Table 3
Relationship between TOP2A or BRCA1 positivity and clinicopathological factors

	TOP2A positivity (%)	*P*-value	BRCA1 positivity (%)	*P*-value
Menopausal status				
Pre-	36	0.11	55	0.43
Post-	52		45	
Tumor size				
⩽5 cm	43	0.99	52	0.84
>5 cm	41		49	
Lymph node metastasis				
Negative	47	0.53	53	0.83
Positive	41		50	
Nuclear grade				
I + II	35	0.10	63	0.07
III	52		44	
Mitotic score				
I + II	29	<0.001	54	0.83
III	67		51	
Tubular formation				
I + II	52	0.45	62	0.46
III	42		51	
Estrogen receptor				
Positive	29	0.08	52	0.99
Negative	49		51	
Progesterone receptor				
Positive	32	0.07	48	0.82
Negative	53		46	
HER-2 status				
Positive	59	<0.05	52	0.99
Negative	35		51	

negative ER and those with negative PR were also more likely, but not significantly so, to show a higher TOP2A positivity than those with, respectively, positive ER (49% vs. 29%, $P = 0.08$) or positive PR (53% vs. 32%, $P = 0.07$) (Table 3). With respect to BRCA1, tumors with a low nuclear grade (I + II) were more likely to show a higher BRCA1 positivity than those with a high nuclear grade (III) (63% vs. 44%, $P = 0.07$).

The relationship between TOP2A or BRCA1 expression and pathological response is shown in Table 4. The pCR rate for TOP2A-positive tumors (17%) was significantly ($P < 0.005$) higher than that for TOP2A-negative tumors (2%). The pCR rate for BRCA1-negative tumors (11%) was higher than that for BRCA1-positive tumors (5%) but the difference was statistically not significant ($P = 0.31$). Multivariate analysis of TOP2A and BRCA1 expression adjusted for menopausal status, tumor size, lymph node metastasis, distant metastasis, nuclear grade, ER, PR, and HER-2 status showed that TOP2A expression was a significant factor which associated with pCR,

Table 4
Relationship between TOP2A or BRCA1 expression and pathological response to epirubicin-based regimens

Pathological response[a]	Non-pCR	pCR	*P*-value
TOP2A			
Positive	38 (83)[b]	8 (17)	<0.005
Negative	61 (98)	1 (2)	
BRCA1			
Positive	52 (95)	3 (5)	0.31
Negative	47 (89)	6 (11)	

[a] Pathological response was classified as described in Section 2.
[b] % of patients.

Table 5
Multivariate analysis of TOP2A and BRCA1 expression with pathological response to epirubicin-based regimens

	Non-pCR[a]	pCR	OR[b] (95% CI[c])	*P*-value
TOP2A				
Negative	61	1	1.00	
Positive	38	8	20.1 (1.44–279)	0.02
BRCA1				
Negative	47	6	1.00	
Positive	52	3	0.44 (0.06–3.15)	0.41

[a] Pathological response was classified as described in Section 2.
[b] Odds ratio adjusted for menopausal status, tumor size, lymph node metastasis, distant metastasis, nuclear grade, ER, PR, and HER2 status.
[c] Confidence interval.

being independent of the other factors (Table 5). Results of the combined analysis of TOP2A and BRCA1 expression are shown in Table 6. The pCR rate for TOP2A-positive and BRCA1-negative tumors (30%) was marginally significantly higher than the rates for TOP2A-positive and BRCA1-positive tumors (8%, $P = 0.06$), and significantly higher than TOP2A-negative and BRCA1-positive tumors (3%, $P < 0.05$), or TOP2A-negative and BRCA1-negative tumors (0%, $P < 0.005$).

Table 6
Relationship between combined TOP2A and BRCA1 expression and pathological response to epirubicin-based regimens

TOP2A	BRCA1	Pathological response[a]		*P*-value
		Non-pCR	pCR	
Positive	Negative	14 (70)[b]	6 (30)	
Positive	Positive	24 (92)	2 (8)	0.06[c]
Negative	Positive	28 (97)	1 (3)	<0.05[c]
Negative	Negative	33 (100)	0 (0)	<0.005[c]

[a] Pathological response was classified as described in Section 2.
[b] % of patients.
[c] *P*-values represent comparison with TOP2A-positive and BRCA1-negative tumors.

4. Discussion

Since TOP2A is a target molecule of epirubicin [14], it has been speculated that TOP2A-positive tumors are more sensitive than TOP2A-negative tumors to epirubicin-based regimens. In this connection, *in vitro* studies using various human cancer cell lines have demonstrated that TOP2A-positive cells are indeed more sensitive to doxorubicin than are TOP2A-negative cells [12]. In addition, some studies have been reported with results that demonstrate a significant association between TOP2A expression and clinical response to epirubicn-based regimens in the neoadjuvant setting [11,16]. However, the relationship between TOP2A expression and pathological response has rarely been investigated [39]. pCR appears to be a better marker than clinical response for the evaluation of sensitivity of breast tumors to chemotherapy because pCR is more closely associated with favorable prognosis than is clinical response [33–35]. For our study, we therefore adopted pCR as an endpoint marker for evaluating the response to epirubicin-based regimens. We were able to show a significantly higher pCR rate (17%) for TOP2A-positive tumors than TOP2A-negative tumors (2% pCR), which is consistent with previously reported findings indicating a significant association between TOP2A expression and clinical response [11,16].

Recently, the importance of TOP2A as a predictive factor for epirubicin-based regimens has also been demonstrated in the adjuvant setting. Knoop et al. reported that patients with *TOP2A* gene amplification show an enhanced recurrence-free survival when treated with CEF than they do when treated with cyclophosphamide plus methotrexate plus 5-fluorouracil (CMF), but a similar increase in recurrence-free survival is not seen in patients with a normal *TOP2A* gene [21]. A similar finding has been reported by Tanner et al., who detected a better relapse-free survival for patients with *TOP2A* gene amplification and treated with tailored and dose-escalated FEC than for those treated with low-dose FEC followed by cyclophosphamide plus thiotepa plus carboplatin (CTCb). This difference was not observed in patients with a normal *TOP2A* gene [23]. These studies further support the notion that TOP2A can serve as a predictive marker of sensitivity to epirubicin-based regimens. Both immunohistochemically determined TOP2A protein expression and *TOP2A* gene amplification have reported to be associated with response to epirubicin-based regimens [21,40,41]. Cardoso et al. conducted a comparative analysis of whether TOP2A expression determined by immunohistochemistry or *TOP2A* gene amplification determined by FISH is more closely associated with response the epirubicin-based regimens, found a stronger association for TOP2A expression [11]. It is further reported that the association between TOP2A overexpression and *TOP2A* gene amplification is not so strong since only 33% of breast tumors with this amplification show TOP2A overexpression, unlike the strong association between HER-2 overexpression and *HER-2* gene amplification [16].

Consistent with previously reported findings [20,42], we found that TOP2A positivity is significantly higher in tumors with a mitotic score of III (67%) or that are ER-negative (49%) or HER-2-positive (59%). Since TOP2A is a key enzyme during cell division and most strongly expressed in the S and G2/M phases [43], TOP2A-positive tumors are thought to have a higher rate of proliferation and a higher proportion of cells in the S or G2/M phases than do TOP2A-negative tumors. It thus seems reasonable to assume that TOP2A-positive tumors are more likely to have a mitotic score of III or to be ER-negative because both types of tumors are highly proliferative. Although HER-2 expression was found to be significantly associated with TOP2A expression, no significant relationship between HER-2 expression and pathological response was observed. In the present study, both Grade 2+ and 3+ were considered to be HER-2 positive but even though HER-2 positive was limited to Grade 3+, we failed to show a significant association of HER-2 status with pathological response (data not shown), indicating that TOP2A rather than HER-2 is a better predictive factor for response to epirubicin-based regimens. Similar results have also been reported [11]. The previously reported association between HER-2 expression and sensitivity to anthracycline-based regimens [3] is thus probably an indirect association mediated through TOP2A.

