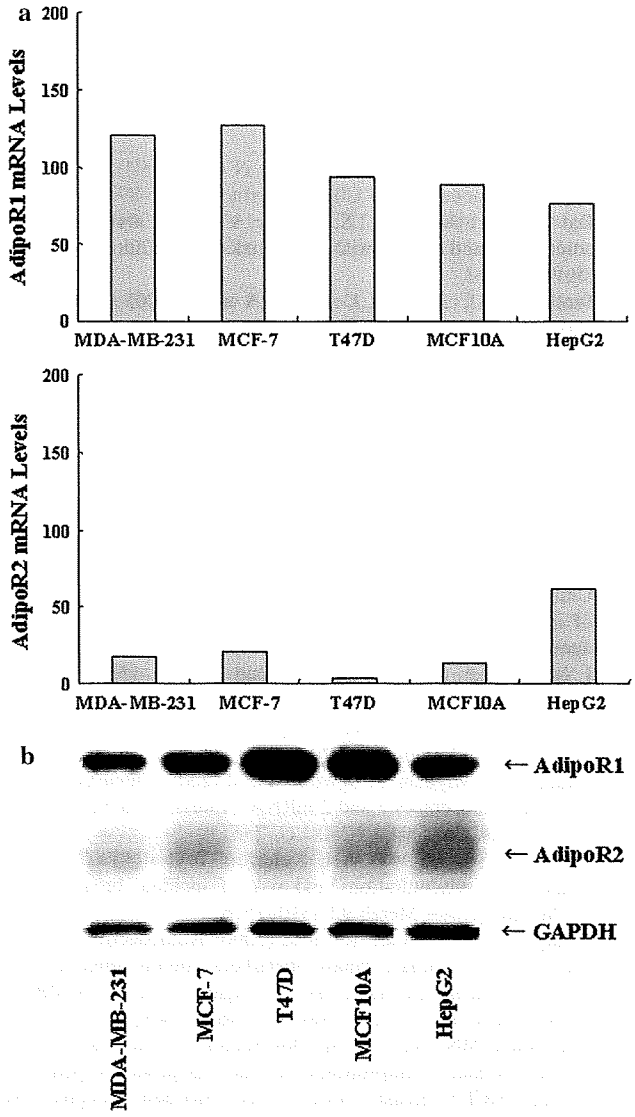

Fig. 3 Expression of AdipoR1 and AdipoR2 in cancer cell lines and a normal epithelial cell line. (**a**) Expression of AdipoR1 and AdipoR2 mRNA in various cell lines was analyzed by real-time PCR with the primers as described in Materials and Methods. (**b**) Protein expression of AdipoR1 and AdipoR2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

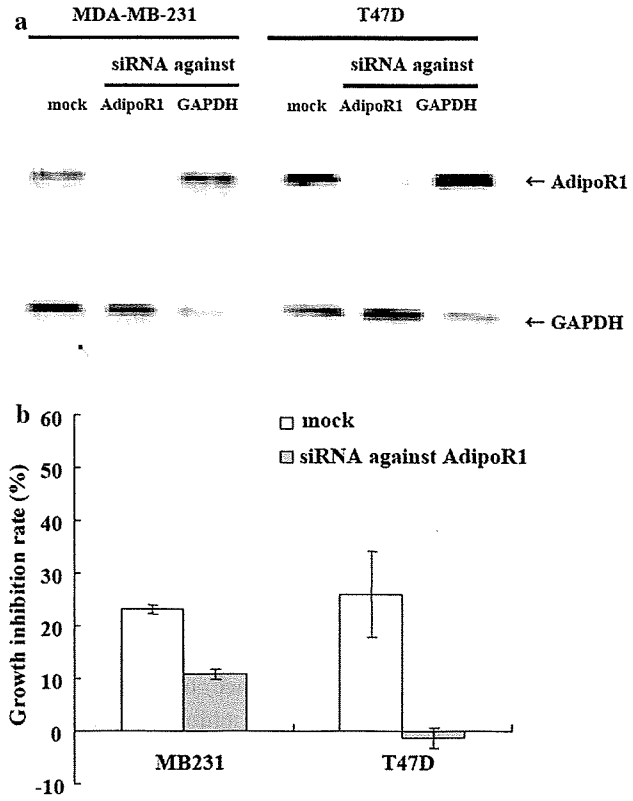

Fig. 4 Influence of siRNA against AdipoR1 on growth inhibition induced by adiponectin in breast cancer cells. (**a**) After 48 h treatment with siRNA against AdipoR1 mRNA, siRNA against GAPDH mRNA or mock treatment, cells were harvested and solubilized in cell lysis buffer. Cell lysates were subjected 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.

Acknowledgments This work was supported in part by a Grant-in-aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1. Miyoshi Y, Funahashi T, Kihara S et al (2003) Association of serum adiponectin levels with breast cancer risk. Clin Cancer Res 9:5699–5704
2. Mantzoros C, Petridou E, Dessypris N et al (2004) Adiponectin and breast cancer risk. J Clin Endocrinol Metab 89:1102–1107
3. Dal Maso L, Augustin LS, Karalis A et al (2004) Circulating adiponectin and endometrial cancer risk. J Clin Endocrinol Metab 89:1160–1163
4. Freedland SJ, Sokoll LJ, Platz EA et al (2005) Association between serum adiponectin, and pathological stage and grade in men undergoing radical prostatectomy. J Urol 174:1266–1270
5. Ishikawa M, Kitayama J, Kazama S et al (2005) Plasma adiponectin and gastric cancer. Clin Cancer Res 11:466–472
6. Yamauchi T, Kamon J, Ito Y et al (2003) Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. Nature 423:762–769
7. Kharroubi I, Rasschaert J, Eizirik DL, Cnop M (2003) Expression of adiponectin receptors in pancreatic beta cells. Biochem Biophys Res Commun 312:1118–1122
8. Chinetti G, Zawadski C, Fruchart JC, Staels B (2004) Expression of adiponectin receptors in human macrophages and regulation by agonists of the nuclear receptors PPARalpha, PPARgamma, and LXR. Biochem Biophys Res Commun 314:151–158
9. Arita Y, Kihara S, Ouchi N et al (1999) Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. Biochem Biophys Res Commun 257:79–83
10. Takahata C, Miyoshi Y, Irahara N et al (2007) Demonstration of adiponectin receptors 1 and 2 mRNA expression in human breast cancer cells. Cancer Lett 250:229–236
11. Tworoger SS, Eliassen AH, Kelesidis T et al (2007) Plasma adiponectin concentrations and risk of incident breast cancer. J Clin Endocrinol Metab 92:1510–1516
12. Wang Y, Lam JB, Lam KS et al (2006) Adiponectin modulates the glycogen synthase kinase-3beta/beta-catenin signaling pathway and attenuates mammary tumorigenesis of MDA-MB-231 cells in nude mice. Cancer Res 66:11462–11470
13. Dieudonne MN, Bussiere M, Dos Santos E et al (2006) Adiponectin mediates antiproliferative and apoptotic responses in human MCF7 breast cancer cells. Biochem Biophys Res Commun 345:271–279
14. Körner A, Pazaitou-Panayiotou K, Kelesidis T et al (2007) Total and high-molecular-weight adiponectin in breast cancer: in vitro and in vivo studies. J Clin Endocrinol Metab 92:1041–1048
15. Arditi JD, Venihaki M, Karalis KP, Chrousos GP (2007) Antiproliferative effect of adiponectin on MCF7 breast cancer cells: a potential hormonal link between obesity and cancer. Horm Metab Res 39:9–13
16. Wang Y, Lam KS, Xu JY et al (2005) Adiponectin inhibits cell proliferation by interacting with several growth factors in an oligomerization-dependent manner. J Biol Chem 280:18341–18347
17. Hug C, Wang J, Ahmad NS et al (2004) T-cadherin is a receptor for hexameric and high-molecular-weight forms of Acrp30/adiponectin. Proc Natl Acad Sci USA 101:10308–10313

