The Breast Journal

LETTER TO THE EDITOR

Successful Endocrine Therapy for Locally Advanced Mucinous Carcinoma of the Breast

To the Editor:

Mucinous carcinoma of the breast is one of the most common special histological subtypes of breast cancer. This cancer is known for a tendency to remain local, showing good prognosis compared with common breast cancers. Modification into inflammatory cancer is very rare (1). Therefore, few reports have evaluated the responses of mucinous carcinoma of the breast to chemotherapy and endocrine therapy (2,3). We describe a case of advanced mucinous breast cancer in which dramatic response to endocrine therapy was demonstrated despite poor response to initial chemotherapy and discuss what induced such a clinical course.

The case was a 49-year-old woman with locally advanced mucinous carcinoma of the breast. She visited our hospital with nodules and redness of the skin on the left breast. Inflammatory breast cancer with satellite skin metastases and silent bilateral mucinous carcinoma were diagnosed using core needle biopsy (CNB). Despite primary systemic chemotherapy with 5-fluorouracil, epirubicin, and cyclophosphamide at 100 mg/m^2 (six courses), and tri-weekly docetaxel at 75 mg/m^2 (four courses), the lesions showed little clinical response. We then tried endocrine therapy as strong positive results were seen for hormonal receptors. Staining rates of estrogen receptor (ER) and progesterone receptor (PgR) were 90% each. After a few months of starting letrozole at 2.5 mg/day, nodules and reddening of the skin were found to be dramatically diminished. The patient is alive and well with no pathological findings after 2.5 years of letrozole intake. Skin appearance and MRI findings have continued to remain stable.

We investigated some biomarkers in this case (Table 1). The results suggested high proliferative capacity, with high levels of Ki67 and vascular endothelial growth factor (VEGF) and positive findings for p53 changes. Use of the 21-gene signature (Oncotype Dx^{TM},Genomic Health, Redwood City, CA) with the CNB specimen before chemotherapy showed intermediate risk of recurrence. We assume that this high proliferative capacity was associated with the rapid growth and advanced lesion shown in this case.

Optimal management for inflammatory carcinoma should involve a combination of therapies including all multidisciplinary modalities, such as systemic chemotherapy, surgery, radiotherapy, and others. The introduction of numerous new chemotherapeutic agents has led to significant improvements in overall survival for patients compared to historic descriptions of outcomes before or after mastectomy and/or radiation (4). However, one recent study of primary endocrine therapy compared to multimodal treatment with chemotherapy for ER-positive/Her2-negative locally advanced primary breast cancer showed no significant difference between groups with regard to 5-year breast cancer-specific survival (primary endocrine therapy, 86%; neoadjuvant chemotherapy, 85%; $p = 0.985$) (5). Other investigations have suggested a lack of significant differences in survival benefit and overall objective response between chemotherapy and endocrine therapy in ER+ groups (6,7).

Some reports have demonstrated that the large amounts of mucus surrounding cancer cells are actually not reduced by treatment. This causes discordances between the clinical and pathological effectiveness of chemotherapy (2). It indicates clinical response seemed to be poorer than pathological response according to residual mucus. However, in the present case, residual cancer cells were seen in CNB and the nodules remained largely unchanged during chemotherapy, although the pathological diagnosis from skin biopsy showed no viable cancer cells within the satellite nodules and the number of nodules actually decreased during endocrine therapy. This does not indicate discordance between the clinical and pathological effectiveness of chemotherapy, but endocrine

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DOI: 10.1111/tbj.12031

The Breast Journal, Volume 18 Number 6, 2012 632–633

Table 1. Changes in Biological Markers Before and After Chemotherapy

	Ki67	VEGF	p53	EGFR	HER2	ER	PgR	Oncotype DX™
Pre-chemotherapy (CNB)	30%	Positive	Positive	Negative	1+	90%	90%	R-score 18
During chemotherapy (CNB)	7%	Negative	Negative	Negative	1+	90%	90%	—
During endocrine therapy (skin biopsy)	No tumor	No tumor	No tumor	No tumor	No tumor	No tumor	No tumor	—

therapy was actually far more effective than chemotherapy.

Table 1 also suggests that the initial chemotherapy might have suppressed Ki67 from 30% to 7% and changed positive status into negative status for both VEGF and p53. These data may indicate that chemotherapy could suppress the proliferative ability of this tumor without reducing the number of cancer cells. Two studies have reported the effectiveness of endocrine therapy in cases with low proliferative capacity and low expression of p53 or Ki67 (8,9). On the basis of such findings, we assume that the initial chemotherapy may have contributed to the effectiveness of endocrine therapy.

In summary, endocrine therapy can have dramatic effects on locally advanced mucinous carcinoma with strongly ER/PgR-positive and HER2-negative results, even after chemotherapy. Such effectiveness might be related to reductions in the proliferative capacity of the tumor. Endocrine therapy should be considered as a viable therapeutic option in appropriately selected patients.

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Breast Cancer
DOI 10.1007/s12282-012-0406-6

ORIGINAL ARTICLE

Comparison of hypofractionated and conventionally fractionated whole-breast irradiation for early breast cancer patients: a single-institute study of 1,098 patients

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Received: 30 May 2012 / Accepted: 17 August 2012

Abstract
Purpose To evaluate the efficacy and safety of hypofractionated whole-breast irradiation (HF-WBI) compared with conventionally fractionated (CF) WBI.
Materials and methods Patients with early breast cancer (stages 0–II and <3 positive lymph nodes) who had undergone breast-conserving surgery were eligible for the HF-WBI study. HF-WBI was administered at 43.2 Gy in 16 fractions over 3.2 weeks to the whole breast with an additional tumor-bed boost of 8.1 Gy in 3 fractions over 3 days for positive surgical margins or those <5 mm. CF-WBI was administered at 50 Gy in 25 fractions over 5 weeks to the whole breast with an additional tumor-bed boost of 16 Gy in 8 fractions over 1.4 weeks to 6 Gy in 3 fractions over 3 days, depending on margin status.
Results From April 1, 2006, to December 31, 2010, 717 patients were registered and 734 breasts were treated by HF-WBI. In the same period, 381 patients and 393 breasts who matched the study criteria chose CF-WBI, so the total number of patients in this comparison was 1,098. Grade 2 acute skin reactions were observed for 24 patients (3 %) in the HF-WBI group and 53 patients (14 %) in the CF-WBI ($p < 0.001$) group. The median follow-up period was 27 months. Two cases of intrabreast tumor recurrence were observed in each treatment group. Regional lymph node recurrence was observed in 1 HF-WBI patient and 2 CF-WBI patients.
Conclusion HF-WBI is superior to CF-WBI in terms of acute skin reaction and has the same short-term efficacy.

Keywords Early breast cancer · Whole-breast irradiation · Hypofractionated radiotherapy · Acute adverse effects · Conventionally fractionated radiotherapy

Introduction

Breast-conserving therapy (BCT) consists of partial mastectomy and whole-breast irradiation (WBI), which is performed after BCT as part of standard care in early breast cancer. Conventional WBI involves administration of 50 Gy in 25 fractions over 5 weeks to the whole breast, with additional tumor-bed boosts [1]. Several meta-analyses have proved the usefulness of conventionally fractionated (CF) WBI [2–10]. However, CF-WBI requires an irradiation period of 5 weeks or more, which is inconvenient for patients, institutes, and systemic treatment schedules. As a result, hypofractionated (HF) WBI has been gaining acceptance in practice. In addition, many studies report equal effectiveness and safety of HF-WBI and CF-WBI [2–10]. The purpose of this clinical trial was to evaluate the efficacy, safety, and convenience of HF-WBI compared with CF-WBI.

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Published online: 12 September 2012

Three years from the beginning of our study, we found the severity of skin reaction was different in the two groups. We checked breast skin dose by skin-dose film dosimetry with radiochromic film and calculated the biologically effective dose (BED) for investigation of the different skin reaction.

Materials and methods

Protocol

We submitted the HF-WBI study protocol to Juntendo University Hospital's Institutional Review Board (IRB) in January 2006, and received study approval in March 2006. The IRB commented that there was not enough evidence for HF-WBI in Japan and we needed to conduct a phase II study before a phase III randomization study of HF-WBI and CF-WBI. We therefore redesigned the HF-WBI study from phase III to phase II and compared the results with those for patients who did not participate in the study and were treated by CF-WBI. We explained to all patients who matched the HF-WBI study criteria that there was some evidence in other countries but not enough evidence for HF-WBI in Japan, and explained the details of HF-WBI and CF-WBI. Patients who were willing to participate in the clinical trial were irradiated by HF-WBI and those who were not willing to participate in the study were irradiated by CF-WBI. Written informed consent was obtained from all patients.