In addition to the clinical significance of TOP2A, we first investigated that of BRCA1 expression for the prediction of response to epirubicin-based regimens in breast cancers. Although BRCA1 expression alone was not significantly associated with pCR rate, combined analysis of TOP2A and BRCA1 expression was found to be very useful for the prediction of pathological response, i.e., TOP2A-positive and BRCA1-negative tumors showed a pCR rate as high as 30% while other

tumors showed a very low pCR rate of 8% or less. These results seem to suggest that, in addition to TOP2A, BRCA1 modulates sensitivity to epirubicin-based regimens. The exact reason why a lack of BRCA1 expression confers resistance to epirubicin-based regimens is currently unknown but we speculate that DNA double-strand breaks are less likely to be repaired in tumor cells defective in BRCA1 expression, resulting in cell cycle arrest and apoptosis.

In conclusion, we were able to demonstrate that a TOP2A-positive and BRCA1-negative phenotype is predictive of a high sensitivity to epirubicin-based regimens, with a pCR rate of up to 30%. Combined determination of TOP2A and BRCA1 expression by means of immunohistochemistry may be clinically useful for the prediction of tumor response to epirubicin-based regimens. Although TOP2A-positive and BRCA1-negative tumors are generally considered to have a biologically aggressive phenotype leading to a high recurrence rate, our finding seems to suggest that prognosis for such breast tumors, if properly treated with eprubicin-based regimens, could be significantly improved. The dose of epirubicin in the present study appears to be lower than that of a current standard (75 or 100 mg/m^2). However, we believe, even in such a lower dose, it is possible to study the association of biomarkers and response to epirubicin-based regimes. But it is possible that higher doses of epirubicin would give the different results though the essential findings are thought not to be affected so much. Our findings, therefore, need to be confirmed by a future study covering a larger number of patients treated with higher doses of epirubicin.

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ORIGINAL PAPER

Low nuclear grade but not cell proliferation predictive of pathological complete response to docetaxel in human breast cancers

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Abstract
Purpose Predictive factors for response to docetaxel in human breast cancers have yet to be identified. The aim of the present study was to investigate the relationship of various clinicopathological and biological parameters with pathological response to docetaxel in the neoadjuvant setting.

Methods The study population comprised 78 patients with primary breast cancers who were treated with docetaxel [60 mg/m^2; four (median) cycles, range 3–6; q3w] as neoadjuvant therapy and subsequently treated with mastectomy or breast conserving surgery. Tumor samples obtained before chemotherapy were subjected to histological examination and immunohistochemistry of HER-2 and Ki-67.

Results The pathological complete response (pCR) rate was significantly ($P = 0.04$) higher for tumors with low nuclear grade (NG-I or -II) (21%) than for tumors with high NG (NG-III) (5%). The pCR rate (20%) of small ($\leq$5 cm) tumors was marginally significantly ($P = 0.05$) higher than that of large (>5 cm) tumors (5%). Combined analysis of NG and tumor size showed that low-NG small tumors have a higher response rate (30%) than high-NG small tumors (11%; $P = 0.13$), low-NG large tumors (11%; $P = 0.15$), and high-NG large tumors (0%; $P = 0.009$). No statistically significant association was observed between pCR rate and menopausal status, lymph node status, ER, PR, HER-2, or Ki-67.

Conclusions Low nuclear grade, but not cell proliferation determined by Ki-67, is associated with a good pathological response to docetaxel. Combination of low nuclear grade and small tumor size may be useful for the selection of breast tumors with a high pCR rate (30%).

Keywords Nuclear grade · Cell proliferation · Breast cancer · Docetaxel · Chemosensitivity

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Introduction

Docetaxel, one of the taxanes, has come into wide use for the treatment of metastatic as well as primary breast

cancers (Seidman et al. 1993; ten Bokkel Huinink et al. 1994; Ravdin et al. 1995; Ravdin and Valero 1995; Bear et al. 2003). In addition to monotherapy, the sequential use of docetaxel and anthracycline-based regimens has been shown to increase the pathological response rate of primary tumors and to improve their prognosis in neoadjuvant and adjuvant settings (Bear et al. 2003). Docetaxel, however, is not effective for all breast cancers, since the response rate of metastatic tumors to docetaxel reportedly ranges from 38 to 67% (Seidman et al. 1993; ten Bokkel Huinink et al. 1994; Ravdin et al. 1995; Ravdin and Valero 1995) and that of primary tumors is 68% (Amat et al. 2003; Estevez et al. 2003). These findings indicate the importance of developing a diagnostic method which can predict the response to docetaxel with high accuracy in order to avoid unnecessary treatment.

Studies of the association of various parameters with the response to docetaxel have reported some significant results. These parameters include p53 status (Bottini et al. 2000), HER-2 overexpression/amplification (Di Leo et al. 2004), *p*-glycoprotein expression (Takamura et al. 2002), *CYP3A4* expression (Miyoshi et al. 2002), and class I and class III *β-tubulin* isotypes expression (Hasegawa et al. 2003). More recently, analysis of gene expression profiles of tumor tissues has been found useful for the prediction of response to docetaxel (Chang et al. 2003; Iwao-Koizumi et al. 2005). However, these reports are preliminary and most of them have investigated docetaxel treatment efficacy in terms of clinical response, but not of pathological response, even though pathological response is believed to be a more reliable indicator than clinical response (Kuerer et al. 1999; Fisher et al. 1998). Thus, the clinical significance of the various predictive factors which have been studied until now remains to be determined and much work needs to be done to develop a reliable predictor of docetaxel response.

Docetaxel binds to β-tubulin and causes kinetic abnormality of microtubules dynamics by enhancing their polymerization and inhibiting their depolymerization (Garcia et al. 1994; Diaz and Andreu 1993). During the metaphase, defective spindle formation induced by docetaxel activates the mitotic checkpoint and leads to cell cycle arrest during the metaphase–anaphase transition, resulting in apoptosis (Murata et al. 1994). Thus, the integrity of the mitotic checkpoint function appears to be very important for the anti-tumor activity of docetaxel to take effect. In fact, disruption of mitotic checkpoint function induced by high expression of Aurora-A has been reported to generate resistance to docetaxel in pancreatic cancer cell lines in vitro (Hata et al. 2005). It was also found that disruption of mitotic checkpoint function leads to the appearance of aneuploid cells with a morphologically characterized high nuclear grade (NG) in various types of human tumors (Tong et al. 2004; Jeng et al. 2004; Fraizer et al. 2004; Hu et al. 2005; Tatsuka et al. 2005). It has therefore been speculated that high-NG tumors are composed of aneuploid tumor cells which represent mitotic checkpoint dysfunction and thus may be resistant to docetaxel.

NG is routinely determined during clinical practice by histological examination of hematoxyline–eosine sections to assess prognosis for breast cancer patients. However, it remains to be determined whether NG is associated with docetaxel sensitivity. In the study presented here we therefore investigated the association between NG and the pathological response to docetaxel monotherapy by breast cancers in the neoadjuvant setting. In addition, we studied the association of cell proliferation determined by immunohistochemistry of Ki-67 with pathological response since it is generally believed that rapidly proliferating tumor cells are more likely to respond to chemotherapy. Since patients who achieved good pathological response, rather than good clinical response, showed improved prognosis (Kuerer et al. 1999; Fisher et al. 1998, van der Hage et al. 2001), in the present study, we have evaluated response to docetaxel pathologically.

Materials and methods

Patients and tumor samples

For this study, 78 female patients with stage II ($n = 44$), III ($n = 19$), and IV ($n = 15$) primary breast cancers were recruited from among patients at Osaka University Hospital and Osaka Medical Center for Cancer and Cardiovascular Diseases. Sixty-nine patients were treated with 3–6 cycles of docetaxel 60 mg/m^2 i.v. q3w (3 cycles for eight patients, 4 cycles for 57 patients, and 6 cycles for four patients) as neoadjuvant therapy followed by mastectomy or breast conserving surgery. The remaining nine patients were treated with docetaxel for only 1 cycle ($n = 1$) or 2 cycles ($n = 8$) because of disease progression. Tumor tissue samples were obtained from the primary tumors by means of vacuum-assisted core needle biopsy prior to chemotherapy and subjected to pathological diagnosis and determination of estrogen receptor (ER), progesterone receptor (PR), HER-2 and Ki-67. On the basis of the cut-off size 5 cm, which distinguish between T2 and T3 in the General Rules for Clinical and Pathological Recording of Breast Cancer 2005 (Inaji and Kobayashi 2005), tumor size was divided into two categories (≤5 cm and >5 cm) in Table 1. NG was determined according to the classification of the General Rules for Clinical and Pathological Recording of Breast Cancer 2005 (Inaji and Kobayashi 2005).