J Cancer Res Clin Oncol (2008) 134:561–567
DOI 10.1007/s00432-007-0319-5

ORIGINAL PAPER

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

Yasuo Miyoshi · Masafumi Kurosumi · Junichi Kurebayashi · Nariaki Matsuura · Masato Takahashi · Eriko Tokunaga · Chiyomi Egawa · Norikazu Masuda · Seung Jin Kim · Masatsugu Okishiro · Tetsu Yanagisawa · Satsuki Ueda · Tetsuya Taguchi · Yasuhiro Tamaki · Shinzaburo Noguchi · The Collaborative Study Group of Scientific Research of the Japanese Breast Cancer Society

Received: 25 July 2007 / Accepted: 21 September 2007 / Published online: 16 October 2007

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 (≤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

Y. Miyoshi · S. J. Kim · M. Okishiro · T. Yanagisawa · S. Ueda · T. Taguchi · Y. Tamaki · S. Noguchi (✉)
Department of Breast and Endocrine Surgery,
Osaka University Graduate School of Medicine,
2-2 Yamadaoka, Suita, Osaka 565-0871, Japan
e-mail: noguchi@onsurg.med.osaka-u.ac.jp

M. Kurosumi
Department of Pathology, Saitama Cancer Center, Saitama, Japan

J. Kurebayashi
Department of Breast and Thyroid Surgery,
Kawasaki Medical School, Matsushima,
Kurashiki, Okayama, Japan

N. Matsuura
Department of Pathology, School of Allied Health Science,
Faculty of Medicine, Osaka University, Osaka, Japan

M. Takahashi
First Department of Surgery,
Hokkaido University School of Medicine, Sapporo, Japan

E. Tokunaga
Department of Surgery and Science, Graduate School of Medical Science, Kyushu University, Fukuoka, Japan

C. Egawa
Department of Surgery, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan

N. Masuda
Department of Surgery, Osaka National Hospital, Osaka, Japan

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 ($\leq$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%; $P = 0.009$).

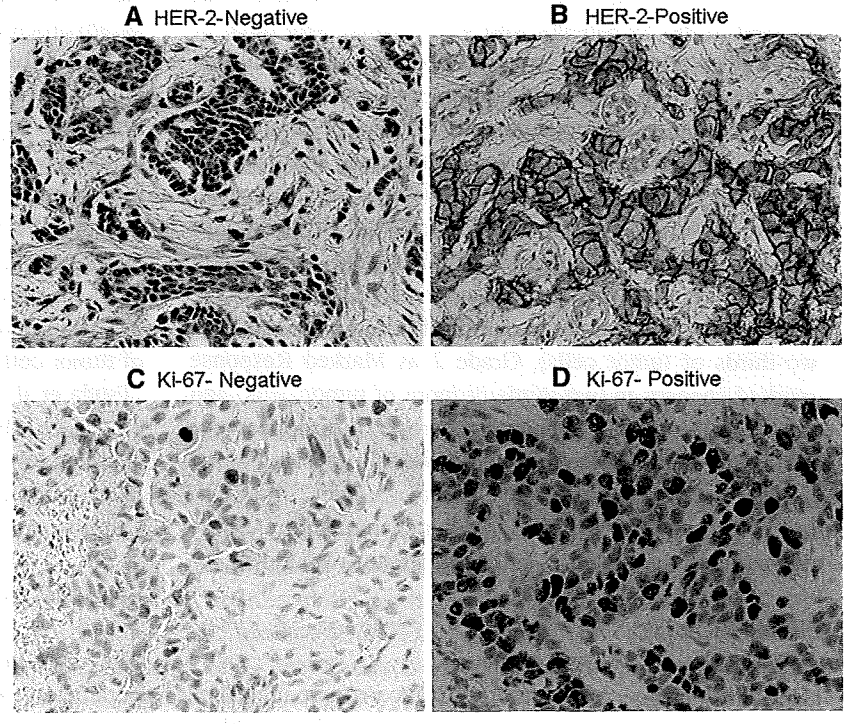

Fig. 1 Immunohistochemical staining of HER-2 and Ki-67 (×400). Strong membranous staining of HER-2 was detected in **b** but none in **a**. High and low frequency of nuclear positivity for Ki-67 were detected in **d** and **c**, respectively

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

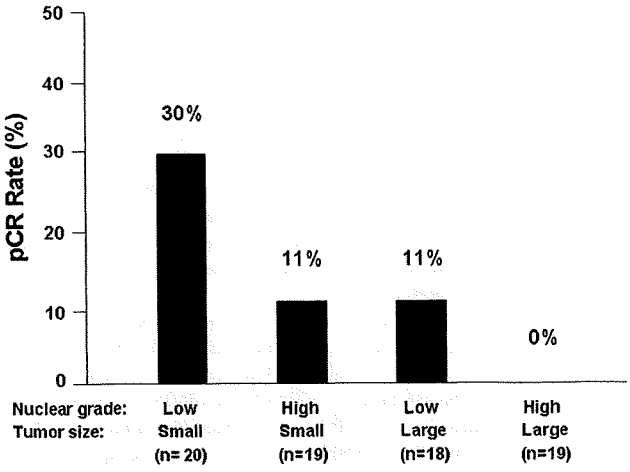

Fig. 2 Pathological complete response (pCR) rates of tumors according to nuclear grade and tumor size

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.

Acknowledgments This study was supported in part by Grants-in-Aid for Scientific Research from the Japanese Breast Cancer Society, for Cancer Research from the Ministry of Health, Labour and Welfare of Japan, and for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

Amat S, Bougnoux P, Penault-Llorca F, Fetissof F, Cure H, Kwiatkowski F, Achard JL, Body G, Dauplat J, Chollet P (2003) Neoadjuvant docetaxel for operable breast cancer induces a high pathological response and breast-conservation rate. Br J Cancer 88:1339–1345

Bear HD, Anderson S, Brown A, Smith R, Mamounas EP, Fisher B, Margolese R, Theoret H, Soran A, Wickerham DL, Wolmark N, National Surgical Adjuvant Breast, Bowel Project Protocol B-27 (2003) The effect on tumor response of adding sequential preoperative docetaxel to preoperative doxorubicin and cyclophosphamide: preliminary results from National Surgical Adjuvant Breast and Bowel Project Protocol B-27. J Clin Oncol 21:4165–4174