Patients with early breast cancer stages 0–II and <3 positive lymph nodes who had undergone partial mastectomy with sentinel lymph node biopsy or axillary node dissection were eligible for this study. Staging procedure and pre-operative examination consisted of general blood test, tumor markers, mammography, ultrasound of breast and regional lymph node area, breast MRI, tumor biopsy, and CT scan of neck to pelvis. Post-operative pathological examination consisted of pathological type, tumor extension, marginal status, estrogen receptor (ER), progesterone receptor (PgR), human epidermal growth factor related 2 (Her2), and pathological status of lymph nodes. Exclusion criteria of this study included any active collagen disease, active double primary cancer, concurrent chemotherapy, previous chest irradiation, patients who needed irradiation of regional lymph node area and pregnancy.

To determine the HF-WBI fractionation schedule, data from the Ontario trial [5] were utilized. HF-WBI involved 43.2 Gy in 16 fractions over 3.2 weeks to the whole breast with an additional tumor-bed boost of 8.1 Gy in 3 fractions over 3 days for cases with positive surgical margins or those <5 mm. Margin criteria were defined as follows:

- positive or close, tumor cells at or within 5 mm of the tumor cell-free margin;
- negative, tumor cells at or beyond 5 mm of the free margin.

CF-WBI involved 50 Gy in 25 fractions over 5 weeks to the whole breast with an additional tumor-bed boost of 6 Gy in 3 fractions over 3 days for cases with negative surgical margins, 10 Gy in 5 fractions over 5 days for cases with positive surgical margins or those within 5 mm of the tumor cell-free margin, or 16 Gy in 8 fractions over 1.4 weeks for cases with positive surgical margins or for cases with more than 3 marginal ducts. This is the standard of care at our institution.

Radiotherapy for all the patients was planned using the Eclipse three-dimensional treatment-planning system (Varian Medical Systems, Palo Alto, CA, USA). To facilitate treatment planning, computed tomography of the chest was obtained with each patient in the upright position, and the body outline, left and right lungs, and heart appeared to be delineated. The clinical target volume (CTV) was defined as the entire palpable breast. The planning target volume (PTV) was obtained by adding a 10-mm margin to the CTV and an additional 15-mm margin for the skin. The WBI treatment technique involved the use of 2 opposing tangential fields. Radiation fields were customized as appropriate by a multileaf collimator. To minimize irradiation of the lungs, the angle of the beams was adjusted to the inner margin. Electronic tissue compensation planning was performed by use of the Eclipse software to ensure uniform dose distribution and to achieve target dose homogeneity within ±7 % of PTV. All breasts were irradiated with 4-MV photon beams (Clinac 21EX; Varian). Tumor-bed boosts were given using 9–15-MeV electrons; field size and electron energy depended on the area which needed to be irradiated, the thickness of breast tissue at the boost site, and tumor depth from the skin.

The primary endpoint was intrabreast tumor control (IBTC). Secondary endpoints were acute adverse effects of the skin, subcutaneous tissue, breast tissue, and lungs, and late adverse effects of the skin, subcutaneous tissue, breast tissue, lungs, ribs, and heart. Acute adverse effects were investigated weekly during treatment and 1 or 2 weeks after completion of treatment. They were scored according to common terminology criteria for adverse events (CTCAE) v 3.0. Late adverse effects and tumor control were assessed at every clinical visit and at least every 6 months after completion of treatment. Late adverse effects were scored on the late effects normal tissue—subjective, objective, management, analytic (LENT-SOMA) scale.

Film dosimetry

Three years from the beginning of study we found the severity of skin reaction was different in the two groups. In 2009 we checked the skin dose for some patients by use of radiochromic films. At that time we did not find any skin dose differences. We therefore decided to evaluate the real skin dose and skin reaction. We submitted a skin dosimetry study protocol to the IRB in April 2011 and it was approved in June 2011. We checked breast skin dose at three points by skin dose film dosimetry, by use of radiochromic film, and calculated the BED to investigate skin reaction differences. Dosimetry was performed by use of the GAFCHROMIC EBT (International Specialty Products, Wayne, NJ, USA). Dosimetric points were:

- point 1, 5 cm away from the nipple in the inner lower quadrant;
- point 2, 5 cm away from the nipple in the outer upper quadrant; and
- point 3, just above the nipple.

Skin doses were measured for 10 patients undergoing HF-WBI and 8 patients undergoing CF-WBI. Fractionation tissue sensitivity was quantified by use of the α/β ratio and linear-quadratic formula $E/\alpha = nd(1 + d/\alpha/\beta)$ to calculate the isoeffect. Acute skin reactions to radiation have an α/β ratio of 10.6 Gy [11].

Statistical analysis

The chi-squared test and Fisher's exact test were used to compare results for the 2 treatment groups. Associations of early toxicity with menopausal status, irradiation technique, concurrent endocrine therapy, and previous chemotherapy were analyzed for the 2 treatment groups by use of the chi-squared test and a logistic regression model. IBTC was calculated by use of the Kaplan–Meier method and the 2 treatment groups were compared by use of the log rank test. Cox's proportional hazards regression model was adjusted to obtain the hazard ratio. A p value of <0.05 was considered to be statistically significant. Statistical analysis was performed by use of the SAS package version 8.02 (SAS Institute, Cary, NC, USA).

Results

Patients

From April 1, 2006, to December 31, 2010, 717 patients who matched in the eligibility criteria for HF-WBI were registered and 734 breasts were treated. The number of control CF-WBI patients was 381, and 393 breasts were treated (Tables 1, 2). The 1,127 breast tumors were categorized into the following T stages: Tis ($n = 191$), T1 ($n = 599$), and T2 ($n = 337$). One hundred and fifty-two tumors were N1 (Table 2). Clinical stages were: stage 0 ($n = 191$), stage I ($n = 543$), stage IIa ($n = 301$), and stage IIb ($n = 92$). The age of the patients ranged from 22

Table 1 Patient characteristics (total no. of participants 1,098)

Characteristics	HF-WBI	CF-WBI
Total no. of patients	717 (65 %)	381 (35 %)
Age (years) (median)	29–85 (54)	22–88 (53)
Menopause	417 (58.2 %)	213 (55.9 %)
Bilateral tumor	17 (2.4 %)	12 (3.1 %)
Neo-adjuvant chemo.	238 (33.2 %)	86 (22.6 %)
Concurrent endo.	88 (12.3 %)	56 (14.7 %)
Adjuvant therapy	426 (59.4 %)	195 (51.2 %)

chemo chemotherapy, *endo* endocrine therapy

Table 2 Tumor characteristics (total no. of tumors 1,127)

Characteristics	HF-WBI	CF-WBI
No. of breasts	734	393
T stage		
Tis	102 (13.9 %[a])	89 (22.6 %)
T1	388 (52.9 %)	211 (53.8 %)
T2	244 (33.2 %)	93 (23.6 %)
N stage		
N0	635 (86.5 %)	340 (86.3 %)
N1	99 (13.5 %)	53 (15.9 %)
Pathology		
DCIS	102	89
IDC	598	284
Other	34	20
ER status		
Positive	577 (78.6 %)	319 (81.2 %)
Negative	151	70
Unknown	6	4
PgR status		
Positive	483 (65.8 %)	274 (69.8 %)
Negative	243	115
Unknown	8	4
HER2 status[b]		
Positive	136 (21.5 %)	64 (21.1 %)
Negative	494	238
Unknown	2	2
Left sided tumor	358 (48.8 %)	196 (49.9 %)
Positive or close margin	236 (32.2 %)	156 (39.7 %)

[a] Patients were informed about the lack of evidence of the efficacy of HF-WBI in Tis

[b] In invasive tumor (exclude DCIS)

to 88 years. No significant differences were found between the 2 groups in terms of age, menopausal status, T1, N1, tumor side (left or right breast), bilateral tumor, ER, PgR, Her 2 status or concurrent endocrine therapy. However, use of neo-adjuvant chemotherapy (HF-WBI group 33.2 % vs. CF-WBI group 22.6 %) and incidence of stage T2 (HF-WBI group 33.2 % vs. CF-WBI group 23.6 %) were significantly different. Incidence of ductal carcinoma in situ (DCIS, Tis) was significantly lower in the HF-WBI group (13.9 vs. 22.6 % in the CF-WBI group). This was because patients had been informed there was less evidence of the efficacy and safety of HF-WBI in DCIS. Tumor margins were positive or close for 32.2 % of patients in the HF-WBI group and 39.7 % of patients in the CF-WBI group.

