with regard to the effects of NAC and the prognosis. To determine whether NAC should be administered for the various sub-types of breast cancer, an accurate histological diagnosis and an appreciation of the individual sub-type's sensitivity and responsiveness to NAC are essential. Favorable chemotherapy regimens should be developed for each sub-type. For the types with poor response to NAC, innovative regimens based on their unique clinicopathological features should be investigated.

Conflict of interest

None declared.

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Efficacy of everolimus, a novel mTOR inhibitor, against basal-like triple-negative breast cancer cells

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Patients with triple-negative breast cancers (TNBCs) typically have a poor prognosis because such cancers have no effective therapeutic targets, such as estrogen receptors for endocrine therapy or human epidermal growth factor receptor 2 (HER2) receptors for anti-HER2 therapy. As the phosphatidylinositol 3′ kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) cascade is activated in TNBCs, mTOR is a potential molecular target for anticancer therapy. In this study, we investigated the antitumor activities of everolimus, an oral mTOR inhibitor, in nine TNBC cell lines. Everolimus effectively inhibited cell growth at concentrations under 100 nM (IC_{50}) in five cell lines and even in the 1-nM range in three of the five cell lines. To identify specific characteristics that could be used as predictive markers of efficacy, we evaluated the expressions of proteins in the mTOR cascade, basal markers, and cancer stem cell markers using western blotting, fluorescent *in situ* hybridization (FISH), or immunohistochemistry. All five of the sensitive cell lines were categorized as a basal-like subtype positive for either epidermal growth factor receptor (EGFR) or CK5/6, although resistant cell lines were not of this subtype and tended to exhibit the characteristics of cancer stem cells, with decreased E-cadherin and the increased expression of Snail or Twist. *In vivo* assays demonstrated antitumor activity in a mouse xenograft model of basal-like breast cancer, rather than non-basal breast cancer. These results suggest that everolimus has favorable activity against basal-like subtypes of TNBCs. Epidermal growth factor receptor and CK5/6 are positive predictive markers of the TNBC response to everolimus, while cancer stem cell markers are negative predictive markers. (*Cancer Sci* 2012; 103: 1665–1671)

Triple-negative breast cancers (TNBCs) are defined as estrogen receptor (ER)-negative, progesterone receptor (PGR)-negative, and human epidermal growth factor receptor 2 (HER2)-negative tumors; these tumors account for 11–23% of all breast cancers.[1–3] Triple-negative breast cancers follow a more aggressive clinical course than other forms of breast cancers and have a poor prognosis.[1] As TNBCs have no indications for endocrine therapy or HER2 inhibitors, which are the main treatment options for breast cancers, novel molecular-targeted therapies against TNBCs are crucially needed.

Triple-negative breast cancers are a heterogeneous population containing a subgroup that is extremely sensitive to chemotherapy, while another subgroup is resistant to such therapy.[2,4–6] For example, familial breast cancers with the *BRCA1/2* germline mutation are frequently included in the TNBC category that is chemosensitive.[5] In contrast, metaplastic carcinoma of the breast, which often lacks ER, PGR, and HER2 expressions, is quite chemoresistant.[6] Gene expression profiling can be used to separate breast cancers into five distinct molecular subtypes: luminal A (ER or PGR positive and HER2 negative); luminal B (ER or PGR positive and HER2 positive); HER2 overexpressing (ER or PGR negative and HER2 positive); normal breast-like; and basal-like.[7–10] Recently, Herschkowitz *et al.*[11] reported a novel subgroup of TNBCs – the claudin-low subgroup, which is characterized by low gene expressions of the tight junction proteins claudin 3, 4, 7, and E-cadherin – which is clearly different from the basal-like subtype. The claudin-low subtype has been shown to have cancer-stem-cell-like features because it exhibits a high CD44/CD24 expression ratio[12] and the upregulation of *snail* and *twist*,[13] which have been described as specific markers for cancer stem cells.[14,15] Clearly, TNBC subtypes must be classified to facilitate the development of effective therapies for individuals and to improve therapeutic outcomes.

Everolimus (RAD001) is an inhibitor of serine-threonine kinase mammalian target of rapamycin (mTOR) and has shown broad antitumor activities in preclinical models.[16,17] Everolimus has been approved for the treatment of refractory renal cell carcinoma,[18] progressive neuroendocrine tumors of pancreatic origin (PNET),[19] and subependymal Giant cell astrocytoma associated with tuberous sclerosis.[20] In addition, several clinical trials have reported the effectiveness of everolimus used in combination with trastuzumab or hormone therapy against HER2-overexpressing or hormone-receptor-overexpressing breast cancers, respectively.[21,22] However, the effect of everolimus against TNBCs has not yet been examined. The loss of function of phosphatase and tensin homolog deleted in chromosome 10 (PTEN) has been reported with varying frequencies in breast cancers[23,24] and has been shown to occur frequently in TNBCs.[23,25] As PTEN dysfunction leads to the activation of the phosphatidylinositol 3′ kinase (PI3K)/Akt/mTOR signaling pathway, mTOR is a potential molecular target for the treatment of TNBCs.