Table 1 Relationship between clinicopathological parameters and pathological response to docetaxel

Pathological response [a]	Non-pCR	pCR	*P* value
Menopausal status			
Pre-	28 (87) [b]	4 (13)	0.94
Post-	40 (87)	6 (13)	
Tumor size			
≤ 5 cm	32 (80)	8 (20)	0.05
> 5 cm	36 (95)	2 (5)	
Lymph node metastasis			
Negative	19 (83)	4 (17)	0.43
Positive	49 (89)	6 (11)	
Distant metastases			
Negative	54 (86)	9 (14)	0.42
Positive	14 (93)	1 (7)	
Nuclear grade			
I + II	30 (79)	8 (21)	0.04
III	36 (95)	2 (5)	

[a] Pathological response was defined as described in the Materials and Methods

[b] % of patients

Assessment of pathological response

Pathological response of breast cancers to docetaxel was assessed in the 69 patients who were treated with three or more cycles of docetaxel and were operated upon. Multiple slides prepared from the primary tumors were examined for the evaluation of chemotherapeutic effect according to the criteria in the General Rules for Clinical and Pathological Recording of Breast Cancer 2005 (Inaji and Kobayashi 2005). These criteria specify Grade 0 as No Response (almost no change in cancer cells), Grade 1 as Slight Response (1a: mild changes in cancer cells regardless of the area; 1b: marked changes in one-third or more but less than two-thirds of tumor cells), Grade 2 as Marked Response (marked changes in two-thirds or more of tumor cells) and Grade 3 as Complete Response (necrosis or disappearance of all tumor cells). Nine patients showed progression of the disease after one cycle ($n = 1$) or two cycles ($n = 8$) of docetaxel, and were switched to other chemotherapy. These nine patients were rated as pathological non-responders.

ER and PR assay

ER and PR protein levels in breast cancers were identified with an enzyme immunoassay using kits from Abbott Research Laboratories (Chicago, IL, USA) according to the manufacturer's instructions (cut-off values were 13 and 10 fmol/mg protein for ER and PR, respectively) or immunohistochemically (cut-off value was 10% for both ER and PR).

Immunohistochemical assessment of HER-2 and Ki-67 expression

The expression of HER-2 and Ki-67 was immunohistochemically evaluated by with the avidin–biotin-peroxidase method HER-2 in the 60 tumors and Ki-67 in the 58 tumors which were available for this study. In brief, endogeneous peroxidases were quenched by incubating the sections for 20 min in 3% H_2O_2, followed by several washes in methanol. In addition, antigen retrieval for Ki-67 was performed by heating the samples in 10 mmol/l citrate buffer (pH 6.0) at 95°C for 30 min. Non-specific binding was blocked by incubating the slides with Block Ace (Dainippon Sumitomo Pharma, Osaka, Japan) for 30 min, after which the samples were incubated with a polyclonal rabbit anti-c-erbB2 antibody (1:100 dilution; Nichirei Biosciences Inc., Tokyo, Japan) for HER-2 or with a mouse anti-human Mib-1 monoclonal antibody (1:100 dilution; Immunotech, Cedex, France) at 4°C overnight for Ki-67. Next, the samples were incubated with biotinylated anti-rabbit immunoglobulin G antibody for HER-2 (Vector Laboratories, Burlingame, CA, USA) or anti-mouse immunoglobulin G antibody (Vector Laboratories) for Ki-67 using the ABC Kit (Vector Laboratories) at room temperature for 30 min. The antibody complex was then visualized with 3, 3′′-diaminobenzidine tetrahydrochloride (Merck KGaA, Darmstadt, Germany).

A positive reaction for HER-2 was scored into four grades according to the intensity and pattern of the staining. Based on a previously reported method, the grades were defined thus: Grade 0: no or less than 10% membrane staining in tumor cells; Grade 1+: faint membrane staining in more than 10% of tumor cells staining of only part of the membrane; Grade 2+: weak-to-moderate staining of complete membrane in more than 10% of tumor cells; Grade 3+: strong complete membrane staining in more than 10% of tumor cells according to the method previously reported (Tsuda et al. 2002). Grade 2+ and 3+ tumors were considered to be HER-2 positive. For Ki-67 identification, nuclear staining was counted in 1,000 cancer cells and 25% was used as the cut-off value as was done in a previous study (Molino et al. 1997).

Statistical methods

The correlation of clinicopathological and biological parameters with pathological response to docetaxel was evaluated using the chi-square test. The relationship between pCR and NG was determined using a logistic regression method to obtain the odds ratio and 95% confidence interval, being adjusted for the menopausal status

and tumor stage. Statistical significance was assumed for $P < 0.05$.

Results

Relationship between clinicopathological or biological parameters and pathological response to docetaxel

Pathological response was divided into two categories, i.e., pathological complete response (pCR, Grade 3) and Non-pCR (Grades 0, 1a, 1b, and 2), to examine its relationship with clinicopathological factors (Table 1). Low-NG (I and II) tumors showed a significantly ($P = 0.04$) higher pCR rate (21%) than high-NG (III) tumors (pCR: 5%). In addition, the pCR rate of small (≤5 cm) tumors (20%) was marginally significantly ($P = 0.05$) higher than that of large (>5 cm) tumors (5%). No statistically significant association was observed between pCR rate and menopausal status, lymph node status or distant disease status. Multivariate analysis including menopausal status, tumor stage, and NG showed that only NG was a significant factor which associated with pCR, being independent of the other factors (Table 2).

The pathological response was studied for its association with biological parameters including ER, PR, HER-2, and Ki-67. Representative results of immunohistochemical examinations of HER-2 and Ki-67 in Fig. 1 show that there was no statistically significant association between pCR rate and any of the parameters (Table 3).

Table 2 Multivariate analysis of various factors

	Non-pCR	pCR	OR [a]	95%CI [b]	*P* value
Menopausal status					
Pre-	28	4	1.00		
Post-	40	6	0.94	0.24–4.47	0.94
Tumor stage					
II	36	8	1.00		
III	18	1	0.20	0.02–1.84	0.15
IV	14	1	0.33	0.03–3.09	0.33
Nuclear grade					
I + II	30	8	1.00		
III	36	2	0.18	0.03–0.99	0.04

[a] Odds ratio adjusted for menopausal status, tumor stage, and nuclear grade

[b] Confidence interval

Combination of NG and tumor size for prediction of pathological response

Since NG and tumor size were, respectively, significantly and marginally significantly associated with pathological response, breast tumors were classified into four groups according to these parameters to determine which tumor subgroup is most likely to respond to docetaxel (Fig. 2). Low-NG small tumors showed a higher response rate (30%) than high-NG small tumors (11%; $P = 0.13$), low-NG large tumors (11%; $P = 0.15$), and high-NG large tumors (0%; $P = 0.009$).

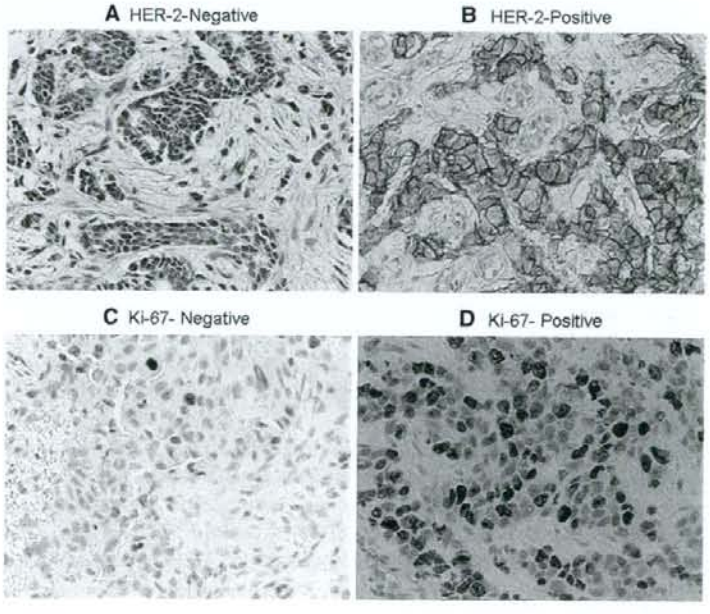

Fig. 1 Immunohistochemical staining of HER-2 and Ki-67 (×400). Strong membranous staining of HER-2 was detected in **b** but none in **a**. High and low frequency of nuclear positivity for Ki-67 were detected in **d** and **c**, respectively