Convenience

Treatment was administered for 21–36 days (median 26 days) in the HF-WBI group and for 38–49 days (median 43 days) in the CF-WBI group. In Japanese health insurance, treatment management fee and external beam irradiation fee per fraction depend on treatment method. We assessed our technique as moving field irradiation. Treatment costs for HF-WBI were 252,800 yen (US$3,224 at 1 US$ = 78 yen) in cases with negative margins and 281,600 yen (US$3,591) in cases with positive margins (3 times electron boost). Treatment costs for CF-WBI were 423,800 yen (US$5,405) in cases with negative margins and 443,000 yen (US$5,649) in cases with positive margins. In cases of bilateral tumor, Japanese health insurance calculates the second site at half-price. For HF-WBI of 16 fractions add 144,000 yen (US$1,836), for CF-WBI of 25 fraction add 225,000 yen (US$2,869), and for tumor-bed boost in 3 fractions add 12,600 yen (US$161) to the first site price. For example, in cases with negative margins in both sides, the total treatment cost is 396,800 yen (US$5,061) in HF-WBI and 648,800 yen (US$8,276) in CF-WBI. Thus, treatment duration and costs were almost one-third lower for HF-WBI for all patients.

Acute adverse effects

Grade 2 acute skin reactions were observed in 24 patients (3.3 %) from the HF-WBI group and 53 patients (13.5 %) from the CF-WBI group (Table 3). Incidence of other grade 2 effects, mastitis and pneumonitis, was almost the same in two groups.

Factors associated with grade 2 dermatitis are provided in Table 4. Fractionation schedule was the most significant factor ($p < 0.001$ in univariate and multivariate analysis) and menopausal status was a marginally significant factor ($p < 0.05$ in univariate analysis, $p = 0.04$ in multivariate analysis).

Table 3 Acute adverse effects

	Grade	HF-WBI	CF-WBI
Skin reaction	0	183	33
	1	529	308
	2	24 (3.3 %)	53 (13.5 %)
Mastitis (soft tissue, others)	2	3 (0.4 %)	1 (0.3 %)
Pneumonitis	2	2 (0.3 %)	2 (0.5 %)

Scored according to the common terminology criteria for adverse events (CTCAE) v 3.0

Table 4 Factors associated with grade 2 dermatitis

Variable	No. (%)	χ^2	Univariate (p)	Multivariate (p)
HF-WBI	3.3	41.97	<0.001	<0.001
CF-WBI	13.5			
Menopause	6.7	4.850	<0.05	0.04
Premenopause	7.2			
Bilateral	11.1	0.029	NS	NS
Unilateral	6.7			
Neo-adj. chemo.	5.7	0.882	NS	NS
No chemo.	7.3			
Conc. endo.	5.9	0.411	NS	NS
No endo.	7.5			

chemo chemotherapy, *endo* endocrine therapy

Table 5 Results of film dosimetry

Arm	Isocenter dose	Average dose		
		Point 1	Point 2	Point 3
Dose ratio				
HF ($n = 10$)	270	164	161	138
CF ($n = 8$)	200	106	112	93
CF/HF	1.35	1.34	1.36	1.35
BED difference				
HF dose		26.2 Gy/16 f	25.8 Gy/16 f	22.1 Gy/16 f
HF-BED	$\alpha/\beta = 10.6$	30.3	29.7	25.0
CF dose		26.5 Gy/25 f	28.0 Gy/25 f	23.3 Gy/25 f
CF-BED	$\alpha/\beta = 10.6$	29.7	31.0	25.3
BED difference		0.6 HF > CF	1.3 CF > HF	0.3 CF > HF

Biologically effective dose (BED) = $E/\alpha = nd(1 + d/\alpha/\beta)$

The average skin dose, dose ratios, and BED differences for the 10 HF-WBI patients and 8 CF-WBI patients at points 1, 2, and 3 are shown in Table 5. In the same way as the isocenter dose of 2.7 Gy was 1.35 times higher than 2.0 Gy, skin dose at points 1, 2, and 3 were 1.34, 1.36, and

1.35 times higher, respectively. Average BED at points 1, 2, and 3 were 30.3, 29.7, and 25.0, respectively, in HF-WBI patients and 29.7, 31.0, and 25.3, respectively, in CF-WBI patients. BED differences at points 1, 2, and 3 were just 0.6, 1.3 and 0.3, respectively. There were no significant differences between the two groups with regard to dose ratio and BED.

Radiation pneumonitis was observed in 2 patients (0.3 %) from the HF-WBI group and in 2 patients (0.5 %) from the CF-WBI group. No organizing pneumonia was observed. No other late adverse effect above grade 2 was observed in either group.

Tumor control

The follow-up period ranged from 8 to 64 months with a median of 27 months. Two cases of intrabreast tumor recurrence (IBTR) were observed in each treatment group (Table 6). The IBTC was 99.7 % in the HF-WBI group and 99.5 % in the CF-WBI group. Surgical margins were positive for all IBTR patients. Margin status was significantly associated with IBTR ($p < 0.01$). Radiation schedule and other factors had no effect on IBTR in this period.

Regional lymph node recurrence was observed in 1 patient from the HF-WBI group and 2 patients from the CF-WBI group. Distant metastasis was observed in 8 patients from the HF-WBI group and 3 patients from the CF-WBI group. Mortality from breast cancer occurred for 3 patients in the HF-WBI group.

Discussion

BCT is a standard treatment in early breast cancer. The major benefit of BCT is preservation of the breast, with all the consequent advantages in respect of the patients' quality of life. In recent years, the amount of surgery has been decreasing, because of advances in diagnostic precision and the extensive use of systemic therapy. However, the 5 to 7-week schedule of CF-WBI used in BCT places a burden on both the patients' quality of life and on radiotherapy departments that receive a large number of breast cancer patients. HF-WBI is one solution used to improve this situation. HF-WBI has been performed for more than 20 years in the UK and other countries influenced by British medical practice. Six randomized trials and more than 30 nonrandomized trials [2–10] of HF-WBI have reported tumor control and damage to normal tissue similar to those for the standard CF-WBI schedule of 50 Gy in 25 fractions over 5 weeks.

Table 6 Intrabreast tumor recurrence cases

	Case 1	Case 2	Case 3	Case 4
Method	HF	HF	CF	CF
Age	44	57	59	51
Stage	TisN0M0	T2N1M0	T1N0M0	TisN0M0
ER	Positive	Negative	Positive	Positive
PgR	Positive	Negative	Positive	Positive
HER2	–	Positive	Positive	–
Dose (Gy)	51.3	54	60	60
Margin	Positive	Positive	Positive	Positive
Rec. period (months)	36	22	24	36
Rec. path.	DCIS	IDC	DCIS	IDC
Treatment	Mastec	Chemo	Mastec	Mastec

HF HF-WBI, *CF* CF-WBI, *ER* estrogen receptor, *PgR* progesterone receptor, *Rec* recurrence, *path.* pathology, *Mastec* mastectomy

The American Society of Radiation Oncology (ASTRO) consensus guideline for HF-WBI [2] states that patients suitable for HF-WBI are those with stage I breast cancer (T1N0M0), favorable histology, and negative surgical margins who are ER-positive and HER2-negative. Our prospective study was a nonrandomized, single-institute study of 2 radiotherapy fractionation schedules for 1,098 patients. One-thousand one-hundred and twenty-seven breasts were irradiated over a period of 5.9 years. Seven-hundred and seventeen (65 %) patients selected HF-WBI as their preferred treatment. At the beginning of the trial, almost the same number of patients chose HF and CF, but the number of HF patients gradually began to increase as a result of breast oncologists' and former patients advising its use because of its convenience and the low occurrence of acute skin reactions. In the HF-WBI group, 102 patients with DCIS, 244 with T2, 236 with positive or close surgical margins, and 151 with ER-negative status, and 136 with HER2-positive status were included. IBTR was recognized in 2 patients in the HF-WBI group—1 case was Tis with ER-positive status and the other was T2 with HER2-positive status. Of the 2 patients with IBTR in the CF-WBI group one was Tis with ER-positive status and the other was T1 with HER2-positive statusd. IBTC in this study was >99 % in both treatment groups. All of the IBTR cases had positive surgical margins (Table 6). The factor that affected IBTR was margin status. However, with median follow up of 27 months, it is too early to discuss tumor control and affected factors. Distant metastases were significantly higher in the HF-WBI group, possibly because the incidence of T2 cancer was higher in HF-WBI group. Longer follow-up is necessary to determine the final outcome.