In this study, we investigated the antitumor activities of everolimus in TNBC cell lines *in vitro* and *in vivo* and identified predictive markers of the response of TNBCs to everolimus.

Material and Methods

Cell lines and reagents. The following TNBC cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) for use in this study: MDA-MB-157, MDA-MB-231, MDA-MB-436, MDA-MB-468, Hs578T, BT20, BT549, HCC38, and HCC1937. All the cell lines were cultured in modified Eagle's medium essential (MEME) or RPMI medium supplemented with 10% FBS at 37°C and in humidified 5% CO_2. Everolimus was a generous gift of Novartis Pharma AG (Basel, Switzerland). GDC0914 bismesylate and

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perifosine were purchased from Selleck Chemicals (Houston, TX, USA). Antibodies against epidermal growth factor receptor (EGFR) (2232; Cell Signaling Technology, Inc, Beverly, MA, USA), phospho-EGFR (p-EGFR; Tyr 1069) (2234; Cell Signaling), PTEN (9552; Cell Signaling), Akt (9272; Cell Signaling), phospho-Akt (p-Akt; Ser473) (9271; Cell Signaling), mTOR (2972; Cell Signaling), phospho-mTOR (p-mTOR; Ser2448) (2971; Cell Signaling), S6 ribosomal protein (2212; Cell Signaling), phospho-S6 ribosomal protein (p–S6; Ser235/236) (2211; Cell Signaling), 4EBP1 (9452; Cell Signaling), phospho-4EBP1 (p-4EBP1; Ser65) (9451; Cell Signaling), E-cadherin (4065; Cell Signaling), Snail (ab17732; Abcam, Cambridge, UK), Twist (Twist2C1a; Bio Matrix Research, Chiba, Japan), and β-actin (4967; Cell Signaling) were also purchased.

Cell proliferation assays. Cell proliferation assays were performed using a Cell Counting Kit-8 assay (CCK-8; Dojindo, Kumamoto, Japan) according to the product protocol. Briefly, cells were plated into 96-well, flat-bottomed plates at 2–3 × 10^3 cells/180 μL/well. After overnight incubation, triplicate wells were treated with varying concentrations of everolimus ranging from 0.1 to 500 nM for 96 h. The existing medium was removed and replaced with 110 μL of fresh medium containing 10 μL of CCK-8 reagent and allowed to incubate for 4 h. Absorbance was measured for each well at a wavelength of 450 nm. The percent survival and IC_{50}-values were calculated as described previously.[26]

Western blotting. Cultured cells were washed with cold PBS and lysed in M-PER buffer (Pierce, Rockford, IL, USA). The protein concentration of the supernatant was measured using the bicinchoninic acid (BCA) protein assay (Pierce). The membrane was probed with the first antibody and then with horseradish-peroxidase-conjugated secondary antibody. The bands were visualized using enhanced chemiluminescence (ECL Plus Western Blotting Detection Kit; Amersham, Piscataway, NJ, USA).

Immunohistochemistry. Cells were cultured in chamber slides for 48 h. The cultured cells were washed with PBS and fixed with 100% ethanol. The slides were then treated with 3% hydrogen peroxide for 30 min. The slides were incubated with primary antibodies against cytokeratin (CK) 5/6 protein (1:40; Dako Cytomation, Glostrup, Denmark) for 60 min at room temperature. Immunoreactions were detected using the EnVison Plus system (Dako).

Fluorescent *in situ* hybridization analysis. All the cell lines were cultured in appropriate media supplemented with 10% FBS; the FISH analyses were outsourced to SRL (Tokyo, Japan).

DNA sequencing. Sequencing was performed to detect the following mutations: in *EGFR*,[27] deletions in exon 19 (del 19) and L858R in exon 21; in *PI3KCA*,[28,29] E542K and E545K in exon 9 and H1047R in exon 20; and in *AKT1*,[30] E17K in exon 4. Briefly, the total RNAs were extracted from each cell line using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 μg of total RNA using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). The first-strand cDNA was amplified by PCR using specific primers for *EGFR*. Genomic DNA was extracted from each cell line using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany), and exon regions were amplified via PCR using specific primers for *PI3KCA* and *AKT1*. DNA sequencing of the PCR products was performed using the dideoxy chain termination method and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Small interfering RNA treatment. Individual small interfering RNA (siRNA) duplexes specific to human PTEN (Invitrogen, Carlsbad, CA, USA) or a control siRNA that does not target any sequence in the human genome (non-target control; Invitrogen) was transfected into MDA-MB-231 and BT20 cells according to the product protocol. Briefly, for each well transfection, we prepared RNAi duplex–Lipofectamine RNAiMax Transfection Reagent (Invitrogen) complexes with final a concentration of 10 nM and diluted the cells in complete growth medium without antibiotics. We added 3 × 10^3 cells to each well with RNAi duplex–Lipofectamine RNAiMax complexes. After 24 h of incubation, the medium was removed and replaced with fresh medium with 10% FBS and antibiotics containing various concentrations of everolimus for the cell proliferation assay.