Table 3 Relationship between biological parameters and pathological response to docetaxel

Pathological response [a]	Non-pCR	pCR	*P* value
Estrogen receptor			
Positive	18 (90) [b]	2 (10)	0.78
Negative	50 (88)	7 (12)	
Progesterone receptor			
Positive	17 (89)	2 (11)	0.85
Negative	51 (88)	7 (12)	
HER-2 status			
Positive	18 (86)	3 (14)	0.64
Negative	35 (90)	4 (10)	
Ki-67			
Positive	34 (89)	4 (11)	0.66
Negative	18 (86)	3 (14)	

[a] Pathological response was defined as described in the Materials and methods

[b] % of patients

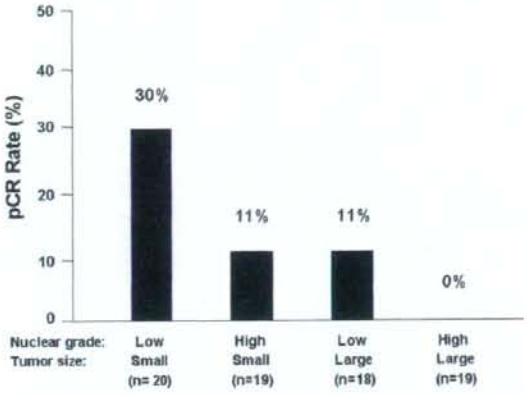

Fig. 2 Pathological complete response (pCR) rates of tumors according to nuclear grade and tumor size

Discussion

It is well established that pCR is the most reliable endpoint of neoadjuvant chemotherapy because reports of a better prognosis for patients who achieve pCR have been consistent (Kuerer et al. 1999; Fisher et al. 1998), whereas conflicting results have been reported for the relationship between clinical response and prognosis (van der Hage et al. 2001). We have been able to show that low NG tumors have a significantly ($P = 0.04$) higher pCR rate (21%) than high-NG (III) tumors (5%). High NG is reportedly associated with DNA aneuploidy (van der Hage et al. 2001), which indicates the presence of disrupted spindle checkpoint function, which is hypothesized to cause tumor resistance to docetaxel (Hata et al. 2005). In line with these findings, we have been able to show in the study presented here that resistance to docetaxel is stronger in high-NG than in low-NG tumors. On the other hand, the lack of an association between Ki-67 expression and pCR seems to indicate that cell proliferation is not an important determinant of sensitivity to docetaxel. Interestingly, it has been reported that high NG and high proliferation are associated with a good response to anthracycline-based regimens (Penault-Llorca et al. 2003; Vincent-Salomon et al. 2004; Prisack et al. 2005; Burcomber et al. 2005; Fernandez-Sanchez et al. 2006). It is clinically well established that taxanes and anthracycles are not cross-resistant and are effective for different spectrums of breast tumors. The findings of our study appear to suggest that low-NG tumors are more likely to respond to taxanes and high-NG tumors to anthracycline-based regimens.

We have also found that the pCR rate for small tumors (20%) is marginally significantly ($P = 0.05$) higher than that for large tumors (5%). The association between a high pCR rate and small tumor size has also been reported for anthracycline-based regimens (Fernandez-Sanchez et al. 2006), suggesting that such an association is not specific to the chemotherapeutic regimen but merely indicates that small tumors are more likely to achieve pCR because of their small tumor burden. When tumors are divided into subgroups according to NG and tumor size, low-NG small tumors show a pCR rate as high as 30% for docetaxel, which is comparable to the pCR rate achieved by sequential therapy with anthrayclince-based regimens and taxanes. At present, however, clinically useful predictors of response to docetaxel are not available. Our findings appear to suggest that NG and tumor size, both of which are very simple parameters that can be obtained with a routine histological examination, could be useful for the prediction of sensitivity to docetaxel.

In conclusion, low NG, but not cell proliferation determined by Ki-67, is associated with a good pathological response to docetaxel. Combination of low NG and small tumor size may prove useful for the selection of breast tumors with a high pCR rate (30%). The observations presented here need to be confirmed by a future study including a larger number of patients.

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PRECLINICAL STUDY

Growth-inhibitory effect of adiponectin via adiponectin receptor 1 on human breast cancer cells through inhibition of S-phase entry without inducing apoptosis

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Abstract Adiponectin is one of the most important adipocytokines secreted from adipose tissue. In addition to its effects on glucose and fatty acid metabolism, it has been reported that adiponectin has a direct growth-inhibitory effect on breast cancer cells. However, it still remains to be established how adiponectin affects cell cycle and apoptosis and whether or not its inhibitory effect is mediated through adiponectin receptors. Here, we demonstrated that adiponectin treatment resulted in a significant dose-dependent growth inhibition of both MDA-MB-231 and T47D cells. In both cell lines, the G0/G1 population significantly increased after adiponectin treatment, but apoptosis was not induced. High expression of mRNA and protein of adiponectin receptor 1 was observed, but expression of adiponectin receptor 2 was very low in both cell lines. Treatment with small interference RNA against adiponectin receptor 1 significantly reduced the growth inhibition induced by adiponectin in both cell lines. Taken together, adiponectin decreases breast cancer cell proliferation by inhibiting the entry into S-phase without inducing apoptosis, and this inhibitory effect is mediated through adiponectin receptor 1.

Keywords Adiponectin · AdipoR1 · AdipoR2 · Breast cancer · Cell cycle

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Abbreviations

AdipoR1	Adiponectin receptor 1
AdipoR2	Adiponectin receptor 2
PBS	Phosphate-buffered saline
SDS	Sodium dodecyl sulfate
TBST	Tris-buffered saline with Tween-20
TUNEL	Terminal deoxynucleotidyl transferase biotin-dUTP Nick End Labeling
WST1	Water-soluble tetrazolium

Introduction

Adiponectin is one of the most important adipocytokines secreted from adipose tissue. In contrast to other adipocytokines such as tumor necrosis factor-α and leptin, serum adiponectin concentration correlates inversely with obesity. This peptide hormone plays a preventive role in the pathogenesis of diabetes through the modulation of glucose and fatty acid metabolism and insulin sensitivity in various stromal and epithelial cells, and in the pathogenesis of atherosclerosis through the inhibition of vascular smooth muscle and endothelial cell proliferation.

In addition, we have recently shown that low adiponectin concentration is significantly associated with an increased risk of breast cancer [1], a finding that was recently confirmed by Mantzoros et al. [2]. It has also been demonstrated that adiponectin concentration is inversely related to the risk of endometrial cancer [3], prostate cancer [4], and gastric cancer [5]. These results seem to suggest that the well-established association of obesity with a high risk for various types of cancer might be explained at least in part by the downregulation of adiponectin, and that adiponectin might have a growth-inhibitory effect on various types of cancer.

Recently, two adiponectin receptor isoforms, AdipoR1 and AdipoR2, have been cloned [6]. In mice, AdipoR1 is expressed in various organs such as skeletal muscle, lung, and spleen, and AdipoR2 is expressed predominantly in the liver [6]. In humans, AdipoR1 and AdipoR2 are expressed in the islet cells of the pancreas, macrophages, adipocytes and vascular smooth muscle [7–9]. Very recently, we have been able to show that both AdipoR1 and AdipoR2 are expressed in breast cancer cells. Although it has been reported that adiponectin inhibits the proliferation of breast cancer cells in vitro, it still remains to be established whether or not this inhibitory effect is mediated through these adiponectin receptors. It also remains to be studied how adiponectin affects cell cycle and apoptosis. In the present study, we have investigated these issues using human breast cancer cells in vitro.

Materials and methods

Cell culture and reagents

MDA-MB-231 cells were maintained in Dulbecco's modified Eagle's medium/Ham's F-12. MCF-7, T47D and HepG2 cells were maintained in RPMI 1640 medium. All media were purchased from Sigma (St. Louis, MO) and supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, UT) and 1% antibiotic–antimycotic solution (Gibco/Invitrogen Carlsbad, CA). One normal breast epithelial cell line (MCF-10A) was maintained in mammary epithelial growth medium (Cambrex Bio Science Walkersville Inc., Walkersville, MD) according to the manufacturer's instructions. All cell lines were maintained in a humidified incubator at 37°C in a 5% CO_2 atmosphere.