PTV dose homogeneity and normal tissue dose reduction are more important in HF-WBI than in CF-WBI. In HF-WBI, three-dimensional intensity-modulated radiation therapy (IMRT) is more appropriate than an open field or a physical wedge filter. Use of an electric compensator or the field-within-a-field technique is easier and results in good dose homogeneity as IMRT. The Eclipse software used in

this study ensured adequate electronic tissue compensation and good dose homogeneity. The total planning time using the Eclipse software was less than 1 h.

Most HF-WBI randomized trials have required the maximum dose to the breast on the central axis plane to be no greater than 105–107 % and no less than 93–95 % of the prescription dose [2]. No stipulations were placed on the homogeneity of dose distribution outside the central axis plane. Most patients in these trials were treated by use of two-dimensional planning techniques without tissue heterogeneity corrections. Optimizing the homogeneity of dose in the off-axis planes and in the central-axis plane reduces acute toxicity. Some studies have reported a correlation between skin reaction and breast volume and one Egyptian study reported a significant correlation between breast volume and the severity of acute skin reactions [12]. This, however, was mainly caused by inhomogeneous distribution of the dose to large-volume breasts by the conventional irradiation technique. In our study with Japanese patients, the number of patients was large enough to eliminate breast volume bias and we used a dose homogeneity-correction technique within the whole PTV. The ASTRO guideline recommends that the minimum dose should be no less than 93 % and that the maximum dose should be no more than 107 % of the prescription dose (±7 %) in the central-axis plane, and also encourages the use of three-dimensional planning techniques for all patients to minimize dose inhomogeneity and reduce toxicity [2].

In our study the percentage of grade 2 acute skin reactions was significantly lower in the HF-WBI group (3.3 %) than in the CF-WBI group (13.5 %; Tables 3, 4). START trials have reported less acute reactions with HF-WBI [9, 10]. The START A trial reported that severe acute reactions were 0.3 % in the 50 Gy arm, 0 % in the 41.6 Gy arm, and 0 % in the 39 % arm (>0.005) [9]. In a retrospective study from the Cancer Institute of the Japanese Foundation for Cancer Research, 9 % of patients in the HF-WBI group (40 Gy in 16 fractions) and 22 % in the CF-WBI group suffered grade 2–3 dermatitis ($p = 0.016$) [13]. Differences between radiation techniques affect skin dose and reaction. Therefore, skin-dose film dosimetry was performed to evaluate skin dose for the 2 treatment groups in our study. However, we found no significant differences of skin dose and BED in the two groups. Thus, we believe skin dose and BED do not correlate simply with skin reaction. Skin reaction might be affected by volume and type of the low-energy X-ray component, and scattered radiation from the multileaf collimator, wedge filter, and other devices. We could not identify a clear reason for the different skin reactions between the 2 groups from this dosimetry. We believe the modern radiation technique to improve PTV dose homogeneity could be affecting skin reaction, or radiobiological uncertainties in skin reaction may exist that are not reflected in the α/β ratio.

The incidence of pneumonitis in both treatment groups was lower than that in other studies. Careful field settings of the inner margin to minimize irradiation to the lungs and use of the Eclipse software for electronic tissue compensation planning contributed to this result. No changes in breast appearance were reported during the follow-up period. No other late adverse effects above grade 2 were observed in either treatment group. However, patients are still being carefully monitored at the time of writing of this manuscript because of the possibility of higher incidence of late adverse effects in the breast, heart, lungs, and brachial plexus in the HF-WBI group.

Conclusion

In this study we compared treatment results for 717 patients and 734 breasts treated by HF-WBI with those for 381 patients and 393 breasts treated with CF-WBI who matched the eligibility criteria of the HF-WBI clinical study. Incidence of acute skin reactions was significantly lower in HF-WBI group. IBTC was 99 % in both groups, with no difference during the median 27-month period of follow up. Cost and treatment period for HF-WBI were two-thirds those for CF-WBI. If acute skin reaction in other studies which use the dose homogeneity technique for the whole PTV is as low as ours, the patient burden of WBI would be reduced further. Our study included ER-negative, HER2-positive, and surgical margin-positive patients. This is the first report to compare the efficacy, safety, and convenience of HF-WBI and CF-WBI for Japanese patients, and this group of patients will provide much information with longer follow up.

Conflict of interest The authors report no conflict of interest or financial support for this study.

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Cancer Chemother Pharmacol (2012) 69:923–930
DOI 10.1007/s00280-011-1777-7

ORIGINAL ARTICLE

Predictive value of MGMT, hMLH1, hMSH2 and BRCA1 protein expression for pathological complete response to neoadjuvant chemotherapy in basal-like breast cancer patients

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Received: 22 May 2011 / Accepted: 26 October 2011 / Published online: 15 November 2011

Abstract
Purpose To evaluate the importance of biological markers to predict pathologic complete response (pCR) to neoadjuvant chemotherapy (NACT) in patients with locally advanced basal-like breast cancers (BLBCs).
Patients and methods Thirty-two BLBC patients receiving NACT with an anthracycline-based regimen plus taxane were included in this study. The immunoreactivities of MGMT, MLH1, MSH2 and BRCA1 before and after NACT were evaluated.
Results A pCR was obtained in 10 of 32 cases (31%). Cancer-related ($P = 0.013$) and disease-free ($P = 0.023$) survival rates were significantly higher in the pCR group than in the non-pCR group. In biopsy samples before NACT, attenuated expression of MGMT, MLH1, MSH2 and BRCA1 was observed in 12/32 (38%), 0/32 (0%), 5/32 (16%) and 28/32 (88%) cases, respectively. On evaluation of pCR, patients' characteristics (patients' age, menopausal status, or clinical and pathological stages) and immunohistochemical patterns, attenuated expression of MGMT was only found to be significantly predictive of a pCR ($P = 0.018$). Paired biopsy sample before NACT and a surgical tumor material after NACT were available for 19 cases of non-pCR. In these cases, decrease in expression during NACT were more frequently observed for MGMT as compared to MLH1, MSH2 or BRCA1 ($P = 0.021$).
Conclusions MGMT status is a predictive factor for pCR with neoadjuvant anthracycline-based plus taxane combination chemotherapy, which may be helpful in the selection of appropriate NACT for Japanese patients with BLBC.

Keywords Breast cancer · Neoadjuvant chemotherapy · Basal-like subtype · MGMT · Immunohistochemistry · Mismatch repair

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Introduction

In recent studies of breast cancer patients with neoadjuvant chemotherapy (NACT), widely used for patients with locally advanced disease over the last few decades, the pathologic complete response (pCR) proved to be an important independent prognostic indicator for prolonged disease-free and overall survival [1, 2]. Basal-like breast cancer (BLBC) is characterized by triple negative of estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor 2 (HER2), and positive for basal cytokeratin, epidermal growth factor receptor (EGFR) or c-Kit [3–5]. The prognosis is generally unfavorable [4, 5]. Although adjuvant hormone therapy has been shown to be effective for ER-positive breast cancers [6] and adjuvant trastuzumab therapy also improves survival with HER2 positive breast cancers [7], no targeted therapy is available for BLBC and chemotherapy is the only option other than surgery. Recent studies have shown that BLBCs are sensitive to NACT [8, 9], but still have an ominous outcome, particularly in patients with poor responses [8].