Transfection of wild-type EGFR. Constructs of wild-type EGFR (EGFRwt) and empty vectors were generously contributed by Dr Kazuto Nishio (Osaka, Japan). The pVSV-G vector (BD Biosciences Clontech, Mountain View, CA, USA) for the constitution of the viral envelope and the pQCXIX constructs were co-transfected into HEK293 cells (BD Biosciences Clontech) using a FuGENE6 transfection reagent (Roche Diagnostics, Basel, Switzerland). Forty-eight hours after transfection, the culture medium was collected, and the viral particles were concentrated by centrifugation. MDA-MB-231 and MDA-MB-436 target cells were infected using a virus-containing medium according to standard procedures and were used for the cell proliferation assay.

Xenograft studies. Experiments were performed in accordance with the United Kingdom Coordinating Committee on Cancer Research Guidelines for the welfare of animals in experimental neoplasia (second edition).

Suspensions of MDA-MB-231 and MDA-MB-468 cells (5 × 10^6) were injected subcutaneously into the backs of 5-week-old BALB/cAJcl-nu/nu mice (CLEA Japan, Tokyo, Japan). After 5 weeks (tumors > 120 mm^3), the mice were randomly allocated into groups of five animals to receive everolimus (10 mg/kg per day, three times per week) or the vehicle only by oral gavage for 3 weeks. The tumor diameter and body weight of each mouse were measured three times weekly. The tumor diameters were measured using calipers three times per week to evaluate the effects of treatment, and the tumor volume was determined using the following equation: tumor volume = $ab^2/2$ (mm^3) (where a is the largest diameter of the tumor and b is the shortest diameter). Day "x" denotes the day on which the effect of the drugs was estimated and day "0" denotes the first day of treatment. All the mice were killed on day 22 after measuring their tumors.

Results

Sensitivity to everolimus in TNBC cell lines. We screened nine TNBC cell lines for sensitivity to everolimus. The IC_{50} values for everolimus in the nine cell lines ranged from 0.7 nM to over 200 nM (Fig. 1). As also shown in Figure 1, everolimus effectively inhibited growth in five of the nine cell lines at an IC_{50} under 100 nM. Among them, MDA-MB-468, Hs578T, and BT549 were highly sensitive to everolimus, with an IC_{50} of around 1 nM. We examined the induction of apoptosis in everolimus-sensitive cell lines using three different assays. However, we did not observe any significant apoptosis events (data not shown). In addition, we examined the growth-inhibitory effect of GDC0914 (a PI3K inhibitor) and perifosine (an Akt inhibitor) using MDA-MB-468 and BT549, two everolimus-sensitive cell lines, and MDA-MB-231 and MDA-MB-157, two everolimus-resistant cell lines. We found no significant differences in sensitivity to either inhibitor between everolimus-sensitive cell lines and resistant cell lines with a sub-μM IC_{50} concentration (data not shown).

Baseline expressions of proteins in the mTOR cascade. We measured the protein expressions of PTEN, p-AKT, Akt,

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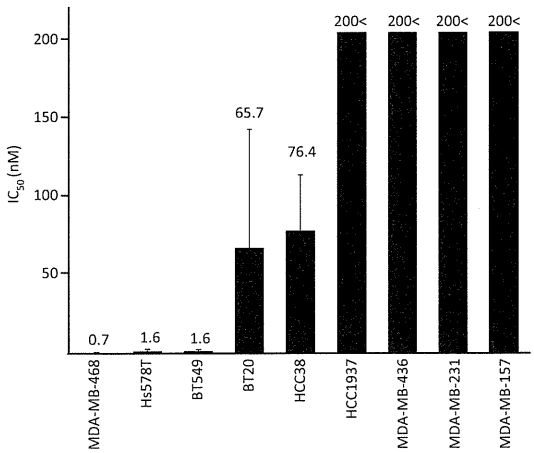

Fig. 1. IC_{50} values for everolimus of nine triple-negative breast cancer (TNBC) cell lines. Each cell line was treated with the indicated concentrations of everolimus for 96 h. Viable cell numbers were relatively quantified using the CCK-8 assay and were expressed as a percent of the untreated control.

p-mTOR, mTOR, p-S6, S6, p-4EBP1, and 4EBP1 using a western blot analysis in the nine TNBC cell lines (Fig. 2a). No differences in the expression levels of p-mTOR and mTOR, which are the targets of everolimus, were seen among the TNBC cell lines. As also shown in Figure 2(a), PTEN was not detected in five cell lines, among which MDA-MB-468, BT549, HCC1937, and MDA-MB-436 have been reported to harbor a somatic mutation of *PTEN*.[31] Among the five cell lines with a loss of PTEN, MDA-MB-468, BT549, and HCC38 were sensitive to everolimus; however, HCC1937 and MDA-MB-436 were resistant to everolimus.