Bottini A, Berruti A, Bersiga A, Brizzi MP, Brunelli A, Gorzegno G, DiMarco B, Aguggini S, Bolsi G, Cirillo F, Filippini L, Betri E, Bertoli G, Alquati P, Dogliotti L (2000) p53 but not bcl-2 immunostaining is predictive of poor clinical complete response to primary chemotherapy in breast cancer patients. Clin Cancer Res 6:2751–2758

Burcombe RJ, Makris A, Richman PI, Daley FM, Noble S, Pittam M, Wright D, Allen SA, Dove J, Wilson GD (2005) Evaluation of ER, PgR, HER-2 and Ki-67 as predictors of response to neoadjuvant anthracycline chemotherapy for operable breast cancer. Br J Cancer 92:147–155

Chang JC, Wooten EC, Tsimelzon A, Hilsenbeck SG, Gutierrez MC, Elledge R, Mohsin S, Osborne CK, Chamness GC, Allred DC, O'Connell P (2003) Gene expression profiling for the prediction of therapeutic response to docetaxel in patients with breast cancer. Lancet 362:362–369

Di Leo A, Chan S, Paesmans M, Friedrichs K, Pinter T, Cocquyt V, Murray E, Bodrogi I, Walpole E, Lesperance B, Korec S, Crown J, Simmonds P, Von Minckwitz G, Leroy JY, Durbecq V, Isola J, Aapro M, Piccart MJ, Larsimont D (2004) HER-2/neu as a predictive marker in a population of advanced breast cancer patients randomly treated either with single-agent doxorubicin or single-agent docetaxel. Breast Cancer Res Treat 86:197–206

Diaz JF, Andreu JM (1993) Assembly of purified GDP-tubulin into microtubules induced by taxol and taxotere: reversibility, ligand stoichiometry, and competition. Biochemistry 32:2747–2755

Estevez LG, Cuevas JM, Anton A, Florian J, Lopez-Vega JM, Velasco A, Lobo F, Herrero A, Fortes J (2003) Weekly docetaxel as neoadjuvant chemotherapy for stage II and III breast cancer: efficacy and correlation with biological markers in a phase II, multicenter study. Clin Cancer Res 9:686–692

Fernandez-Sanchez M, Gamboa-Dominguez A, Uribe N, Garcia-Ulloa AC, Flores-Estrada D, Candelaria M, Arrieta (2006) Clinical and pathological predictors of the response to neoadjuvant anthracycline chemotherapy in locally advanced breast cancer. Med Oncol 23:171–183

Fisher B, Bryant J, Wolmark N, Mamounas E, Brown A, Fisher ER, Wickerham DL, Begovic M, DeCillis A, Robidoux A, Margolese RG, Cruz AB Jr, Hoehn JL, Lees AW, Dimitrov NV, Bear HD (1998) Effect of preoperative chemotherapy on the outcome of women with operable breast cancer. J Clin Oncol 16:2672–2685

Fraizer GC, Diaz MF, Lee IL, Grossman HB, Sen S (2004) Aurora-A/STK15/BTAK enhances chromosomal instability in bladder cancer cells. Int J Oncol 25:1631–1639

Garcia P, Braguer D, Carles G, el Khyari S, Barra Y, de Ines C, Barasoain I, Briand C (1994) Comparative effects of taxol and Taxotere on two different human carcinoma cell lines. Cancer Chemother Pharmacol 34:335–343

Hasegawa S, Miyoshi Y, Egawa C, Ishitobi M, Taguchi T, Tamaki Y, Monden M, Noguchi S (2003) Prediction of response to docetaxel by quantitative analysis of class I and III beta-tubulin isotype mRNA expression in human breast cancers. Clin Cancer Res 9:2992–2997

Hata T, Furukawa T, Sunamura M, Egawa S, Motoi F, Ohmura N, Marumoto T, Saya H, Horii A (2005) RNA interference targeting aurora kinase a suppresses tumor growth and enhances the taxane chemosensitivity in human pancreatic cancer cells. Cancer Res 65:2899–2905

Hu W, Kavanagh JJ, Deaver M, Johnston DA, Freedman RS, Verschraegen CF, Sen S (2005) Frequent overexpression of STK15/Aurora-A/BTAK and chromosomal instability in tumorigenic cell cultures derived from human ovarian cancer. Oncol Res 15:49–57

Inaji H, Kobayashi K (2005) The Journal of the Japanese Breast Cancer Society: Ggeneral rules for clinical and pathological recording of breast cancer. Breast Cancer Suppl 12:S9

Iwao-Koizumi K, Matoba R, Ueno N, Kim SJ, Ando A, Miyoshi Y, Maeda E, Noguchi S, Kato K (2005) Prediction of docetaxel response in human breast cancer by gene expression profiling. J Clin Oncol 23:422–431

Jeng YM, Peng SY, Lin CY, Hsu HC (2004) Overexpression and amplification of Aurora-A in hepatocellular carcinoma. Clin Cancer Res 10:2065–2071

Kuerer HM, Newman LA, Smith TL, Ames FC, Hunt KK, Dhingra K, Theriault RL, Singh G, Binkley SM, Sneige N, Buchholz TA, Ross MI, McNeese MD, Buzdar AU, Hortobagyi GN, Singletary SE (1999) Clinical course of breast cancer patients with complete pathologic primary tumor and axillary lymph node response to doxorubicin-based neoadjuvant chemotherapy. J Clin Oncol 17:460–469

Miyoshi Y, Ando A, Takamura Y, Taguchi T, Tamaki Y, Noguchi S (2002) Prediction of response to docetaxel by CYP3A4 mRNA expression in breast cancer tissues. Int J Cancer 97:129–132

Molino A, Micciolo R, Turazza M, Bonetti F, Piubello Q, Bonetti A, Nortilli R, Pelosi G, Cetto GL (1997) Ki-67 immunostaining in 322 primary breast cancers: associations with clinical and pathological variables and prognosis. Int J Cancer 74:433–437

Murata K, Sato T, Kanamaru R (1994) Effect of a new anticancer drug, docetaxel (RP56976), on human leukemia cell lines. Gan To Kagaku Ryoho 21:307–313

Penault-Llorca F, Cayre A, Bouchet Mishellany F, Amat S, Feillel V, Le Bouedec G, Ferriere JP, De Latour M, Chollet P (2003) Induction chemotherapy for breast carcinoma: predictive markers and relation with outcome. Int J Oncol 22:1319–1325

Prisack HB, Karreman C, Modlich O, Audretsch W, Danae M, Rezai M, Bojar H (2005) Predictive biological markers for response of invasive breast cancer to anthracycline/ cyclophosphamide-based primary (radio-)chemotherapy. Anticancer Res 25:4615–4621

Ravdin PM, Valero V (1995) Review of docetaxel (Taxotere), a highly active new agent for the treatment of metastatic breast cancer. Semin Oncol Suppl 4:17–21

Ravdin PM, Burris HA 3IIIrd, Cook G, Eisenberg P, Kane M, Bierman WA, Mortimer J, Genevois E, Bellet RE (1995) Phase II trial of docetaxel in advanced anthracycline-resistant or anthracenedione-resistant breast cancer. J Clin Oncol 13: 2879–2885

Seidman AD, Hudis C, Crown JPA (1993) Phase II evaluation of Taxotere (RP56976 NSC 628503) as initial chemotherapy for metastatic breast cancer. Proc Am Soc Clin Oncol 12:63