The mismatch repair genes human mut L homolog (MLH)1 and human mut S homolog (MSH)2 are components of the DNA mismatch repair pathway, whose activation may trigger DNA damage signaling, a process which

induces cell cycle arrest and can lead to cell death [10]. O6-Methylguanine-DNA methyltransferase (MGMT) rapidly reverses alkylation (including methylation) at the O6 position of guanine by transferring the alkyl-group to the active site of the enzyme, constituted by a cysteine [11]. An inactivated MGMT gene allows accumulation of O6-alkylguanine that is the most cytotoxic lesion of alkylating agents, which subsequent to incorrect pairing with thymidine triggers mismatch repair, thereby inducing DNA damage and eventually cell death [12]. In accordance with this mechanism, inactivation of MGMT renders MLH1 and/or MSH2-deficient cells more sensitive to alkylating agents. In sporadic breast cancer, MLH1 and MSH2, but not MGMT are targets of epigenetic silencing and subsequently reduced expression at the protein level in the mismatch repair pathway [13–17].

Breast cancer susceptibility protein (BRCA)1 and BRCA2 are essential for repair of double strand breaks and stalled replication forks by homologous recombination. Accumulated evidence suggests that dysfunction of BRCA1 is a crucial mechanism underlying BLBC tumorigenesis [18, 19], and cells deficient for BRCA1 have been shown to be exceedingly sensitive to poly (ADP-ribose) polymerase (PARP) inhibitors [20, 21]. In addition, PARP inhibitors may also sensitize to alkylating agents such as temozolomide, as suggested in recent trials [22].

The purpose of this study was to evaluate the immunoreactivities of MGMT, MLH1, MSH2 and BRCA1 before neoadjuvant anthracycline-based plus taxane combination chemotherapy and to clarify the potential roles of these proteins in predicting pCR to NACT in Japanese patients with BLBC.

Materials and methods

Patient and materials

Locally advanced breast cancer was defined as breast cancer histologically and/or cytologically documented as stage IIA (T2 $\geq$ 3 cm), IIB, IIIA, IIIB or IIIC using the UICC/TNM classification [23]. A cohort of 412 patients thus selected, receiving preoperative NACT and surgically treated at Juntendo University Hospital (Tokyo, Japan) between 2005 and 2010, were first screened for expression of ER, PgR and HER2 in tru-cut biopsy samples before NACT. In all, 40 cases (9.7%) were negative for three markers (triple negative). Second, triple-negative cases were examined for expression of basal markers (cytokeratin 5/6/14/17 cocktail used), EGFR and c-Kit. Thirty-two cases (80%) of 40 triple-negative cases were positive for at least one of these markers and were therefore considered to be BLBCs [3]. The characteristics of these patients are listed in Table 1.

Table 1 Pretreatment patient and tumor characteristics

Characteristics	No.	%
Total patients	32	
Patient age[a], years		
≤50	13	40.6
>50	19	59.4
Menopausal status		
Premenopausal	12	37.5
Postmenopausal	20	62.5
Initial tumor size		
T1	1	3.1
T2	23	71.9
T3	4	12.5
T4	4	12.5
Initial lymph node status		
N0	13	40.6
N1	14	43.8
N2	3	9.4
N3	2	6.2
Initial clinical stage		
IIA	12	37.5
IIB	13	40.6
IIIA	4	12.5
IIIB	1	3.1
IIIC	2	6.3

[a] Median/mean ± SD (range), years 55.0/52.7 ± 10.8 (33–69)

Neoadjuvant chemotherapy, surgery and follow-up

Patients received NACT with anthracycline-based chemotherapy in 4 cycles of triweekly 80 mg/m^2 of epirubicin and 600 mg/m^2 of cyclophosphamide (EC), or 4–6 cycles of triweekly 500 mg/m^2 of fluorouracil, 75 or 100 mg/m^2 of epirubicin and 500 mg/m^2 of cyclophosphamide (FEC). EC or FEC was followed by taxane chemotherapy in 12 cycles of weekly 80 mg/m^2 of paclitaxel, or 4 cycles of triweekly 75 mg/m^2 of docetaxel. Clinical evaluation of the response to NACT was evaluated prior to surgery after the last cycle of chemotherapy according to the product of primary tumor diameters and the axillary clinical status and was determined by clinical findings, ultrasound, computed tomography and magnetic resonance imaging examinations according to RECIST (Response Evaluation Criteria In Solid Tumors) [24]. The patients were classified into four groups: complete response (CR), partial response (PR), stable disease (SD) or progressive disease (PD). After neoadjuvant chemotherapy, patients underwent appropriate surgery according to the size of their residual tumor.

Surgically resected materials as well as tru-cut biopsy samples taken at the time of diagnosis were routinely fixed in 15% formalin and embedded in paraffin wax, according

Table 2 List of primary antibodies used for immunohistochemistry

Antigen	Clone	Source	Dilution	Retrieval	Staining type
ER	SP1	Roche diagnostics	Prediluted	Heat	Nuclear
PgR	1E2	Roche diagnostics	Prediluted	Heat	Nuclear
Her2/neu	4B5	Roche diagnostics	Prediluted	Heat	Membrane
CK5/6	D5/16 B4	DakoCytomation	1:50	Heat	Cytoplasmic and membrane
CK14	LL002	Leica microsystems	1:30	Heat	Cytoplasmic and membrane
CK17	E3	DakoCytomation	1:40	Heat	Cytoplasmic and membrane
EGFR	EGFR 113	Leica microsystems	1:15	Heat	Cytoplasmic and membrane
c-Kit (C-19)	polyclonal (sc-168)	Santa Cruz biotechnology	1:100	Heat	Cytoplasmic and membrane
MGMT	MT 3.1	Thermo scientific	1:40	Heat	Nuclear
MLH1	G168-15	Diagnostic BioSystem	1:50	Heat	Nuclear
MSH2	polyclonal	Calbiochem	1:30	Heat	Nuclear
BRCA1	MS110	Oncogene research products	1:150	Heat	Nuclear

to routine procedures, and sections were cut and stained with hematoxylin and eosin (H&E). All slides stained with H&E were independently examined by two of the authors (H.M. and Y.A.) without knowledge of the demographic or treatment response information. Pathological responses to NACT were evaluated according to previously described criteria [1] in the surgical specimens. In particular, the absence of invasive cancer in both the primary breast tumor and axillary lymph nodes qualified for pCR. Cases of residual in situ cancer only were also considered to be pCR.

In patients who underwent a segmental mastectomy with axillary lymph node dissection with ≤ 3 positive nodes, postoperative irradiation treatment was delivered to residual breast, or in those with >4 positive nodes, delivered to both residual breast and supraclavicular lymph region. In patients who underwent a modified radical mastectomy with >4 positive axillary nodes, postoperative irradiation treatment was delivered to the chest wall and supraclavicular region.

Locoregional radiotherapy was instituted within 8 weeks of the completion of surgery. All patients were followed up regularly by physical and blood examinations with mandatory screening by chest X-ray, ultrasound, computed tomography and bone scan. The median and mean duration of follow-up were 33 and 30 months (range 5–68) for the survivors alive at the date of their last visit ($n = 22$).

All procedures were carried out with the prior informed consent of the patients. This study was approved by the Institutional Review Board and the ethical committee of our hospital (registration #22–217).

Immunohistochemistry

Briefly, 4-μm-thick paraffin sections were dewaxed in xylene, rehydrated through a series of graded alcohols, placed in Target Retrieval Solution (pH 9.0, DakoCytomation, Kyoto, Japan) and submitted to heat retrieval using an autoclave for 30 min. After heating, the slides were allowed to cool to room temperature and endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 5 min. The slides were then incubated overnight at 4°C with the primary antibodies detailed in Table 2. To detect basal cytokeratins, a cytokeratin 5/6/14/17 antibody cocktail was used. Immunohistochemical staining was performed using an Envision Kit (DAKO). The slides were incubated with horseradish peroxidase-labeled polymers? conjugated with secondary antibodies and then with substrate-chromogen (3,3-diaminobenzidine tetrahydrochloride) solution, followed by light counterstaining with Mayer's hematoxylin.

Assessment of immunostaining

All slides were reviewed and scored independently by two of the authors (H.M. and Y.A.) without knowledge of the demographic or treatment response information. Interobserver variations were resolved by re-evaluation and discussion to reach consensus. The cutoff for ER, PgR, CK5/6/14/17, EGFR and c-Kit was 10% positive cells, irrespective of intensity. A HER2-negative result was defined as either HER2 0 or 1+ (strongly positive in <10% of cancer cells); a positive was concluded with HER2 2+ (moderately positive in >10% of cancer cells) and gene amplified <twofold in fluorescence in situ hybridization. Distinct nuclear staining for MGMT, MLH1, MSH2 and BRCA1 was considered to be positive, regardless of the staining intensity. Normal ductal epithelium which exhibited nuclear staining for these proteins was considered as an internal positive control. The percentage of positive tumor cells was assessed and classified into four categories: <10; 10–50; 51–90; >90%.