Recombinant adiponectin protein was obtained from Biovendor (Heidelberg, Germany). Antibodies against AdipoR1 and AdipoR2 were obtained from Immuno-Biological Laboratories (Gunma, Japan).

Cell viability assay

Cells were seeded at a concentration of 2×10^3 cells/well in 100 µl of culture medium into 96-well culture plates and incubated for 24 h. Cells were washed and fresh culture medium containing various concentrations of adiponectin (10 ng/ml–30 µg/ml) was added. After adiponectin treatment, 10 µl of the cell proliferation reagent WST-1 (Chemicon International, Inc., Temecula, CA) was added in 100 µl of culture medium to each well, and the plates were incubated for 1.5 h. Absorbance of the samples was measured at 440 nm using a microplate reader (Molecular Devices, Sunnyvale, CA).

Flow cytometry

Cells were exposed to 10 µg/ml adiponectin for 24, 48, or 72 h, harvested by trypsinization, and centrifuged at 200*g* for 5 min. Cells were washed with phosphate-buffered saline (PBS), fixed in 70% ethanol at −20°C for 1 h, and stained with 50 µg/ml propidium iodide in PBS-glucose containing ribonuclease A (Sigma, 2 kU/ml) for 1 h. The DNA content of the cells was measured using a FACSCalibur flow cytometer and analyzed with ModFit software (BD Biosciences, San Jose, CA).

RNA extraction and quantitative RT-PCR for adiponectin receptors

Total RNA was extracted from the cultured cell with TRIZOL reagent according to the manufacturer's protocol (Molecular Research Center, Cincinnati, OH). Three micrograms of total RNA underwent RT for single-strand cDNA using oligo(dT)15 primer and Superscript II (Life Technologies, Inc., Bethesda, MD) and was scaled up to a final volume of 50 µl. The RT reaction was performed at 42°C for 50 min, followed by heating at 70°C for 15 min.

Real-time PCR for AdipoR1 and AdipoR2 was carried out using the ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, CA). The sequence of the primers and probes for AdipoR1 and AdipoR2 as well as the reaction conditions were described in our previous report [10]. β-Glucoronidase transcripts for quantitative control were used to normalize the transcript content of the sample. The primer and probe mixture for β-glucoronidase was purchased from Perkin-Elmer Applied Biosystems and used according to the manufacturer's protocol. The standard curves for AdipoR1, AdipoR2 and β-glucoronidase mRNA were generated using serially diluted solutions of each PCR product (10^{-12} µg PCR product for AdipoR1 and AdipoR2 and 10^{-8} µg PCR product for β-glucoronidase) as a template. Real-time PCR assays were conducted in duplicate for each sample, and the mean value was used for calculation of the relative expression levels. The final expression levels of AdipoR1 and AdipoR2 mRNA were expressed as ratios to those of β-glucoronidase.

Protein expression analysis of adiponectin receptors

At 60–70% confluence, cells were harvested by trypsinization and centrifuged at 200*g* for 5 min. The pellets were

lysed with lysis buffer (RIPA buffer: 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 2 mm phenylmethylsulfonyl fluoride, 1 mm sodium orthovanadate), and centrifuged at 10,000*g* for 5 min at 4°C. The resulting membrane extracts were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA) and blocked in blocking buffer for 1 h at room temperature. Membranes were incubated overnight at 4°C with rabbit polyclonal anti-AdipoR1 antibody or with rabbit polyclonal anti-AdipoR2 antibody (1:500 dilution, Immuno-Biological Laboratories) in Tris-buffered saline with Tween-20 (TBST). Membranes were then incubated with the secondary HRP-conjugated anti-rabbit IgG (1:5,000 dilution, DakoCytomation, Glostrup, Denmark) in TBST. Signal detection for each protein was performed using an ECL Western Blotting Reagents kit (GE Healthcare Bio-Science, Uppsala, Sweden).

Detection of apoptosis by Terminal deoxynucleotidyl transferase biotin-dUTP Nick End Labeling (TUNEL) assay

MDA-MB-231 and T47D cells were plated in slide flasks and grown for 24 h before adiponectin treatment. Cells were treated with or without 10 μg/ml of adiponectin for 48 h, then rinsed twice with PBS, and subjected to the TUNEL assay using the DeadEnd Colorimetric TUNEL System (Promega, Madison, WI). Paclitaxel (PTX) treatment (20 nM) was used as a positive control for the induction of apoptosis.

Small interfering RNA (siRNA) transfection

We obtained siRNA against AdipoR1 and against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from Dharmacon Research, Inc. (Lafayette, CO). Each siRNA was transfected into breast cancer cell lines with DharmaFECT transfection reagents in accordance with the manufacturer's instructions.

Results

Effect of adiponectin on cell proliferation and cell cycle progression

MDA-MB-231 and T47D cells were treated with various concentrations of adiponectin for 24, 48, or 72 h. Adiponectin treatment resulted in a significant dose-dependent growth inhibition of both MDA-MB-231 and T47D cells (Fig. 1a). DNA flow cytometry was performed on cells treated with 10 μg/ml adiponectin for 24, 48, or 72 h. In both cell lines, the G0/G1 population increased to about 80% after 72 h (Fig. 1b).

Influence of adiponectin on apoptosis

We examined whether adiponectin could induce apoptosis in breast cancer cell lines (MDA-MB-231 and T47D). Cells were treated with adiponectin or paclitaxel for 48 h and then subjected to the TUNEL assay. Apoptotic cells were frequently observed after treatment with paclitaxel in both cell lines, whereas apoptotic cells were rarely observed after treatment with adiponectin (Fig. 2).

Expression of adiponectin receptors (AdipoR1 and AdipoR2)

Expression of AdipoR1 and AdipoR2 mRNA was determined in five cell lines, i.e., three human breast cancer cell lines (MDA-MB-231, MCF-7, T47D), one normal breast epithelial cell line (MCF-10A), and one human hepatocellular carcinoma cell line (HepG2). AdipoR1 and AdipoR2 mRNA expression was detected in all cell lines (Fig. 3a). The expression level of AdipoR1 mRNA was similar for all cell lines. The expression level of AdipoR2 mRNA was much lower than that of AdipoR1 mRNA in MDA-MB-231, MCF-7, T47D, and MCF10A similar to that of AdipoR1 in HepG2.

Expression of AdipoR1 and AdipoR2 protein was determined in these cell lines by western blot analysis. Consistent with the expression levels of mRNA, AdipoR1 protein expression was observed at similar levels in all cell lines while AdipoR2 protein expression was much lower than AdipoR1 protein expression in MDA-MB-231, MCF-7, T47D, and MCF10A, but similar in HepG2 (Fig. 3b).

Effect of AdipoR1 knock-down on adiponectin-induced growth-inhibition

Since AdipoR1 was preferentially expressed in human breast cancer cell lines, we attempted to investigate the influence of AdipoR1 knock-down by siRNA on the growth-inhibitory effect of adiponectin. Treatment with siRNA against AdipoR1 mRNA resulted in a decrease in AdipoR1 protein expression after 48 h in MDA-MB-231 and T47D (Fig. 4a). Treatment with siRNA against GAPDH decreased GAPDH protein level but not AdipoR1