mTOR cascade modulation by everolimus. To observe the effect of everolimus, we measured the protein expressions of p-S6 and S6 before and after everolimus treatment in MDA-MB-468 and BT549, two everolimus-sensitive cell lines, and MDA-MB-231 and MDA-MB-157, two everolimus-resistant cell lines. Equivalent reductions of p-S6 and S6 in response to only 0.5 nM of everolimus were observed in all four cell lines (Fig. 2b), indicating that everolimus acted on all the cell lines. We also measured the protein expressions of p-Akt and Akt and found an elevation in p-Akt after treatment with everolimus in BT549, MDA-MB-231, and MDA-MB-157 cells; however, this result was not observed in MDA-MB-468 cells.

Basal markers and cancer stem cell markers. Nielsen *et al.*[32] defined basal-like breast cancers as those showing a positive expression of EGFR or CK5/6 in TNBCs. In our results, EGFR was overexpressed in MDA-MB-468, Hs578T, BT549, and BT20 cells (Fig. 3a), while MDA-MB-468 and BT20 cells exhibited the gene amplification of *EGFR* (Fig. 3b). No mutations in the *EGFR* gene (del E746–A750, L858R) were detected in any of the cell lines (data not shown). CK5/6 was positive in MDA-MB-468, BT20, and HCC38 cells based on an immunocytochemical analysis (Fig. 3c). The status of basal-cell-like markers in the nine TNBC cell lines is shown in Table 1. According to the definition by Nielsen *et al.*,[32] we were able to categorize all five of the sensitive cell lines – MDA-MB-468, Hs578T, BT549, BT20, and HCC38 – as basal-like breast cancer. In contrast, the four resistant cell lines were not characterized as basal-like breast cancer. We also measured the expressions of the cancer stem cell marker proteins, E-cadherin, Snail, and Twist in all of the cell lines. E-cadherin was decreased in Hs578T, BT549, MDA-MB-436, MDA-MB-231, and MDA-MB-157. The expression of Snail gradually increased in the more resistant cells (HCC1937, MDA-MB-436, MDA-MB-231, and MDA-MB-157). Twist was overexpressed in MDA-MB-436 and MDA-MB-157 cells. In summary, the resistant cell lines tended to show characteristics of cancer stem cells, with decreased E-cadherin expression and the increased expression of Snail or Twist.

Effect of PTEN knockdown or EGFR overexpression on everolimus sensitivity. We used siRNA oligonucleotides to silence the expression of PTEN in the BT20 and MDA-MB-231 cell lines to test whether PTEN expression confers everolimus sensitivity. Before the cell proliferation assay, we determined that the siRNA oligonucleotides against PTEN selectively reduced the mRNA expression levels by 80% or more after 48 h and that the PTEN protein levels were reduced after 72 h, compared with a nonspecific control siRNA. However, the sensitivity of these cells to everolimus did not change with the loss of PTEN expression. The results for MDA-MB-231 are shown in Figure 4(a).

We transfected construct expressing EGFRwt into the EGFR-silenced cell lines MDA-MB-231 and MDA-MB-436 to determine the effect of EGFR expression on everolimus sensitivity. However, the overexpression of EGFR did not affect the sensitivity to everolimus. The results for MDA-MB-231 are shown in Figure 4(b).

***In vivo* antitumor effects.** To determine whether everolimus is also effective against basal-like breast cancer *in vivo*, the growth inhibitory effect was evaluated against MDA-MB-468, basal-like breast cancer cell line, and MDA-MB-231, non-basal-like breast cancer cell line, tumor xenografts. Everolimus treatment (10 mg/kg day, three times per week for 3 weeks) significantly suppressed the tumor volumes of the MDA-MB-468 xenografts, with T/C values of 38.3% ($P = 0.016$) on day 22 (Fig. 5a). On the other hand, everolimus treatment did not significantly suppress the tumor volumes of the MDA-MB-231 xenografts, with T/C values of 58.7% ($P = 0.35$) on day 22 (Fig. 5b). Body weight loss after treatment was not observed in the MDA-MB-468 and MDA-MB-231 xenograft groups (data not shown).

Discussion

Patients with TNBCs have relatively poor outcomes and cannot be treated with endocrine therapy or therapies targeted to HER2 receptors.[1] The lack of tailored therapies is problematic for the treatment of TNBCs, and the development of novel therapies is crucial. In this study, everolimus effectively inhibited growth in some TNBC cell lines with a sub-nM IC_{50} concentration *in vitro*. In previous reports, everolimus has shown limited growth-inhibitory activities against several human cancer cell lines, compared with TNBC cell lines.[33,34] Our results suggest that everolimus is a promising therapy for TNBCs.