Takamura Y, Kobayashi H, Taguchi T, Motomura K, Inaji H, Noguchi S (2002) Prediction of chemotherapeutic response by collagen gel droplet embedded culture-drug sensitivity test in human breast cancers. Int J Cancer 98:450–455

Tatsuka M, Sato S, Kitajima S, Suto S, Kawai H, Miyauchi M, Ogawa I, Maeda M, Ota T, Takata T (2005) Overexpression of Aurora-A potentiates HRAS-mediated oncogenic transformation and is implicated in oral carcinogenesis. Oncogene 24:1122–1127

ten Bokkel Huinink WW, Prove AM, Piccart M, Steward W, Tursz T, Wanders J, Franklin H, Clavel M, Verweij J, Alakl M, Bayssas M, Kaye SB (1994) A phase II trial with docetaxel (Taxotere) in second line treatment with chemotherapy for advanced breast cancer. A study of the ORTC Early Clinical Trials Group. Ann Oncol 5:527–532

Tong T, Zhong Y, Kong J, Dong L, Song Y, Fu M, Liu Z, Wang M, Guo L, Lu S, Wu M, Zhan Q (2004) Overexpression of Aurora-A contributes to malignant development of human esophageal

squamous cell carcinoma. Clin Cancer Res 10:7304–7310 Erratum in (2005) Clin Cancer Res 11:4635

Tsuda H, Sasano H, Akiyama F, Kurosumi M, Hasegawa T, Osamura RY, Sakamoto G (2002) Evaluation of interobserver agreement in scoring immunohistochemical results of HER-2/neu (c-erbB-2) expression detected by HercepTest, Nichirei polyclonal antibody, CB11 and TAB250 in breast carcinoma. Pathol Int 52:126–134

van der Hage JA, van de Velde CJ, Julien JP, Tubiana-Hulin M, Vandervelden C, Duchateau L (2001) Preoperative chemotherapy in primary operable breast cancer: results from the European Organization for Research and Treatment of Cancer trial 10902. J Clin Oncol 19:4224–4237

Vincent-Salomon A, Rousseau A, Jouve M, Beuzeboc P, Sigal-Zafrani B, Freneaux P, Rosty C, Nos C, Campana F, Klijanienko J, Al Ghuzlan A, Sastre-Garau X, Breast Cancer Study Group (2004) Proliferation markers predictive of the pathological response and disease outcome of patients with breast carcinomas treated by anthracycline-based preoperative chemotherapy. Eur J Cancer 40:1502–1508

Available online at www.sciencedirect.com
ScienceDirect
Cancer Letters 264 (2008) 44–53
CANCER Letters
www.elsevier.com/locate/canlet

Topoisomerase IIalpha-positive and BRCA1-negative phenotype: Association with favorable response to epirubicin-based regimens for human breast cancers

Yasuo Miyoshi [a], Masafumi Kurosumi [b], Junichi Kurebayashi [c], Nariaki Matsuura [d], Masato Takahashi [e], Eriko Tokunaga [f], Chiyomi Egawa [g], Norikazu Masuda [h], Seung Jin Kim [a], Masatsugu Okishiro [a], Tetsu Yanagisawa [a], Satsuki Ueda [a], Tetsuya Taguchi [a], Yasuhiro Tamaki [a], Shinzaburo Noguchi [a,*], From the Collaborative Study Group of Scientific Research of the Japanese Breast Cancer Society

[a] *Department of Breast and Endocrine Surgery, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan*
[b] *Department of Pathology, Saitama Cancer Center, Saitama, Japan*
[c] *Department of Breast and Thyroid Surgery, Kawasaki Medical School, Matsushima, Kurashiki, Okayama, Japan*
[d] *Department of Pathology, School of Allied Health Science, Faculty of Medicine, Osaka University, Osaka, Japan*
[e] *First Department of Surgery, Hokkaido University School of Medicine, Sapporo, Japan*
[f] *Department of Surgery and Science, Graduate School of Medical Science, Kyushu University, Fukuoka, Japan*
[g] *Department of Surgery, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan*
[h] *Department of Surgery, Osaka National Hospital, Osaka, Japan*

Received 20 October 2007; received in revised form 4 January 2008; accepted 8 January 2008

Abstract

Epirubicin exerts its anti-tumor effect through binding to topoisomerase IIalpha (TOP2A) and inducing DNA double-strand breaks. BRCA1 is involved in the repair of these breaks. We investigated the relationship between TOP2A or BRCA1 immunohistochemical expression and pathological response in 108 primary breast cancers treated with epirubicin-based regimens. The pCR (pathological complete response) rate for TOP2A-positive (17%) was significantly ($P < 0.005$) higher than for TOP2A-negative (2%), while the pCR rate for BRCA1-negative (11%) was non-significantly higher than for BRCA1-positive (5%). The pCR rate of TOP2A-positive and BRCA1-negative (30%) was significantly higher than for TOP2A-negative and BRCA1-positive (3%; $P < 0.05$), or TOP2A-negative and BRCA1-negative (0%; $P < 0.005$). The TOP2A-positive and BRCA1-negative phenotype associates with a favorable response to epirubicin-based regimens.

Keywords: BRCA1; Breast cancer; Epirubicin; Pathological response; Topoisomerase IIalpha

* Corresponding author. Tel.: +81 6 6879 3772; fax: +81 6 6879 3779.
E-mail address: noguchi@onsurg.med.osaka-u.ac.jp (S. Noguchi).

0304-3835/$ - see front matter © 2008 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.canlet.2008.01.015

1. Introduction

Epirubicin, which belongs to the anthracycline family, is one of the most aggressive drugs against breast cancer and epirubicin-based regimens such as 5-FU plus epirubicin pulse cyclophosphamide (FEC) and epirubicin plus cyclophosphamide (EC) are widely used in adjuvant and neoadjuvant as well as in metastatic settings. These epirubicin-based regimes, however, although very active, are not necessarily effective for all patients. In fact, response rates of metastatic breast cancers to epirubucin-based regimens reportedly range from 50% to 60% [1,2]. On the other hand, adverse events such as leucopenia and alopecia are observed in virtually all patients treated with these regimens although their severity differs from patient to patient. In addition, a small but significant proportion of patients develop serious adverse events such as cardiac failure and myeloproliferative diseases. In order to increase the efficiency of chemotherapy and avoid unnecessary adverse events, it is therefore very important to administer chemotherapy to those patients who are likely to respond and not to those who are unlikely to respond. For purpose, reliable predictive factors for response to chemotherapy need to be developed. Until now, various biological parameters, including HER-2 [3], p-glycoprotein [4], p53 [5], estrogen receptor (ER) [6], S-phase fraction [7], Ki-67 [7], have been proposed as candidate predictive factors for response to epirubicin-based and doxorubicin-based regimens (doxorubicin is another anthracycline) but their clinical value remains controversial so that they have not yet been integrated in daily practice.