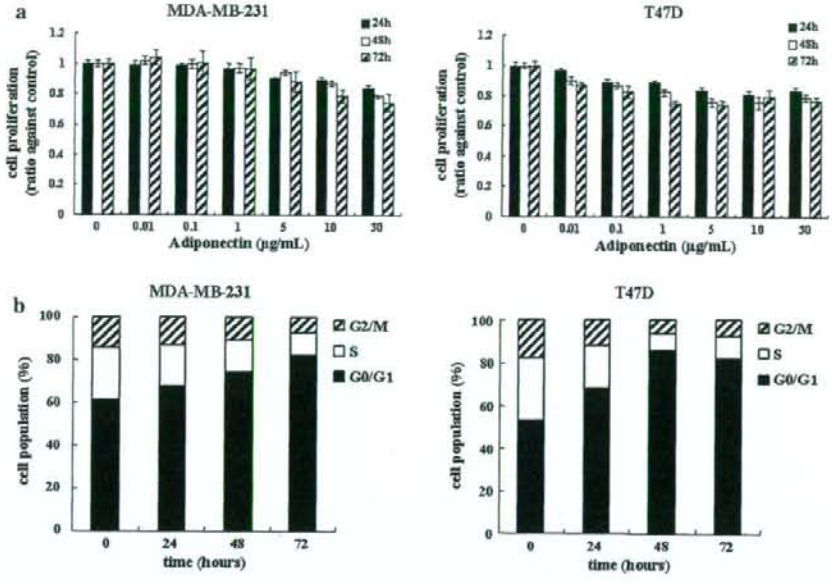

Fig. 1 Effect of adiponectin on cell proliferation and cell cycle in human breast cancer cell lines. (**a**) MDA-MB-231 and T47D cells were plated at 2×10^3 into 96-well plates and left overnight. Cells were treated with concentrations of adiponectin from 10 ng/ml to 30 μg/ml for the times indicated. The relative number of viable cells was estimated using the WST1 assay. A value of 100% was assigned to the absorbance value of each cell culture without adiponectin. Bars: mean + SD of three determinations. (**b**) Cells were treated with 10 μg/ml adiponectin and harvested at various times as indicated, fixed, and stained with propidium iodide. The cell cycle distribution of MDA-MB-231 and T47D cells was detected by DNA flow cytometry. The protocol for DNA analysis is described in Materials and Methods

protein level, and mock treatment did not affect either AdipoR1 or GAPDH protein levels.

Adiponectin treatment induced a growth inhibition in both mock-treated cell lines showing 23% reduction in MDA-MB-231 cells and 26% reduction in T47D cells. Treatment with siRNA against AdipoR1 significantly reduced the growth inhibition induced by adiponectin in both cell lines. The extent of reduction was more prominent in T47D cells than MDA-MB-231 cells (Fig. 4b).

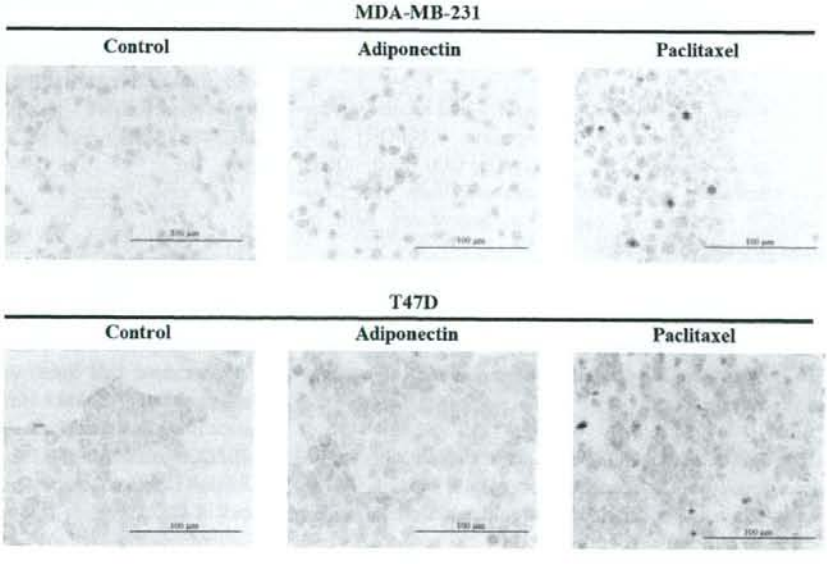

Fig. 2 Immunocytochemical staining of human breast cancer cells treated with adiponectin. Cells were treated with or without 10 μg/ml adiponectin for 48 h and fixed in ethanol. Cells undergoing apoptosis were detected using the DeadEnd TUNEL System as described in Materials and Methods. Paclitaxel (PTX) treatment (20 nM) was used as a positive control for the induction of apoptosis

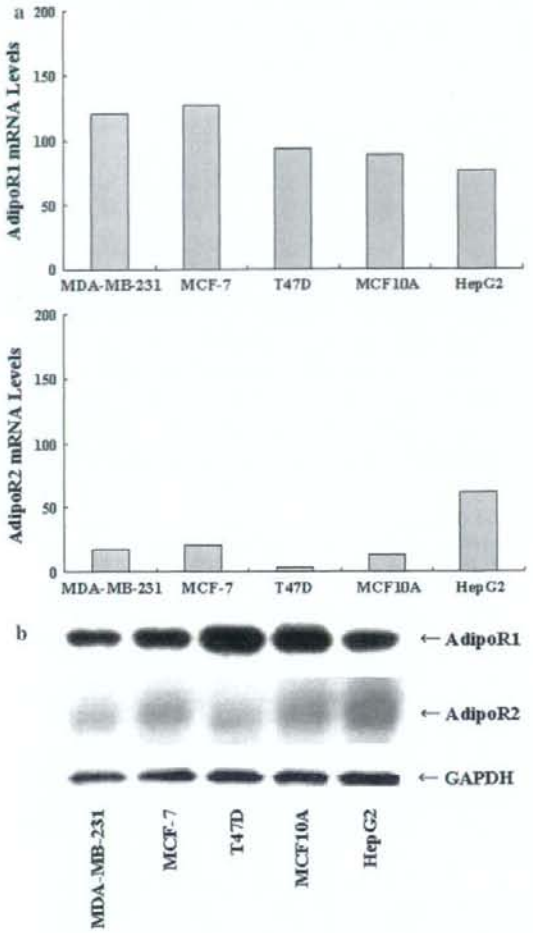

Fig. 3 Expression of AdipoR1 and AdipoR2 in cancer cell lines and a normal epithelial cell line. (**a**) Expression of AdipoR1 and AdipoR2 mRNA in various cell lines was analyzed by real-time PCR with the primers as described in Materials and Methods. (**b**) Protein expression of AdipoR1 and AdipoR2 in various cell lines was analyzed by western blotting using antibodies to AdipoR1, AdipoR2, and GAPDH

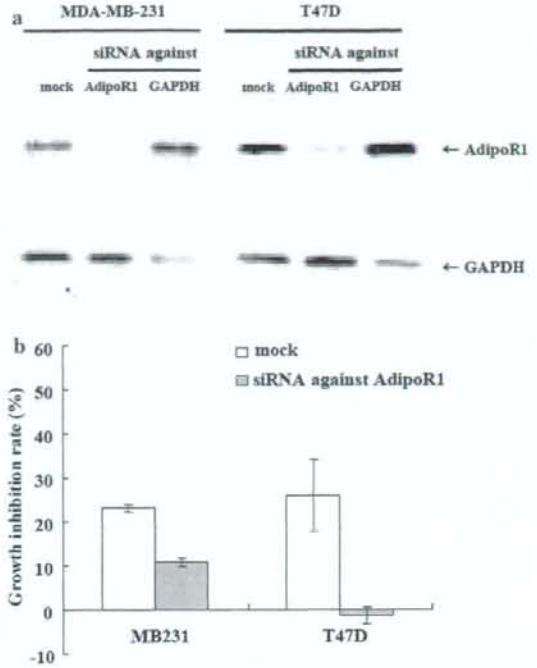

Fig. 4 Influence of siRNA against AdipoR1 on growth inhibition induced by adiponectin in breast cancer cells. (**a**) After 48 h treatment with siRNA against AdipoR1 mRNA, siRNA against GAPDH mRNA or mock treatment, cells were harvested and solubilized in cell lysis buffer. Cell lysates were subjected to western blotting analysis using anti-AdipoR1 and anti-GAPDH antibodies as described in Materials and Methods. (**b**) After 48 h treatment with siRNA against AdipoR1 mRNA or mock treatment in the presence of adiponectin, WST-1 assay was performed to analyze cell proliferation in two breast cancer cell lines. Reduction in rates of cell growth is shown on the vertical axis as a percent of the absorbance in cells treated without any siRNA in the absence of adiponectin. Bars: mean + SD of three determinations

Discussion

We have recently shown that low adiponectin concentration is significantly associated with an increased risk of breast cancer [1]. This association was also confirmed by the recent study [11]. Moreover, recent in vitro studies have shown that adiponectin is a potent inhibitor of breast cancer cell proliferation [12, 13]. In the present study, we have confirmed that adiponectin inhibits breast cancer cell proliferation in a time- and dose-dependent manner. Since the influence of adiponectin on cell cycle and apoptosis has yet to be studied, we have investigated this in the present study. We found that adiponectin inhibits cell proliferation by increasing the proportion of cells in the G0/G1 fraction and decreasing the proportion of cells in S-phase and G2/M. TUNEL assay clearly indicates that adiponectin treatment is unlikely to induce apoptosis. Together, these results demonstrate that adiponectin decreases cell proliferation by inhibiting the transition of tumor cells into S-phase without inducing apoptosis. Our results are consistent with the recent reports that adiponectin significantly inhibited cell proliferation whereas the induction of apoptosis was not observed [14, 15]. However, the effect of adiponectin on the induction of apoptosis is controversial. A few studies reported that adiponectin could induce apoptosis in MDA-MB-231 [12] or MCF-7 [13]. The reason for this discrepancy is currently unknown but the different methodology, e.g., different culture condition and

time points in cell viability assay, might explain, at least in part, such a discrepancy.