The classification of TNBC subgroups is necessary for the future development of therapies. In this study, we found that TNBC cell lines classified as basal-like breast cancer were highly sensitive to everolimus, while cell lines characterized as cancer stem-cell-like were less sensitive to everolimus. Similar to the results of the *in vitro* assay, we found that treatment with everolimus significantly inhibited tumor growth in basal-like breast cancers *in vivo*. In a previous report, EGFR expression was associated with a poor prognosis and was a significant independent negative prognostic factor in a multivariate analysis.[32] Voduc *et al.*[35] reported that the risk of local and regional relapse in basal-like breast cancer was higher than those in other breast cancer subtypes. Our results suggest that everolimus is a promising therapy targeting basal-like TNBC.

Previous studies have suggested that the loss of PTEN may predict sensitivity to everolimus, since PTEN dysfunction leads to the activation of the PI3K/Akt/mTOR signaling

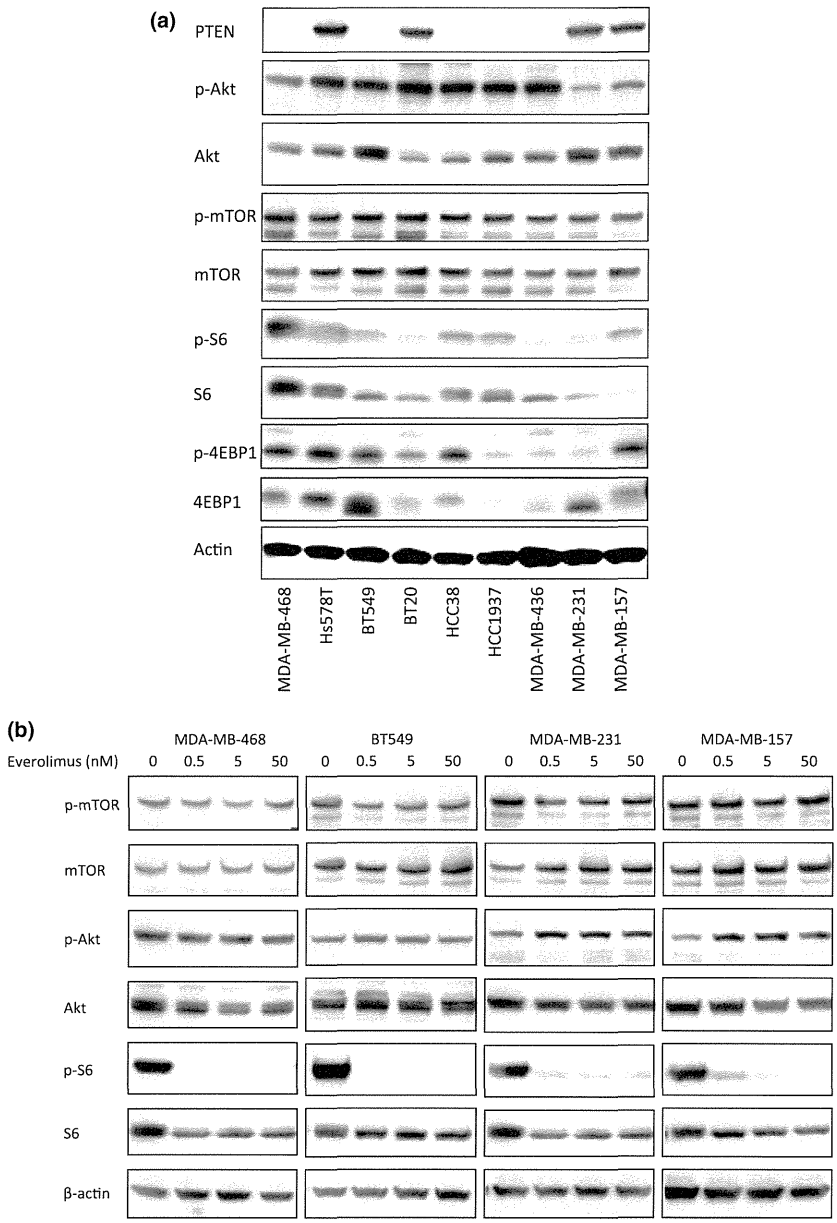

Fig. 2. Baseline expressions of mammalian target of rapamycin (mTOR) cascade proteins and mTOR cascade modulation by everolimus in nine triple-negative breast cancer (TNBC) cell lines. (a) Baseline protein expressions of PTEN, p-Akt, Akt, p-mTOR, mTOR, p-S6, S6, p-4EBP1, 4EBP1, and β-actin in nine TNBC cell lines. Ten micrograms of protein were prepared from the indicated cell lines at 60–70% confluence. The cell lines are arranged in decreasing order of sensitivity to everolimus, from left to right. β-Actin was used as a loading control. (b) Cells were untreated or treated with 0.5, 5, or 50 nM of everolimus for 1 h. Five micrograms of protein were prepared from the indicated cell lines. Equivalent reductions of p-S6 and S6 were observed with only 0.5 nM in the two sensitive cell lines (MDA-MB-468 and BT549) and the two resistant cell lines (MDA-MB-231 and MDA-MB-157).