Table 3 Relationship between clinical and pathological responses to neoadjuvant chemotherapy

	pCR		Non-pCR	
	No.	%	No	%
CR	3	100	0	0
PR	6	37.5	10	62.5
SD	1	12.5	7	87.5
PD	0	0	5	100

pCR pathological complete response, *CR* complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease

$P = 0.015$

Statistical analysis

Categorical analysis of variables was performed using either the Chi-squared test (with Yates' correction) or the Fisher's exact test, as appropriate. Cancer-related survival time was measured from the date of surgery to the end of follow-up or death due to breast cancer or other causes. Recurrence-free survival time was defined as the time from surgery to recurrent disease (alive) or death, with or without recurrence. Survival curves were generated by the Kaplan–Meier method and differences assessed by the log-rank test. A P value of <0.05 was considered statistically significant. The statistical data were obtained using StatView, Version 5.0 (SAS Institute Inc., Cary, NC, USA).

Results

Clinical and pathological responses to neoadjuvant chemotherapy and survival

Regarding clinical responses to NACT, CR was observed in 3 patients (9.4%), PR in 16 (50.0%), SD in 8 (25.0%), and PD in 5 (15.6%). With pathological responses, pCR was obtained in 10 of 32 cases (31%). In the twenty-two non-pCR cases, ten demonstrated 60–99% reduction of primary tumor and twelve <60% reduction. All of the patients with clinical CR featured pCR but none of the patients with PD ($P = 0.015$; Table 3).

Cancer-related ($P = 0.013$) and disease-free ($P = 0.023$) survival rates were significantly higher in the pCR than the non-pCR group. Kaplan–Meier plots illustrating associations of pathological response to NACT with survival are shown in Fig. 1.

Expression of MGMT, MLH1, MSH2 and BRCA-1

The immunohistochemical findings for these proteins are summarized in Table 4. When results were stratified into two groups, i.e., attenuated (≤50%) and normal (>51%), attenuated expression of MGMT, MLH1, MSH2 and BRCA1 was observed in 12/32 (38%), 0/32 (0%), 5/32 (16%) and 28/32 (88%) cases, respectively. The frequencies significantly varied ($P < 0.001$), with no significant relationships to be found. In addition, no significant associations were detected between patient characteristics (patient's age, menopausal status, or clinical and pathological stages) and protein expression (data not shown).

Paired samples consisting of a tru-cut biopsy taken at the time of diagnosis (before NACT) and surgically resected material after NACT were available for 19 of the non-pCR cases. Changes in expression of MGMT, MLH1, MSH2 and BRCA1 during NACT are detailed in Table 5. In the 19 cases, decrease in expression were more frequently observed for MGMT than for MLH1, MSH2 or BRCA1 ($P = 0.021$). Cases with decreased expression in MGMT during NACT (4/9 cases, 44%) frequently tended to show ≥60% tumor reduction, as compared to those with no decrease (2/10, 20%; not statistically significant).

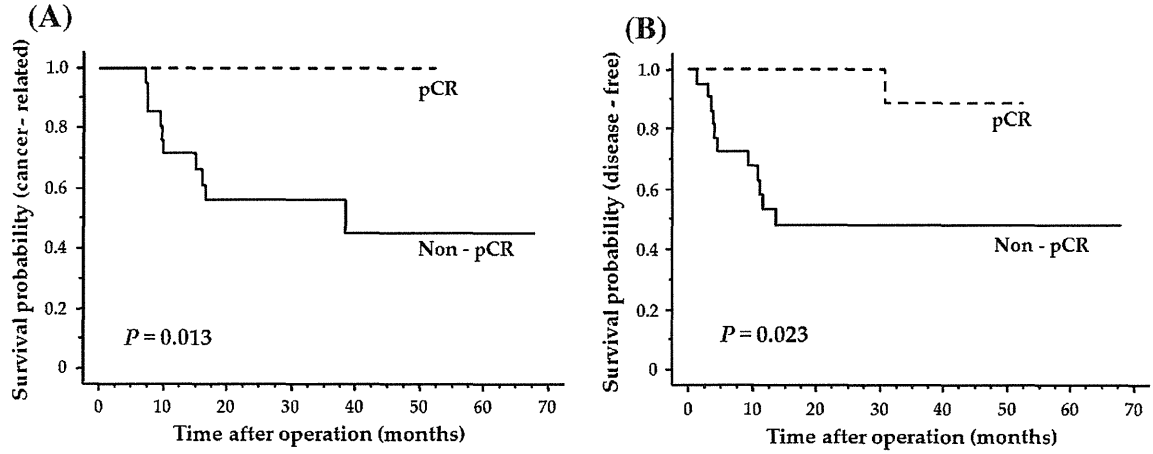

Fig. 1 Cancer-related (**a**) and disease-free (**b**) survival curves with reference to pathological response to neoadjuvant chemotherapy

Table 4 Distribution of expression of MGMT, MLH1, MSH2 and BRCA1 before chemotherapy in biopsy samples

Immunoreactivity	MGMT		MLH1		MSH2		BRCA1		*P* value
	No.	%	No.	%	No.	%	No.	%	
<10%	0	0	0	0	2	6.2	21	65.6	<0.001
10–50%	12	37.5	0	0	3	9.4	7	21.9	
51–90%	6	18.7	5	15.6	12	37.5	4	12.5	
>90%	14	43.8	27	84.4	15	46.9	0	0	
Attenuated (≤50%)	12	37.5	0	0	5	15.6	28	87.5	<0.001
Normal (>50%)	20	62.5	32	100	27	84.4	4	12.5	

Table 5 Change in expression of MGMT, MLH1, MSH2 and BRCA1 before and after neoadjuvant chemotherapy

Change in expression	MGMT		MLH1		MSH2		BRCA1	
	No.	%	No.	%	No.	%	No.	%
Increase	2	10.5	1	5.3	1	5.3	1	5.3
No change	8	42.1	18	94.7	15	78.9	14	73.7
Decrease	9	47.4	0	0	3	15.8	4	21.0

$P = 0.021$

Expression in a representative case (#5) is illustrated in Fig. 2.

Patients' characteristics and immunohistochemistry and their relationship with pCR

On evaluation of associations among pCR, patients' characteristics and immunohistochemical patterns, only attenuated expression of MGMT was found to be significantly predictive of a pCR ($P = 0.018$; Table 6).