We have been able to show that both AdipoR1 and AdipoR2 mRNA are expressed in all tested cell lines including three breast cancer cell lines (MDA-MB-231, T47D, MCF-7), one normal breast epithelial cell line (MCF-10A), and one hepatocellular carcinoma cell line (HepG2). The level of AdipoR1 mRNA is much higher than that of AdipoR2 in MDA-MB-231, T47D, MCF-7, and MCF-10A, but they are expressed at a similar level in HepG2. Western blot analysis results were consistent in that AdipoR1 protein is expressed at a high level in all five cell lines while AdipoR2 protein expression is very low in MDA-MB-231, T47D, MCF-7, and MCF-10A, but is as high as AdipoR1 protein expression in HepG2. These results are consistent with the report that AdipoR2 is predominantly expressed in the liver [6], and seem to suggest that the preferentially used adiponectin receptor in breast cancer cells and normal breast epithelial cells is AdipoR1. Actually, in our previous report, the level of AdipoR1 mRNA was about 100-fold higher than that of AdipoR2 in breast tumors [10].

Thus, in order to study whether or not the growth-inhibitory effect of adiponectin is mediated through AdipoR1, we investigated the influence of siRNA against AdipoR1 mRNA on the growth inhibition induced by adiponectin in two breast cancer cell lines (T47D and MDA-MB-231). We have been able to show that the growth inhibitory effect of adiponectin is significantly cancelled by siRNA treatment in both cell lines, indicating that adiponectin exerts its growth-inhibitory effect through AdipoR1. The observation that the growth-inhibitory effect of adiponectin is almost completely abolished by siRNA in T47D but only partially abolished in MDA-MB-231 might suggest that the effect of adiponectin is mediated exclusively through AdipoR1 in T47D cells, but that other pathways, which might include the interaction with growth factors [16] and T-cadherin [17], may be operative in MDA-MB-231.

In conclusion, we have found that adiponectin decreases breast cancer cell proliferation by inhibiting the entry of cells into S-phase without inducing apoptosis, and that this inhibitory effect is mediated through AdipoR1. Our present observation is consistent with our recent report that breast tumors developing in patients with high serum adiponectin level are more likely to be small and of low histological grade [1], suggesting a possibility that measures to increase the serum adiponectin level might be useful as a new treatment of breast cancer, especially in patients with low serum adiponectin levels. The mechanism of action of adiponectin in inhibiting growth of breast cancer cells needs to be investigated in more detail in future studies.

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Epidermal growth factor receptor lacking C-terminal autophosphorylation sites retains signal transduction and high sensitivity to epidermal growth factor receptor tyrosine kinase inhibitor

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Constitutively active mutations of epidermal growth factor receptor (EGFR) (delE746_A750) activate downstream signals, such as ERK and Akt, through the phosphorylation of tyrosine residues in the C-terminal region of EGFR. These pathways are thought to be important for cellular sensitivity to EGFR tyrosine kinase inhibitors (TKI). To examine the correlation between phosphorylation of the tyrosine residues in the C-terminal region of EGFR and cellular sensitivity to EGFR TKI, we used wild-type (wt) EGFR, as well as the following constructs: delE746_A750 EGFR; delE746_A750 EGFR with substitution of seven tyrosine residues to phenylalanine in the C-terminal region; and delE746_A750 EGFR with a C-terminal truncation at amino acid 980. These constructs were transfected stably into HEK293 cells and designated HEK293/Wt, HEK293/D, HEK293/D7F, and HEK293/D-Tr, respectively. The HEK293/D cells were found to be 100-fold more sensitive to EGFR TKI (AG1478) than HEK293/Wt. Surprisingly, the HEK293/D7F and HEK293/D-Tr cells, transfected with EGFR lacking the C-terminal autophosphorylation sites, retained high sensitivity to EGFR TKI. In these three high-sensitivity cells, the ERK pathway was activated without ligand stimulation, which was inhibited by EGFR TKI. In addition, although EGFR in the HEK293/D7F and HEK293/D-Tr cells lacked significant tyrosine residues for EGFR signal transduction, phosphorylation of Src homology and collagen homology (Shc) was spontaneously activated in these cells. Our results indicate that tyrosine residues in the C-terminal region of EGFR are not required for cellular sensitivity to EGFR TKI, and that an as-yet-unknown signaling pathway of EGFR may exist that is independent of the C-terminal region of EGFR. (*Cancer Sci* 2009)

Epidermal growth factor receptor (EGFR), also termed HER1/ErbB-1, is overexpressed and activated in many cancers.[1–3] Small-molecule inhibitors of EGFR tyrosine kinase and antibodies have been shown to exhibit antitumor activity in several tumors.[4–6] Somatic mutations of EGFR tyrosine kinase in non-small cell lung cancer have been shown to be associated with hyperresponsiveness to gefitinib, a selective EGFR tyrosine kinase inhibitor (TKI).[7,8] Many investigators have subsequently reported that EGFR mutations are strong determinants of the tumor response to EGFR TKI.[9,10] Approximately 90% of non-small cell lung cancer-associated EGFR mutations in two reports consisted of two major EGFR mutations, namely, delE746_A750 in exon 19 and L858R in exon 21.[11] We previously reported hypersensitivity to EGFR TKI of a PC-9 cell line with delE746_A750 in exon 19, one of the commonly encountered mutations mentioned above, and this deletion mutant of EGFR was constitutively active and activated the ERK and Akt pathway.[12–16] Binding of the receptor with its ligand leads to homodimerization and heterodimerization of the receptor tyrosine kinase.[17,18] Thus, EGFR is a ligand-activated tyrosine kinase that ultimately delivers cellular growth signals.

Tyr-1068, Tyr-1148, and Tyr-1173 in the C-terminal region are the major autophosphorylation sites in human EGFR. These C-terminal phosphorylation sites of EGFR interact with adaptor proteins.[19,20] Phosphorylation of the C-terminal autophosphorylation sites of EGFR, triggered by epidermal growth factor (EGF), in turn trigger an intracellular signal cascade involving proteins such as ERK, Akt, Janus kinase, and signal transducer and activator of transcription.[15,21,22] Src homology and collagen homology (Shc) is a molecular adaptor protein that binds phosphorylated tyrosines within activated EGFR, and is itself phosphorylated on tyrosine residues upon stimulation of EGFR. The phosphorylated CH1 site of Shc then engages the binding site for the SH2 domain of growth factor receptor-bound protein (Grb) 2. The SH3 domain of Grb2 directly interacts with the guanyl nucleotide exchange factor son of sevenless homolog (Sos).[23,24] Sos catalyzes the conversion of GDP to GTP on Ras, resulting in Ras activation. Activated GTP-Ras recruits Raf kinase to the plasma membrane, resulting in Raf activation and phosphorylation of its downstream target ERK kinase.[25,26]

Phosphorylation of tyrosine residues at the C-terminal region of EGFR is believed to be important in cell signaling triggered by wild-type EGFR.[27,28] However, the role of this region in an active mutant of EGFR (delE746_A750) has yet to be elucidated in detail. To clarify the biological functions of the tyrosine residues at the C-terminal region of EGFR, we constructed several mutants with C-terminal-truncated or substitution of tyrosine residues to phenylalanine in the C-terminal region. We showed that EGFR lacking C-terminal autophosphorylation sites still generated signals, with retention of cellular hypersensitivity to EGFR TKI.