pathway.[36,37] In the present study, three of the five cell lines that were sensitive to everolimus were PTEN-deficient cells, and the other two sensitive cell lines exhibited normal levels of PTEN protein expression. Two of the four everolimus-resistant cell lines were PTEN-deficient cells. Furthermore, in the siRNA experiment, although silencing the expression of PTEN activated Akt in BT20 and MDA-MB-231 cells, the sensitivities of these cell lines to everolimus did not change. Our results indicate that PTEN deficiency does not predict the response to everolimus in TNBCs. Furthermore, in a clinical trial with glioblastoma patients, no correlation between PTEN deficiency and the response to everolimus was observed.[38] Thus, these results suggest that mTOR was not controlled only by Akt, but also by multiple factors and signaling pathways. Furthermore, the role of PTEN in the response to everolimus may differ according to the type of cancer.

In this study, the expression of EGFR was correlated with the sensitivity to everolimus, and we considered the possibility that EGFR may be a key molecule in determining efficacy. EGFR overexpression in breast cancer has been reported in approximately 20–30% of all cases.[39,40] In TNBCs, EGFR expression was reported in 41–57% of cases and *EGFR* amplification was reported in 18%.[32,41] However, we found that EGFR transfection did not affect the sensitivity to everolimus, suggesting that there is another cascade that affects everolimus sensitivity. We observed an elevation in phosphor EGFR after everolimus treatment in a sensitive cell line, suggesting that a feedback mechanism might influence the sensitivity to everolimus. We examined the induction of apoptosis using three different assays; however, we did not observe any significant apoptosis events. The difference in the sensitivity of the TNBCs can likely be explained by some mechanism of action other than apoptosis. Further studies are needed to clarify these mechanisms and to elucidate the mechanism responsible for the sensitivity to everolimus in TNBCs. Furthermore, we suggest that combination strategies including mTOR inhibitor and PI3K or MEK inhibitor are needed in future clinical trials to overcome the multiple cascades or compensatory feedback systems resulting in cell survival.

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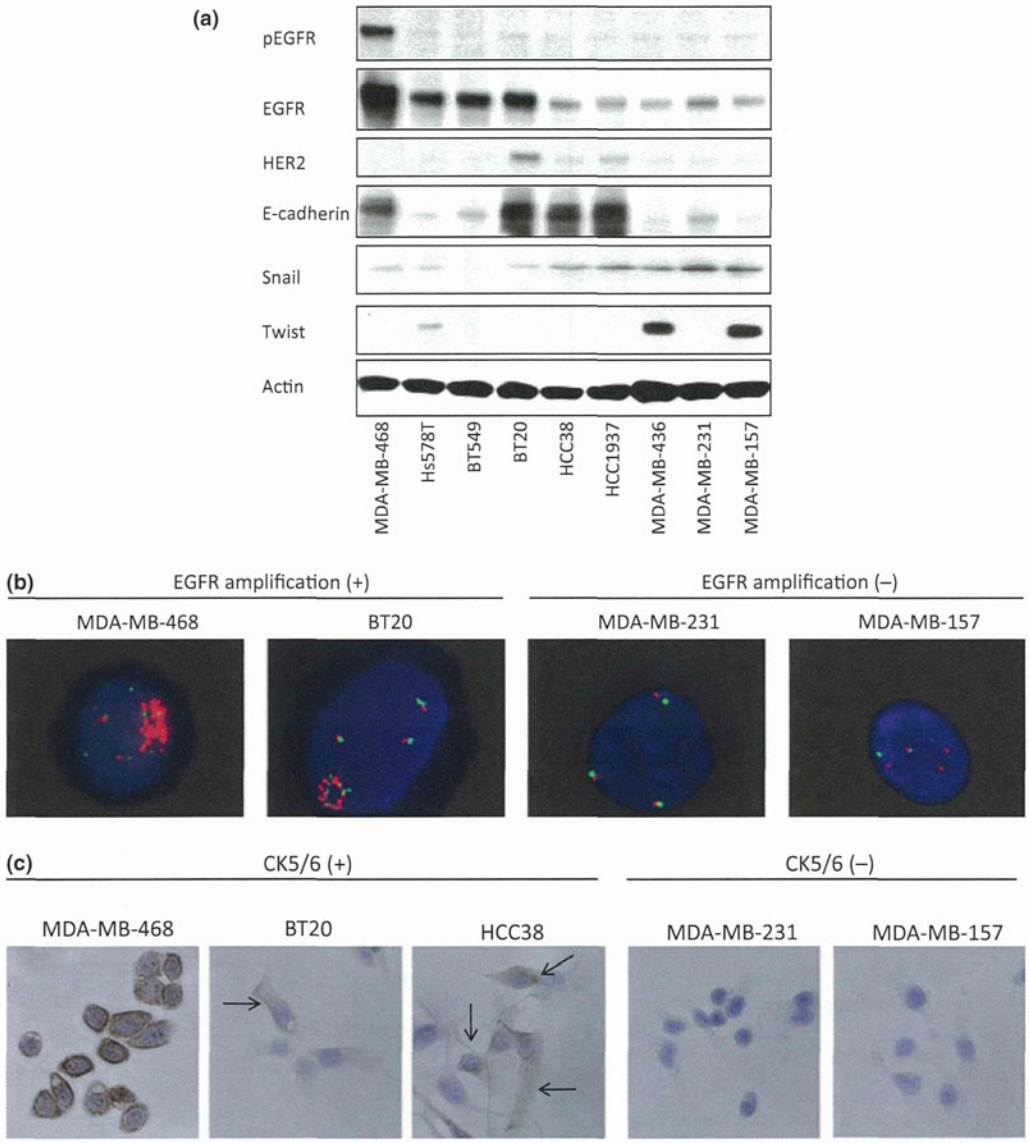