Among the predictive factors so far studied, *HER-2* gene amplification and HER-2 overexpression have been attracting a great deal of attention, and a significant association between *HER-2* gene amplification or HER-2 overexpression and a favorable response to epirubicin-based regimens has been reported [3,8,9]. However, recent studies have shown that such an association between response and HER-2 is indirect and that the direct association occurs between response and the expression of topoisomerase IIalpha (TOP2A), which is a target molecule of epirubicin [10,11]. The *TOP2A* gene is localized close to the *HER-2* gene and is often coamplified with the *HER-2* gene [12]. TOP2A plays a pivotal role in DNA replication and catalyzes the transport of one DNA double helix through another by the transient introduction of DNA double-strand breaks [13]. Anthracyclines including epirubicin and doxorubicin bind to TOP2A and stabilize the DNA double-strand breaks, resulting in cell cycle arrest and apoptosis [13,14]. In fact, an in vitro study has shown that breast cancer cells with TOP2A overexpression are more sensitive to doxorubicin [12]. It has also been reported that TOP2A expression is observed in 20–62% [11,15–19] and TOP2A gene amplification in 12–24% of human breast cancers [11,16,18,20,21]. Several lines of evidence have suggested that anti-tumor activity of the epirubicin-based regimens is associated with TOP2A expression or *TOP2A* gene amplification, although the contradictory results have also been reported [21–24].

In addition to TOP2A, BRCA1 has recently been gaining attention as a predictive factor for response to epirubicin-based regimens. BRCA1 plays an important role in double-strand DNA repair [25], and because epirubicin induces DNA double-strand breaks, it is possible that BRCA1 may modulate the response to epirubicin. In this connection, it has been reported that a mouse cell line deficient in BRCA1 displayed an increased sensitivity to the agents, including doxorubicin, which cause double-strand DNA breaks, and that induction of wild-type BRCA1 resulted in a reduced level of apoptotic cell death after treatment with DNA-damaging agents [26]. It has also been found that overexpression of BRCA1 in murine ovarian cancer cells increased the resistance to doxorubicin [27]. Furthermore, Delaloge et al. reported that 53% of locally advanced breast cancers carrying a BRCA1 mutation showed complete response to the anthracycline-based regimens while only 14% of sporadic breast cancers did, indicating that breast cancers lacking a BRCA1 function due to its mutation are more sensitive to anthracycline-based regimens [28]. Although BRCA1 mutation is rare, a significant proportion of sporadic breast cancers lack BRCA1 expression due to hypermethylation of the promoter region of the *BRCA1* gene [29], overexpression of HMGA1 [30], or overexpression of ID4 [31]. Thus, it is possible that BRCA1 expression may influence the sensitivity of sporadic breast cancers to epirubicin-based regimens. However, this possibility has hardly been investigated.

As mentioned earlier, it has been speculated that TOP2A and BRCA1 may be associated with sensitivity to epirubicin-based regimens, and thus are potentially useful as predictive factors for these regimens. Nevertheless, the association between

BRCA1 and response to epirubicin-based regimens in sporadic breast cancers has yet to be reported. This prompted us to immunohistochemically investigate TOP2A and BRCA1 expression simultaneously in breast cancer tissues obtained before the administration of epirubicin-based regimens (preoperative setting), and to study the relationship between the expression of these two markers and pathological response.

2. Materials and methods

2.1. Patients and tumor samples

For this study, 108 primary breast cancer patients at stage II ($n = 73$), III ($n = 22$), and IV ($n = 13$) were consecutively recruited. They were treated with epribucin-based regimens in the preoperative setting during the period between September 1999 and April 2004 at Osaka University Hospital, Osaka Medical Center for Cancer and Cardiovascular Diseases, and Kyushu University Hospital. Treatment with EC was used for 97 and with FEC for 11 patients and all of them were subsequently treated with breast conserving surgery or mastectomy. The epirubicin-based regimens were administered every 3 weeks for 3–6 cycles (3 cycles for 47 patients, 4 cycles for 45 patients, 5 cycles for one patient, and 6 cycles for seven patients). The remaining eight patients were treated with only 2 cycles of EC ($n = 5$) or FEC ($n = 3$) because of disease progression, and were switched to other chemotherapy (paclitaxel or docetaxel) before surgery. The dose of epirubicin for both the EC and FEC regimens was 60 mg/m^2 epirubicin for 107 patients and 100 mg/m^2 for one patient. Tumor tissue samples were obtained from primary tumors by means of vacuum-assisted core needle biopsy prior to preoperative chemotherapy. The samples were subjected to pathological diagnosis for determination of ER, PR, and HER-2 status as well as immunohistochemical study of TOP2A and BRCA1. This study was approved by the IRB of Osaka University Graduate School of Medicine.

2.2. Assessment of tumor grade and pathological response

Nuclear grade, mitotic score, and tubular formation were determined according to the criteria specified by Elston and Ellis [32]. Since the association between pathological response and patient prognosis is much stronger than that between clinical response and patient prognosis [33–35], we adopted pathological response, but not clinical response, to evaluate the effect of epirubicin-based regimens in the present study. Pathological response of breast tumors was evaluated in 100 patients who were treated with three or more cycles of the epirubicin-based regimens alone. Multiple slides prepared from primary breast tumors after preoperative chemotherapy were examined and chemotherapeutic effect was determined as for the breast tumors according to the criteria specified in the General Rules for Clinical and Pathological Recording of Breast Cancer 2005 [36]. These criteria define 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). The eight patients who showed a progressive disease after 2 cycles of the epirubicin-based regimens and were switched to other types of chemotherapy were classified as pathological non-responders.

2.3. Immunohistochemistry of HER-2, TOP2A, and BRCA1 expression

The expression of HER-2, TOP2A, and BRCA1 was evaluated immunohistochemically by using the tumor specimens obtained as described under patients and tumor samples. Sections prepared from the formalin-fixed paraffin-embedded tumor specimens were deparaffinised and rehydrated in graded alcohol. Antigens were retrieved by incubating the sections in 10 mmol/l citrate buffer (pH 6.0) at 95 °C for 50 min for TOP2A or by boiling for 15 min in a microwave oven for BRCA1. After quenching endogenous peroxidase with 3% H_2O_2 in methanol for 20 min, the resultant slides were treated with Block Ace (Dainippon Sumitomo Pharmaceutical, Osaka, Japan) for 30 min at room temperature. The samples were then incubated overnight at 4 °C with a polyclonal rabbit anti-c-erbB2 antibody (1:100 dilution; Nichirei Biosciences Inc., Tokyo, Japan) for HER-2, with a mouse monoclonal anti-TOPOIIα antibody (1:70 dilution; KiS1, DakoCytomation Inc., Carpinteria, CA) for TOP2A, or with a mouse monoclonal anti-BRCA1 antibody (1:70 dilution; Ab-1, Oncogene Science, Cambridge, MA) for BRCA1. They were subsequently incubated at room temperature for 30 min with the ABC Kit (Vector Laboratories, Burlingame, CA) using biotinylated anti-rabbit immunoglobulin G antibody for HER-2 or biotinylated anti-mouse immunoglobulin G (IgG) antibody for BRCA1. For TOP2A, incubation was performed with EnVision+ System Peroxidase (DakoCytomation) according to the manufacturer's instructions. Finally, the antibody complex was visualized with 3,3′-diaminobenzidine tetrahydrochloride (Merck, Darmstadt, Germany) and the sections were counter-stained with hematoxylin.