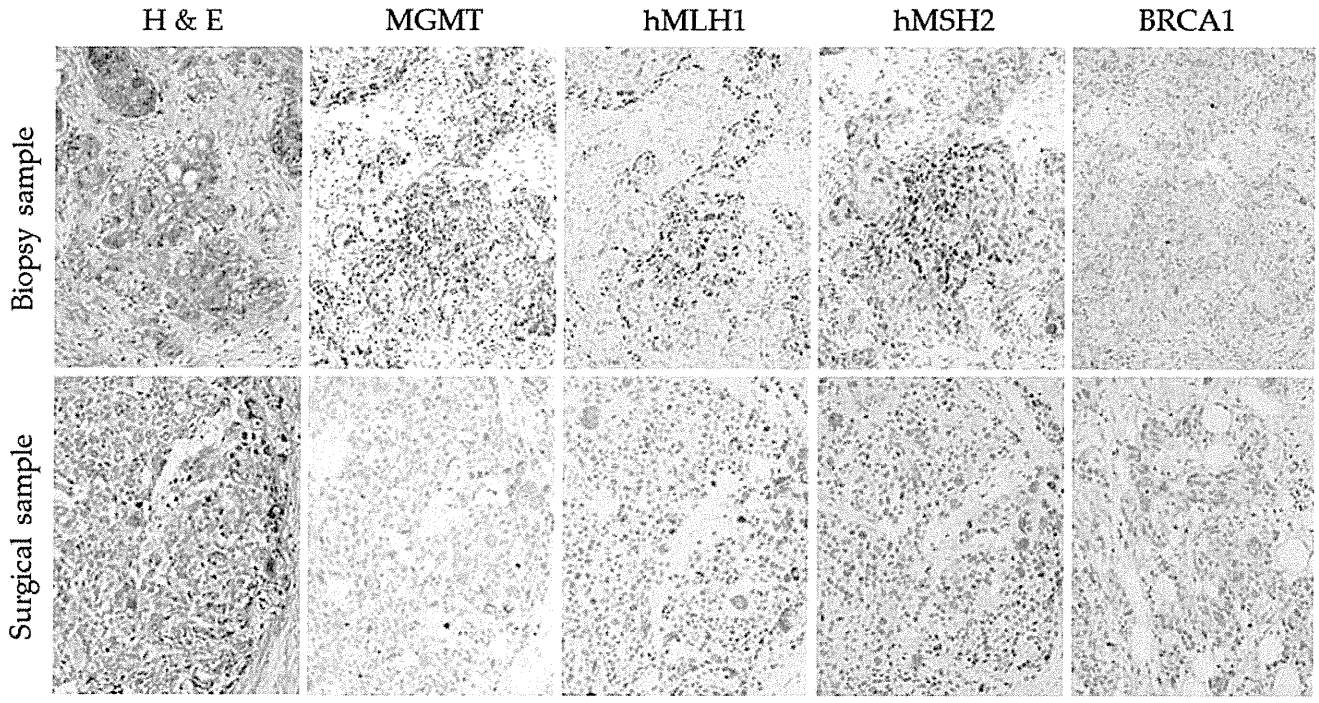

Fig. 2 H&E staining and immunohistochemistry of serial sections of paired samples obtained from a biopsy (original magnification, ×70) and surgical material (original magnification, ×82) in non-pCR from case No. 5. BLBC, shown as expansive and medullary growth pattern, consists of tumor cells with high nuclear grade and pleomorphism. Note marked decrease of MGMT expression; 50–90% nuclear immunopositivity for MGMT in tumor cells in a tru-cut biopsy sample before neoadjuvant chemotherapy and <10% nuclear immunopositivity in tumor cells in the surgically resected sample after neoadjuvant chemotherapy. No change is apparent in immunopositivity for MLH1 (>90% nuclear expression), MSH2 (50–90% nuclear immunopositivity) or BRCA1 (<10% nuclear expression) expression before and after neoadjuvant chemotherapy

Table 6 Univariate analysis of patient characteristics and expression of MGMT, MLH1, MSH2 or BRCA1 associated with pCR versus non-pCR to neoadjuvant chemotherapy

Characteristics	pCR		Non-pCR		*P* value
	No.	%	No.	%	
Total patients	10	31.3	22	68.7	
Age, years					
≤50	4	30.8	9	69.2	NS
>50	6	31.6	13	68.4	
Menopausal status					
Premenopausal	5	41.7	7	58.3	NS
Postmenopausal	5	25.0	15	75.0	
Initial tumor size					
T1 or T2	8	33.3	16	66.7	NS
T3 or T4	2	25.0	6	75.0	
Initial lymph node status					
N0 or N1	8	29.6	19	70.4	NS
N2 or N3	2	40.0	3	60.0	
Initial clinical stage					
II	8	32.0	17	68.0	NS
III	2	28.6	5	71.4	
MGMT expression					
Attenuated	7	58.3	5	41.7	0.018
Normal	3	15.0	17	85.0	
MLH1 expression					
Attenuated	0	0	0	0	NS
Normal	10	31.3	22	68.7	
MSH2 expression					
Attenuated	3	60.0	2	40.0	NS
Normal	7	25.9	20	74.1	
BRCA1 expression					
Attenuated	10	35.7	18	64.3	NS
Normal	0	0	4	100	

Discussion

Regardless of the breast cancer subtype, pCR is a powerful indicator of prolonged survival in patients receiving NACT [1, 2]. We therefore tested survival impact of pathological response to NACT especially in BLBCs reported to be more sensitive to NACT than luminal lesion [8, 9]. In the present study, the pCR rate in BLBC was 31%, consistent with the previous reports stated 21–45% [9, 25, 26]. We also found a significant benefit of pCR on survival in 32 patients with BLBC, in line with earlier finding that none of 34 patients with pCR to NACT relapsed or died [8]. Conversely, two other studies failed to show statistically significant differences between pCR and non-pCR groups with 50 and 22 cases of BLBC [25, 26]. The reasons remain unclear and point to the necessity for larger scale comprehensive studies.

Since our patients with BLBC who achieved pCR showed improved prognosis, the current study was undertaken to determine whether the expression of MGMT, MLH1, MSH2 and BRCA1 could affect the pCR to NACT. Although patients' characteristics and TNM classification were not significantly predictive of a pCR, attenuated expression of MGMT before NACT did serve as a significant predictor. In contrast, MGMT gene expression measured by reverse transcription-PCR was not predictive of response to neoadjuvant chemotherapy [27]. MGMT immunoreactivity has been reported to be correlated with local recurrence, distant metastasis and prognosis in breast carcinoma [28, 29]. To the best of our knowledge, there is no report of relationship between MGMT immunoreactivity and response to neoadjuvant chemotherapy.

As a result of defective MGMT function through promoter methylation, evidence has been provided for prediction of benefit from alkylating agent therapy in glioma [30] and glioblastoma [31], and from multidrug regimens with cyclophosphamide for B-cell lymphomas [32]. In an experimental study, mice overexpressing MGMT proved more resistant to carcinogenesis induced by alkylating agents, whereas knock-out mice were more sensitive [33]. Methylating agents could be shown to effectively inactivate MGMT in human breast carcinoma cells and xenografts, resulting in substantial increase in sensitivity to growth inhibition by alkylating agent [34]. Furthermore, acquisition of doxorubicin resistance by a human breast carcinoma cell line was associated with MGMT hypomethylation of the promoter region [35]. Interestingly, the current study demonstrated that NACT resulted in decreased expression of MGMT in 40% of cases. Moreover, decreased expression in MGMT during NACT enhanced tumor reduction. In chemosensitive BLBC, NACT might selectively alter MGMT expression through mechanisms including promoter methylation.

An attenuated expression of MLH1 and MSH2 may preferentially occur in breast carcinomas with a poorer differentiation [16], but our frequencies of inactivation for these proteins were low at 0 and 16%. In sporadic breast cancer, approximately 20–40% exhibited decreased expression of these proteins [14–16]. In line with our results, no significant correlation was identified between MLH1 and MSH2 with age, tumor size and lymph node metastasis [16]. Current study is discordant with some observations that attenuated MLH1 expression is closely associated with results of NACT [13, 15]. Absent or reduced BRCA1 expression has been observed in approximately 80% of BLBCs, linked with advanced lymph node stage, large size and vascular invasion [36]. In a study of sporadic breast

cancer patients, those with low levels of BRCA1 mRNA expression attained better response to anthracycline-based chemotherapy [37]. Furthermore, the presence of BRCA1-negative foci in biopsy specimens before neoadjuvant epirubicin plus cyclophosphamide treatment was inversely correlated with tumor response [38]. However, there is conflicting evidence as to whether tumors with inactivation of BRCA1 demonstrate particular clinical or pathological features or obtain greater benefit from DNA-damage-based chemotherapy, in line with our findings.

Immunohistochemistry, which is the most practical method for assessing protein expression changes, not only provides a semiquantitative assessment of protein abundance but also defines the cellular localization. Because no special processing of tissue samples is needed and labor-intensive and expensive diagnostic techniques are avoided, immunohistochemistry is perhaps the most readily adaptable technique to clinical practice. Interobserver reproducibility of immunohistochemical scoring is sometimes problematic. However, interobserver variations were easily resolved by re-evaluation and discussion to reach consensus because MGMT immunostaining is distinct and reliable in the present study as well as previous reports [28, 29]. In this study, patients with attenuated MGMT expression in their lesions had a 2.3 times higher probability of achieving a pCR than those with preserved expression. In our limited study, MGMT status might be a predictive factor for pCR to neoadjuvant anthracycline-based plus taxane combination chemotherapy, and a potential aid to selection of appropriate NACT for Japanese patients with BLBC.

Acknowledgments Drs. Nakai and Mitomi contributed equally to this study. The work was supported in part by a Grant-in-Aid from the Japan Society for the Promotion of Science (#21590394) to H. Mitomi and by a Grant-in-Aid from the Juntendo University School of Medicine for the Project Research (#1002) to Y. Alkam.