Fig. 3. Determination of breast cancer subtypes using basal markers and stem cell-like characteristics. (a) Protein expressions of p-EGFR, EGFR, HER2, E-cadherin, Snail, Twist, and β-actin in nine triple-negative breast cancer (TNBC) cell lines. Ten micrograms of protein were prepared from the indicated cell lines at 60–70% confluence. The cell lines are arranged in order of decreasing sensitivity to everolimus, from left to right. β-Actin was used as a loading control. (b) Epidermal growth factor receptor (EGFR) gene fluorescence *in situ* hybridization (FISH) analysis. MDA-MB-468 and BT20 showed the gene amplification of EGFR. The other seven TNBC cell lines did not exhibit the gene amplification of EGFR. Two positive cell lines and two negative cell lines are shown. (c) Immunohistochemical analysis of CK5/6. MDA-MB-468, BT20, and HCC38 were positive for CK5/6, and the other six cell lines were negative. Three positive cell lines and two negative cell lines are shown. Cell membranes were stained by the CK 5/6 antibody in all MDA-MB-468 cells and in some BT20 and HCC38 cells (indicated by arrows).

Table 1. The status of basal-cell-like markers in the triple-negative breast cancer (TNBC) cell lines

	MDA-MB-468	Hs578t	BT549	BT20	HCC38	HCC1937	MDA-MB-436	MDA-MB-231	MDA-MB-157
EGFR protein	+++	++	++	++	±	±	±	±	±
EGFR amplification	+++	–	–	++	–	–	–	–	–
EGFR mutation†	–	–	–	–	–	–	–	–	–
CK5/6 protein	++	–	–	+	+	–	–	–	–
PIK3CA mutation‡	–	–	–	H1047R	–	–	–	–	–
AKT1 mutation§	–	–	–	–	–	–	–	–	–

†*EGFR* mutations: deletions in exon 19 (del 19) and L858R in exon 21. ‡*PI3KCA* mutations: E542K and E545K in exon 9 and H1047R in exon 20. §*AKT1* mutation: E17K in exon 4.