Materials and Methods

Expression constructs. The method used to obtain full-length cDNA of wild-type *EGFR* has been described previously.[12] Wild-type *EGFR* cDNA and 15 bp-deletion *EGFR* (delE746_A750) were introduced into pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) with a myc-tag at its C-terminus. The *EGFR* cDNA with substitution of seven tyrosine residues to phenylalanine in the C-terminal region was amplified by mutagenesis; the QuikChange®

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Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) was used for the polymerase chain reaction and a primer set was synthesized (Supporting Information Table S1). The cDNA of the C-terminal-truncated *EGFR* with 15-bp deletion (*EGFR-D-Tr*) was amplified using the following primer set: forward, CCT CCT CTT GCT GCT GGT GGT G; reverse, GAA CAAGCT TGA CAA GGT AGC GCT GGG GGT CTC. After the polymerase chain reaction products were cut with *Cla*I and *Hind*III, they were ligated to the *Cla*I and *Hind*III sites of the pcDNA3.1 expression vector containing EGFR-D cDNA. The cDNA of wild-type *EGFR* with the C-terminal truncation at amino acid 980 (*EGFR-Wt-Tr*) was made from the *Cla*I and *Xho*I fragments of the pcDNA3.1 expression vector containing wild-type EGFR and the *Cla*I and *Xho*I fragments of the pcDNA3.1 expression vector containing *EGFR-D-Tr*.

Epidermal growth factor receptor cDNA with the myc-tag in pcDNA3.1 was cut and introduced into a pQCLIN retroviral vector (BD Biosciences Clontech, San Diego, CA, USA) together with enhanced green fluorescent protein (EGFP) followed by the internal ribosome entry sequence, to monitor the expression of the inserts indirectly. A pVSV-G vector (Clontech, Palo Alto, CA, USA) for constitution of the viral envelope, pGP vector (Takara, Yotsukaichi, Japan), and the pQCXIX constructs were cotransfected into HEK293 cells using FuGENE6 transfection reagent (Roche Diagnostics, Basel, Switzerland). Briefly, 80% confluent cells cultured in a 10-cm dish were transfected with 2 μg pVSV-G vector plus 6 μg pQCXIX vector. Forty-eight hours after the transfection, the culture medium was collected and the viral particles were concentrated by centrifugation at 15 000*g* for 3 h at 4°C. The viral pellet was then resuspended in fresh Dulbecco's modified Eagle's medium (DMEM; Sigma, St Louis, MO, USA). The titer of the viral vector was calculated by counting the EGFP-positive cells that were infected in serial dilutions of a virus-containing medium and then determining the multiplicity of infection. HER2 and HER3 introduced retrovirally into HEK293 cells were used as positive controls in western blotting.

Cell culture and transfection. The human embryonic kidney HEK293 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM supplemented with 10% fetal bovine serum, penicillin, and streptomycin (Sigma) in a humidified atmosphere of 5% CO_2 at 37°C. The HEK293 cells were transfected with the viral vectors.

***In vitro* growth-inhibition assay.** The growth-inhibitory effects of AG1478 (Biomol International, Plymouth Meeting, PA, USA) in HEK293/Wt, HEK293/Wt-Tr, HEK293/D, HEK293/D7F, and HEK293/D-Tr cells were examined using a 3, 4, 5-dimethyl-2H-tetrazolium bromide (MTT) assay as described previously.[29]

Immunoprecipitation. The culture cells were washed twice with ice-cold phosphate-buffered saline (PBS) (–), and lysed with a lysis buffer containing 20 mM Tris-HCl (pH 7.0), 50 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 10 mM Na pyrophosphate, 50 mM NaF, 1 mM Na orthovanadate, 1% TritonX-100, and the Complete Mini protease inhibitor mix (Roche Diagnostics). The lysates were cleared by centrifugation at 15 000 *g* for 10 min and the protein concentrations of the supernatants were measured using a bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL, USA).

The cell lysates (500 μg) were immunoprecipitated by overnight incubation with 3 μg anti-EGFR antibody, anti-HER3 antibody (Upstate Biotechnology, Lake Placid, NY, USA), anti-HER2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-c-Myc (Roche Diagnostics), followed by further incubation with protein-G agarose (Santa Cruz Biotechnology) for 1 h. Bound proteins were washed three times with lysis buffer and eluted in Laemmli sample buffer containing 2-mercaptoethanol. The eluted proteins were subjected to 2–15% gradient sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immmunoblotted as described above.

Immunoblotting. Whole-cell lysates and the immunoprecipitates were separated using 2–15% gradient SDS-PAGE and blotted on to a polyvinylidene fluoride membrane. The membrane was probed with anti-EGFR, anti-HER3 (Upstate Biotechnology), anti-phospho(Tyr845)-EGFR, anti-phospho(Tyr1068)-EGFR, anti-phospho(Tyr1173)-EGFR, anti-HER2, anti-phospho-tyrosine, anti-p44/42 mitogen-activated protein (MAP) kinase, anti-phospho-p44/42 MAP kinase, anti-Shc, anti-phospho-Shc (Cell Signaling, Beverly, MA), anti-Sos (Santa Cruz), anti-Grb2 (BD Biosciences, San Jose, CA), and anti-c-Myc (Roche Diagnostics) antibodies by incubation for 2 h at room temperature and then with horseradish peroxidase-conjugated anti-rabbit IgG antibody or anti-mouse IgG antibody for 1 h at room temperature. Finally, the proteins were visualized with an enhanced chemiluminescence western blotting detection system (GE Healthcare, Piscataway, NJ, USA).

Chemical crosslinking assay. After treatment or no treatment with EGF (R&D Systems, Minneapolis, MN, USA) the chemical crosslinking assay was carried out in intact cells as described previously.[13] The transfected cells were washed with ice-cold PBS (+) and incubated for 30 min at room temperature in PBS (+) containing 2 mM crosslinker bis(sulfosuccinimidyl)suberate (Pierce Biotechnology). The reaction was terminated with 20 mM Tris (pH 7.5) for 15 min at room temperature. The cells were washed with PBS (+), and 15 μg protein was resolved by 2–15% gradient SDS-PAGE and then immunoblotted with anti-EGFR and anti-phospho-EGFR antibodies.

Results

Epidermal growth factor receptor lacking C-terminal autophosphorylation sites (EGFR-D-Tr and EGFR-D7F) retains signal transduction. To examine the role of the tyrosine residues in the C-terminal region of EGFR in signal transduction, we constructed vectors containing wild-type EGFR, a deletion mutant (delE746_A750 EGFR) with C-terminal truncation, or a mutant with substitution of seven tyrosine residues in the C-terminal region (Fig. 1a), and transfected these vectors into HEK293 cells with rather low expression levels of endogenous EGFR. The expression of exogenous EGFR in the transfectants was confirmed by immunoblotting with anti-EGFR antibodies (Fig. 1b).

In order to examine the signal transduction of EGFR in the transfectants, we analyzed the phosphorylation status of EGFR and its downstream molecules. Phosphorylation of EGFR at the Y845 and Y1173 tyrosine residues was detected in HEK293/Wt and HEK293/D cells cultivated in medium containing 10% fetal bovine serum (Fig. 2a). Enhanced phosphorylation of the Y1068 tyrosine residue was observed specifically in the HEK293/D cells, suggesting that Y1068 is constitutively active in delE746_A750 EGFR. This phenomenon is consistent with our previous reports.[29,30] On the other hand, no significant phosphorylation of Y845, Y1068, or Y1173 was observed in the HEK293/D7F and HEK293/D-Tr cells. ERK and Akt are major downstream pathways of EGFR. We examined the phosphorylation of ERK and Akt in the transfectants. Increased phosphorylation of ERK was observed in the HEK293/D7F, HEK293/D-Tr, and HEK293/D cells, even though HEK293/D7F and HEK293/D-Tr cells were transfected with EGFR lacking the C-terminal autophosphorylation sites.

We also examined ligand-dependent signals in these cells under the 1% serum starve medium (Fig. 2b). Ligand-stimulated phosphorylation of EGFR was observed in the HEK293/Wt cells transfected with wild-type EGFR. Constitutive phosphorylation of EGFR and a further increase in the EGFR phosphorylation response to EGF were observed in the HEK293/D cells. On the other hand, no significant phosphorylation in response to EGF binding was observed in the HEK293/D7F and HEK293/D-Tr cells. Downstream of EGFR, increased phosphorylation of ERK and Akt was observed in response to EGF in the HEK293/D7F

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