Positive reactions for HER-2 were scored as four grades, as previously reported [37], according to the intensity and pattern of the staining. The four grades were: 0

(no or less than 10% membrane staining in tumor cells); 1+ (faint membrane staining in more than 10% of tumor cells, partial staining of the membrane); 2+ (weak-to-moderate but complete membrane staining in more than 10% of tumor cells); 3+ (strong and complete membrane staining in more than 10% of tumor cells) Grade 2+ and 3+ tumors were considered to be HER-2 positive. The most actively stained lesions were selected microscopically and nuclear staining was counted in 1000 cancer cells without knowledge of patients outcome, and 5% and 10% were used as the respective cut-off values for TOP2A and BRCA1 according to the method described previously [17,38].

2.4. ER and PR assay

ER and progesterone receptor (PR) protein levels in the tumor specimens obtained before preoperative chemotherapy were determined in 83 cases with immunohistochemistry (cut-off value was 10% for both ER and PR) or in 21 cases with an enzyme immunoassay using kit from Abbott Research Laboratories (Chicago, IL) according to the manufacturer's instructions (cut-off values for ER and PR were 13 and 10 fmol/mg, respectively).

2.5. Statistical methods

The relationship between clinicopathological or biological parameters and pathological response was evaluated with the Fisher's exact test. Multivariate analysis of the relationship of TOP2A and BRCA1 expression with pCR was determined using a logistic regression method to obtain the odds ratio and 95% confidence interval, being adjusted for menopausal status, tumor size, lymph node metastasis, distant metastasis, nuclear grade, ER, PR, and HER-2 status. Statistical significance was assumed for $P < 0.05$.

3. Results

3.1. Relationship between clinicopathological or biological parameters and pathological response to epirubicin-based regimens

Pathological response was divided into two categories, i.e., pathological complete response (pCR, Grade 3) and non-pCR (Grades 0, 1a, 1b, and 2) for evaluation of its relationship with clinicopathological parameters (Table 1). The pCR rate (13%) of small tumors (≤5 cm) was significantly ($P < 0.05$) higher than that (0%) of large tumors (>5 cm). No statistically significant association was observed between pCR rate and menopausal status, lymph node status, distant disease status, nuclear grade, mitotic score, or tubular formation.

Table 1
Relationship between clinicopathological factors and pathological response to epirubicin-based regimens

Pathological response[a]	Non-pCR	pCR	*P*-value
Menopausal status			
Pre-	62 (94)[b]	4 (6)	0.30
Post-	37 (88)	5 (12)	
Tumor size			
≤5 cm	58 (87)	9 (13)	<0.05
>5 cm	41 (100)	0 (0)	
Lymph node metastasis			
Negative	31 (91)	3 (9)	0.99
Positive	68 (92)	6 (8)	
Distant metastases			
Negative	86 (91)	9 (9)	0.59
Positive	13 (100)	0 (0)	
Nuclear grade			
I + II	45 (94)	3 (6)	0.71
III	45 (90)	5 (10)	
Unknown	9 (90)	1 (10)	
Mitotic score			
I + II	56 (95)	3 (5)	0.25
III	34 (87)	5 (13)	
Unknown	9 (90)	1 (10)	
Tubular formation			
I + II	19 (90)	2 (10)	0.67
III	71 (92)	6 (8)	
Unknown	9 (90)	1 (10)	

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

The pathological response was further studied in terms of its relationship with biological parameters including ER, PR, and HER-2, but no significant association with any of these parameters was detected (Table 2).

Table 2
Relationship between biological parameters and pathological response to epirubicin-based regimens

Pathological response[a]	Non-pCR	pCR	*P*-value
Estrogen receptor			
Positive	28 (90)[b]	3 (10)	0.99
Negative	67 (92)	6 (8)	
Unknown	4 (100)	0 (0)	
Progesterone receptor			
Positive	28 (90)	3 (10)	0.99
Negative	51 (89)	6 (11)	
Unknown	20 (100)	0 (0)	
HER-2 status			
Positive	24 (89)	3 (11)	0.43
Negative	70 (93)	5 (7)	
Unknown	5 (83)	1 (17)	

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

3.2. TOP2A and BRCA1 expression and their relationship with clinicopathological and biological parameters or pathological response

Expression of TOP2A and BRCA1 was examined immunohistochemically in 108 tumor samples obtained before preoperative chemotherapy. Representative immunohistochemical results are shown in Fig. 1. Tumors with a high mitotic score (III) were significantly more likely to show a higher TOP2A positivity than tumors with a low mitotic score (I + II) (67% vs. 29%, $P < 0.001$). Tumors with positive HER-2 were significantly more likely to show a higher TOP2A positivity than those with negative HER-2 (59% vs. 35%, $P < 0.05$) (Table 3). Tumors with