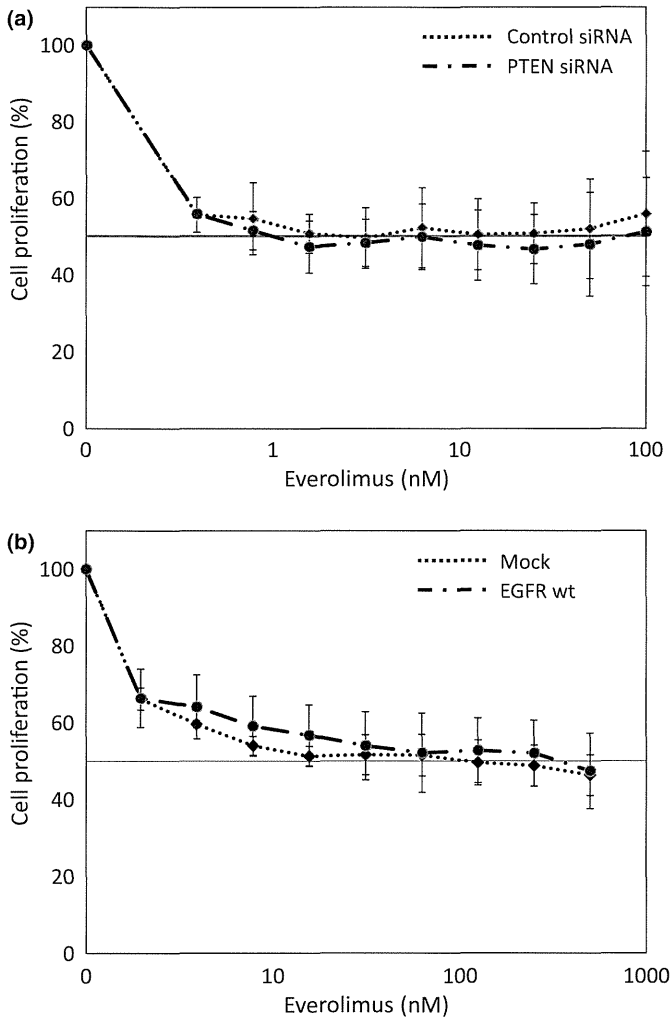

Fig. 4. Effect of PTEN or epidermal growth factor receptor (EGFR) modulation on everolimus sensitivity. (a) MDA-MB-231 and BT20 cells were transfected with siRNA specific to human PTEN or nonspecific control siRNA. After 24 h, the cells were treated with the indicated concentrations of everolimus for 72 h. The results for MDA-MB-231 are shown. (b) MDA-MB-231 and MDA-MB-436 cells were transfected with retrovirus containing either an empty vector or an EGFRwt vector and then were treated with the indicated concentrations of everolimus for 96 h. The results for MDA-MB-231 are shown.

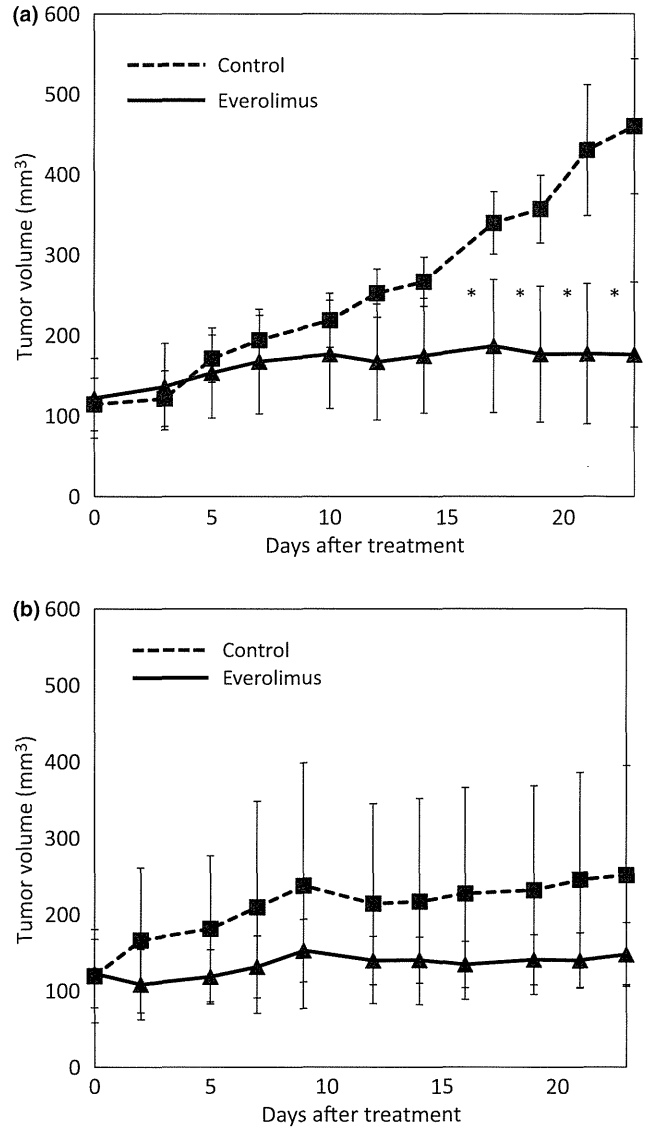

Fig. 5. Effect of RAD001 on the growth of breast cancer cell lines *in vivo*. Athymic nude mice were inoculated with MDA-MB-468 cells (a) or MDA-MB-231 cells (b). When the tumors reached an average size of 120 mm^3, mice were treated with placebo or 10 mg/kg per day RAD001, three times per week for 3 weeks. The tumors were measured twice weekly and tumor size was averaged for each treatment group. Points, mean; bars, standard deviation (SD); $*P < 0.05$, significantly different from placebo-treated mice.

Recent reports have indicated that the emergence of cancer stem cells occurs, in part, as a result of the epithelial-mesenchymal transition (EMT).[42,43] The EMT is characterized by a decrease in epithelial-specific gene expression, including E-cadherin, and a gain in mesenchymal-specific gene expression, including *twist* and *snail*.[14,15] Our data shows that cancer stem cell-positive TNBC cells tend to be resistant to everolimus. These EMT-rich TNBCs do not respond to traditional cytotoxic drugs or targeted therapies that act on signal transduction. Thus, other therapeutic strategies against these TNBCs, such as stem cell- or EMT-targeted drugs, are urgently needed.

This study suggests that everolimus is a promising agent for the treatment of TNBCs, especially basal-like breast cancers. Basal markers (EGFR and CK5/6) or cancer stem cell markers (E-cadherin, snail, or twist) may be predictive markers of the response to everolimus in TNBCs.

Acknowledgments

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Disclosure Statement

The authors have no conflicts of interest.

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