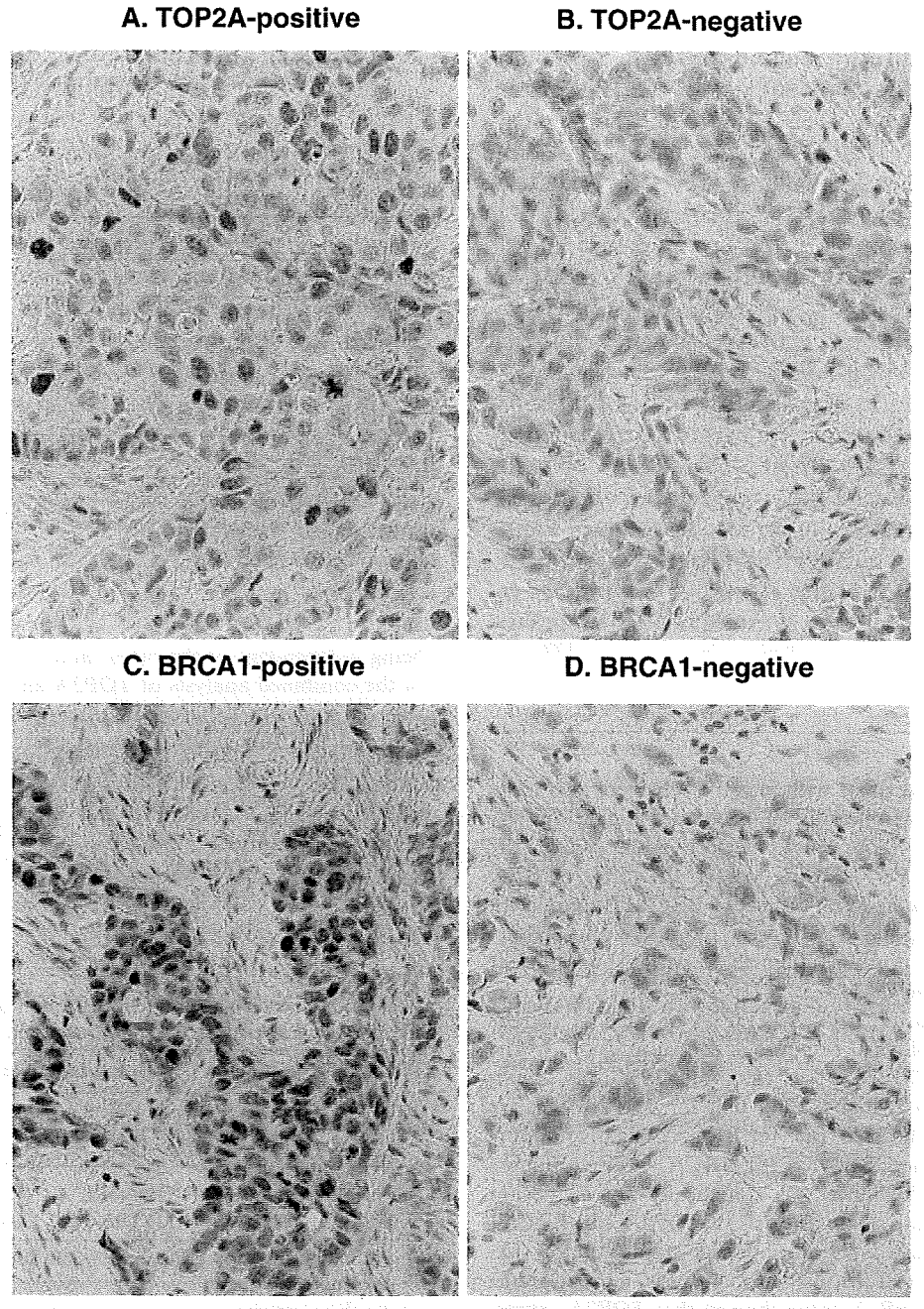

Fig. 1. Immunohistochemical staining of TOP2A and BRCA1. Representative results of immunohistochemical staining of TOP2A and BRCA1 (400×). Nuclear staining of TOP2A-positive (A), TOP2A-negative (B), BRCA1-positive (C), and BRCA1-negative (D) is seen in tumor cells.

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.

Acknowledgements

This study was supported in part by Grants-in-Aid for Scientific Research from the Japanese Breast Cancer Society, for Cancer Research from the Ministry of Health, Labour and Welfare of Japan, and for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

[1] French Epirubicin Study Group, A prospective randomized phase III trial comparing combination chemotherapy with cyclophosphamide, fluorouracil, and either doxorubicin or epirubicin, J. Clin. Oncol. 6 (1988) 679–688.

[2] Italian Multicentre Breast Study with Epirubicin, Phase III randomised study of fluorouracil, epirubicin and cyclophosphamide v fluorouracil, doxorubicin, and cyclophosphamide, in advanced breast cancer: an Italian Multicentre Trial, J. Clin. Oncol. 6 (1988) 976–982.

[3] F. Penault-Llorca, A. Cayre, F. Bouchet Mishellany, S. Amat, V. Feillel, G. Le Bouedec, J.P. Ferriere, M. De Latour, P. Chollet, Induction chemotherapy for breast carcinoma: predictive markers and relation with outcome, Int. J. Oncol. 22 (2003) 1319–1325.

[4] S. Veneroni, N. Zaffaroni, M.G. Daidone, E. Benini, R. Villa, R. Silvestrini, Expression of P-glycoprotein and in vitro or in vivo resistance to doxorubicin and cisplatin in breast and ovarian cancers, Eur. J. Cancer 30 (1994) 1002–1007.

[5] D. Kandioler-Eckersberger, C. Ludwig, M. Rudas, S. Kappel, E. Janschek, C. Wenzel, H. Schlagbauer-Wadl, M. Mittlbock, M. Gnant, G. Steger, R. Jakesz, TP53 mutation and p53 overexpression for prediction of response to neoadjuvant treatment in breast cancer patients, Clin. Cancer Res. 6 (2000) 50–56.

[6] A.E. Ring, I.E. Smith, S. Ashley, L.G. Fulford, S.R. Lakhani, Oestrogen receptor status, pathological complete response and prognosis in patients receiving neoadjuvant chemotherapy for early breast cancer, Br. J. Cancer 91 (2004) 2012–2017.

[7] A. Vincent-Salomon, A. Rousseau, M. Jouve, P. Beuzeboc, B. Sigal-Zafrani, P. Freneaux, C. Rosty, C. Nos, F. Campana, J. Klijanienko, A. Al Ghuzlan, X. Sastre-Garau, Breast Cancer Study Group, Proliferation markers predictive of the pathological response and disease outcome of patients with breast carcinomas treated by anthracycline-based preoperative chemotherapy, Eur. J. Cancer 40 (2004) 1502–1508.

[8] K. Park, J. Kim, S. Lim, S. Han, Topoisomerase II-alpha (topoII) and HER2 amplification in breast cancers and response to preoperative doxorubicin chemotherapy, Eur. J. Cancer 39 (2003) 631–634.

[9] K.I. Pritchard, L.E. Shepherd, F.P. O'Malley, I.L. Andrulis, D. Tu, V.H. Bramwell, M.N. LevineNational Cancer Institute of Canada Clinical Trials Group, HER2 and responsiveness of breast cancer to adjuvant chemotherapy, N. Engl. J. Med. 354 (2006) 2103–2111.

[10] V. Durbecq, C. Desmed, M. Paesmans, F. Cardoso, A. Di Leo, M. Mano, G. Rouas, J.Y. Leroy, C. Sotiriou, M. Piccart, A.D. Larsimont, Correlation between topoisomerase-IIalpha gene amplification and protein expression in HER-2 amplified breast cancer, Int. J. Oncol. 25 (2004) 1473–1479.

[11] F. Cardoso, V. Durbecq, D. Larsimont, M. Paesmans, J.Y. Leroy, G. Rouas, C. Sotiriou, N. Renard, V. Richard, M.J. Piccart, A. Di Leo, Correlation between complete response to anthracycline-based chemotherapy and topoisomerase II-alpha gene amplification and protein overexpression in locally advanced/metastatic breast cancer, Int. J. Oncol. 24 (2004) 201–209.

[12] T.A. Jarvinen, M. Tanner, V. Rantanen, M. Barlund, A. Borg, S. Grenman, J. Isola, Amplification and deletion of topoisomerase IIalpha associate with ErbB-2 amplification and affect sensitivity to topoisomerase II inhibitor doxorubicin in breast cancer, Am. J. Pathol. 156 (2000) 839–847.

[13] J.C. Wang, DNA topoisomerases, Annu. Rev. Biochem. 65 (1996) 635–692.
[14] J. Cummings, J.F. Smyth, DNA topoisomerase I and II as targets for rational design of new anticancer drugs, Ann. Oncol. 4 (1993) 533–543.
[15] P. Rudolph, G. MacGrogan, F. Bonichon, S.O. Frahm, I. de Mascarel, M. Trojani, M. Durand, A. Avril, J.M. Coindre, R. Parwaresch, Prognostic significance of Ki-67 and topoisomerase IIalpha expression in infiltrating ductal carcinoma of the breast. A multivariate analysis of 863 cases, Breast Cancer Res. Treat. 55 (1999) 61–71.
[16] J.S. Coon, E. Marcus, S. Gupta-Burt, S. Seelig, K. Jacobson, S. Chen, V. Renta, G. Fronda, H.D. Preisler, Amplification and overexpression of topoisomerase IIalpha predict response to anthracycline-based therapy in locally advanced breast cancer, Clin. Cancer Res. 8 (2002) 1061–1067.
[17] V. Durbecq, M. Paesmans, F. Cardoso, C. Desmedt, A. Di Leo, S. Chan, K. Friedrichs, T. Pinter, S. Van Belle, E. Murray, I. Bodrogi, E. Walpole, B. Lesperance, S. Korec, J. Crown, P. Simmonds, T.J. Perren, J.Y. Leroy, G. Rouas, C. Sotiriou, M. Piccart, D. Larsimont, Topoisomerase-II alpha expression as a predictive marker in a population of advanced breast cancer patients randomly treated either with single-agent doxorubicin or single-agent docetaxel, Mol. Cancer Ther. 3 (2004) 1207–1214.
[18] R. Bhargava, P. Lal, B. Chen, HER-2/neu and topoisomerase IIa gene amplification and protein expression in invasive breast carcinomas: chromogenic in situ hybridization and immunohistochemical analyses, Am. J. Clin. Pathol. 123 (2005) 889–895.
[19] P. Fritz, C.M. Cabrera, J. Dippon, A. Gerteis, W. Simon, W.E. Aulitzky, H. van der Kuip, c-erbB2 and topoisomerase IIalpha protein expression independently predict poor survival in primary human breast cancer: a retrospective study, Breast Cancer Res. 7 (2005) 374–384.
[20] R.E. Mueller, R.K. Parkes, I. Andrulis, F.P. O'Malley, Amplification of the TOP2A gene does not predict high levels of topoisomerase II alpha protein in human breast tumor samples, Genes Chromosomes Cancer 39 (2004) 288–297.
[21] A.S. Knoop, H. Knudsen, E. Balslev, B.B. Rasmussen, J. Overgaard, K.V. Nielsen, A. Schonau, K. Gunnarsdóttir, K.E. Olsen, H. Mouridsen, B. Ejlertsen, Danish Breast Cancer Cooperative Group: retrospective analysis of topoisomerase IIa amplifications and deletions as predictive markers in primary breast cancer patients randomly assigned to cyclophosphamide, methotrexate, and fluorouracil or cyclophosphamide, epirubicin, and fluorouracil: Danish Breast Cancer Cooperative Group, J. Clin. Oncol. 23 (2005) 7483–7490, Erratum in, J. Clin. Oncol. 24 (2006) 1015.
[22] A. Di Leo, D. Gancberg, D. Larsimont, M. Tanner, T. Jarvinen, G. Rouas, S. Dolci, J.Y. Leroy, M. Paesmans, J. Isola, M.J. Piccart, HER-2 amplification and topoisomerase IIalpha gene aberrations as predictive markers in node-positive breast cancer patients randomly treated either with an anthracycline-based therapy or with cyclophosphamide, methotrexate, and 5-fluorouracil, Clin. Cancer Res. 8 (2002) 1107–1116.
[23] Scandinavian Breast Group Trial 9401 M. Tanner, J. Isola, T. Wiklund, B. Erikstein, P. Kellokumpu-Lehtinen, P. Malmström, N. Wilking, J. Nilsson, J. Bergh, Topoisomerase IIalpha gene amplification predicts favorable treatment response to tailored and dose-escalated anthracycline-based adjuvant chemotherapy in HER-2/neu-amplified breast cancer: Scandinavian Breast Group Trial 9401, J. Clin. Oncol. 24 (2006) 2428–2436.
[24] F.P. O'Malley, S. Chia, D. Tu, L.E. Shepherd, M.N. Levin, D.G. Huntsman, V.H. Bramwell, I.L. Andrulis, K.I. Pritchard, Prognostic and predictive value of topoisomerase II alpha in a randomized trial comparing CMF to CEF in premenopausal women with node positive breast cancer (NCIC CTG MA.5), ASCO Proc. 24 (18 Suppl.) (2006) 11s.
[25] Q. Zhong, C.F. Chen, S. Li, Y. Chen, C.C. Wang, J. Xiao, P.L. Chen, Z.D. Sharp, W.H. Lee, Association of BRCA1 with the hRad50-hMre11-p95 complex and the DNA damage response, Science 285 (1999) 747–750.
[26] A. Fedier, R.A. Steiner, V.A. Schwarz, L. Lenherr, U. Haller, D. Fink, The effect of loss of Brca1 on the sensitivity to anticancer agents in p53-deficient cells, Int. J. Oncol. 22 (2003) 1169–1173.
[27] V. Sylvain, S. Lafarge, Y.J. Bignon, Dominant-negative activity of a Brca1 truncation mutant: effects on proliferation, tumorigenicity in vivo, and chemosensitivity in a mouse ovarian cancer cell line, Int. J. Oncol. 20 (2002) 845–853.
[28] S. Delaloge, P. Pautier, I. Kloos, BRCA1-linked breast cancer (BC) is highly more chemosensitive than its BRCA2-linked or sporadic counterparts, Paper presented at: 27th Congress of the European Society for Medical Oncology, Nice, France, 18–22 October, 2002, Abstract 120.
[29] K. Miyamoto, T. Fukutomi, K. Asada, K. Wakazono, H. Tsuda, T. Asahara, T. Sugimura, T. Ushijima, Promoter hypermethylation and post-transcriptional mechanisms for reduced BRCA1 immunoreactivity in sporadic human breast cancers, Jpn. J. Clin. Oncol. 32 (2002) 79–84.
[30] G. Baldassarre, S. Battista, B. Belletti, S. Thakur, F. Pentimalli, F. Trapasso, M. Fedele, G. Pierantoni, C.M. Croce, A. Fusco, Negative regulation of BRCA1 gene expression by HMGA1 proteins accounts for the reduced BRCA1 protein levels in sporadic breast carcinoma, Mol. Cell. Biol. 23 (2003) 2225–2238.
[31] N.C. Turner, J.S. Reis-Filho, A.M. Russell, R.J. Springall, K. Ryder, D. Steele, K. Savage, C.E. Gillett, F.C. Schmitt, A. Ashworth, A.N. Tutt, BRCA1 dysfunction in sporadic basal-like breast cancer, Oncogene 26 (2007) 2126–2132.
[32] C.W. Elston, I.O. Ellis, Pathological prognostic factors in breast cancer. The value of histological grade in breast cancer: experience from a large study with long-term follow-up, Histopathology 19 (1991) 403–410.
[33] B. Fisher, J. Bryant, N. Wolmark, E. Mamounas, A. Brown, E.R. Fisher, D.L. Wickerham, M. Begovic, A. DeCillis, A. Robidoux, R.G. Margolese, A.B. Cruz Jr., J.L. Hoehn, A.W. Lees, N.V. Dimitrov, H.D. Bear, Effect of preoperative chemotherapy on the outcome of women with operable breast cancer, J. Clin. Oncol. 16 (1998) 2672–2685.
[34] H.M. Kuerer, L.A. Newman, T.L. Smith, F.C. Ames, K.K. Hunt, K. Dhingra, R.L. Theriault, G. Singh, S.M. Binkley, N. Sneige, T.A. Buchholz, M.I. Ross, M.D. McNeese, A.U. Buzdar, G.N. Hortobagyi, S.E. Singletary, Clinical course of breast cancer patients with complete pathologic primary tumor and axillary lymph node response to doxorubicin-based neoadjuvant chemotherapy, J. Clin. Oncol. 17 (1999) 460–469.
[35] J.A. van der Hage, C.J. van de Velde, J.P. Julien, M. Tubiana-Hulin, C. Vandervelden, L. Duchateau, Preopera-