Conflict of interest The authors have no commercial, proprietary, or financial interest in the products or companies described in this article.

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Breast Cancer Res Treat (2012) 133:427–436
DOI 10.1007/s10549-011-1758-x

PRECLINICAL STUDY

Estrogen receptor-α directly regulates sensitivity to paclitaxel in neoadjuvant chemotherapy for breast cancer

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Received: 19 May 2011 / Accepted: 24 August 2011 / Published online: 11 September 2011

Abstract Neoadjuvant chemotherapy (NAC) has become the standard treatment for advanced breast cancer. Several prognostic markers, including estrogen receptor-α (ERα), are used to predict the response to NAC. However, the molecular significance of ERα expression in the efficacy of chemotherapy is not yet fully understood. To examine this issue, we first evaluated ERα transcriptional activity in breast cancer cells derived from pre-NAC specimens using estrogen response element–green fluorescent protein (ERE–GFP) as a reporter gene, and found that, in the cases for which ERα activities determined by GFP expression were not detected or low, pCR (pathological complete response) could be achieved even though ERα protein was expressed. Next, we examined the effects of alterations in ERα expression levels on sensitivity to paclitaxel, a key drug in NAC, by stable expression of ERα in ER-negative SKBR3 cells and by siRNA-mediated down-regulation of ERα in ER-positive MCF-7 cells, and showed that ERα expression and sensitivity to paclitaxel showed an inverse correlation. We also established paclitaxel-resistant MCF-7 cell clones and found that they have higher estrogen-induced ER activity than parent cells. Paclitaxel is a microtubule-stabilizing agent, while HDAC6 (histone deacetylase 6), which we previously identified as an estrogen-regulated gene, enhances cell motility by destabilizing microtubules via deacetylation of α-tubulin. Finally, we demonstrate herein that ERα knockdown in MCF-7 cells prevents deacetylation of α-tubulin, thereby increasing sensitivity to paclitaxel. Taken together, these results suggest that ERα expression directly regulates sensitivity to paclitaxel in NAC for breast cancer via the effect on microtubule stability.

Keywords Breast cancer · Paclitaxel · Neoadjuvant chemotherapy · ERα · HDAC6

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Introduction

Several recent trials have suggested that adjuvant chemotherapy is less effective in patients with estrogen receptor (ER)-positive breast cancers [1–3]. For example, the Cancer and Leukemia Group B9344 trial evaluated the efficacy of anthracycline regimens with or without paclitaxel in adjuvant therapy and found them to be effective in 24% of ER-negative but only 11% of ER-positive patients [2]. Neoadjuvant chemotherapy (NAC) trials of anthracyclines combined with paclitaxel also showed higher pathological complete response (pCR) rates in ER-negative than in ER-positive patients [4, 5].

Recently, NAC has become the standard treatment for advanced breast cancer. NAC has numerous advantages, including down-staging of an inoperable cancer to an operable one, the availability of breast conservation for

patients who would otherwise undergo a mastectomy, and the use of pathological response data as a surrogate marker for long-term clinical outcome [6–9]. Another advantage of NAC is that responsiveness to the particular chemotherapy regimen can be assessed, possibly allowing for individualized therapy [10].

Taxanes are potent antimicrotubule agents, which act by inducing tubulin polymerization and promoting the formation of unusually stable microtubules, thereby inhibiting the normal dynamic reorganization of the microtubular network required for mitosis and cell proliferation [11]. Paclitaxel was the first marketed taxane drug affecting the integrity of microtubules. In recent studies, paclitaxel-based combination chemotherapy has resulted in an improved pCR rate as compared with single-agent taxane treatments [12, 13]. Several prognostic markers of NAC, including ERα, are used to predict responses to paclitaxel. For example, patients with ER-positive breast cancers reportedly gain little benefit from the administration of paclitaxel [14, 15]. However, the molecular mechanisms underlying the role of ERα in the efficacy of paclitaxel are not yet fully understood.

We have studied the roles of ERα expression and estrogen-regulated genes as predictors of the efficacy of systemic therapy for ER-positive breast cancer. In our previous studies, we identified histone deacetylase 6 (HDAC6) as an estrogen-regulated gene using cDNA microarray analysis in the ER-positive breast cancer cell line MCF-7 [16]. In the setting of breast cancer, high HDAC6 expression was also detected [17]. HDAC6 was originally cloned as a member of the histone deacetylase family, is expressed in several tissues, and functions as a deacetylase for tubulin [18]. Furthermore, tubulin is an important component of the microtubule network that regulates cell motility, which appears to be enhanced by HDAC6. Accordingly, we hypothesized that microtubule conformation is the common target for estrogen signals and paclitaxel.

To our knowledge, this is the first study designed to clarify the significance of ERα in tumor sensitivity to paclitaxel, via estrogen-induced deacetylation of tubulin mediated by HDAC6.

Materials and methods

Cell culture and reagents

The human breast cancer cell lines MCF-7 and SKBR3 were cultured in RPMI 1640 (Sigma-Aldrich, MO) supplemented with 10% fetal calf serum (FCS; Tissue Culture Biologicals, CA) and antibiotics. All cells were incubated at 37°C under 5% CO_2 in air.

MCF-7–E10 cells (E10 cells) were established from the MCF-7 by introducing a plasmid carrying the ERE (estrogen responsive element) fused with tk-GFP (green fluorescent protein) gene, as described previously [19, 20]. E2 (17β-estradiol) was purchased from Sigma-Aldrich. Paclitaxel was provided by Bristol-Myers Squibb (NY, USA). The paclitaxel-resistant MCF-7–E10 clones (PAC-1–PAC-3) were developed by increasing the paclitaxel dose, stepwise, from 2 to 10 nM, over 2 months.

Assays of cell growth

The cells (5×10^4/well) were seeded in 24-well plates and cultured. After 48 h, the cells were treated with paclitaxel (0–30 nmol/l) for 3 days. Cell numbers were determined using a cell counter (Coulter Counter ZBI; Beckman Coulter, CA) and presented as percentages relative to those of control cells cultured in the absence of anticancer agents. IC50 values (drug dosages producing 50% inhibition of cell growth) were determined from growth inhibition curves.

Knockdown of ERα by siRNA transfection in MCF-7 cells

Cells (1×10^5/well) were cultured in RPMI medium for 24 h. In accordance with the manufacturer's instructions, 10 nmol/l small interfering RNA [ESR1-7255 (Sigma; si-1) and ESR1-7257 (Sigma; si-2) siRNAs for knockdown of ERα] or scramble siRNA (SC-37007, Santa Cruz Biotechnology, CA) was mixed with siLentFect Lipid Reagent (Bio-Rad, CA) in serum-free RPMI. After 20 min, solutions were added to the cells. To investigate the effects of ERα knockdown, cells were harvested and ERα expression was determined by western blotting using anti-ERα antibody (sc-7207, 1/200, Santa Cruz).

Establishment of SKBR3 cells expressing ERα

ER-negative and HER2-positive SKBR3 cells were transfected with an expression plasmid vector for ERα using Trans IT-LT1 (Mirus Bio, WI) following the manufacturer's specifications, and were subjected to selection in growth medium containing geneticin (Sigma). We confirmed ERα expression employing real-time RT-PCR. We use the term SKBR3-cont to indicate cells transfected with control vector, while SK-ERpos cells were transfected with ERα.

Western blot analysis

Cell lysates were prepared using Lysis-M Reagent (Roche Diagnostics, Germany) according to the manufacturer's

instructions. Total proteins (40 μg) were run on SDS-PAGE using 10% acrylamide gels (SuperSep™ace; Wako, Japan) and proteins were transferred onto a PVDF membrane (Bio-Rad). After blocking the membrane with 10% dry milk for 1 h, blots were probed with an antibody to ERα (sc-7207), α-tubulin (sc-5546, 1/200, Santa Cruz), β-tubulin (#2146, 1/1000, Cell Signaling Technology, MA), or acetylated α-tubulin (6-11B-1, 1/2000, Sigma-Aldrich) in 0.05% Tween-20 in TBS (TTBS) at 4°C overnight. Secondary antibodies used at room temperature for 1 h were as follows: goat anti-rabbit IgG (Immun-Star Goat Anti-Rabbit-AP, 1/3000; Bio-Rad) for ERα, α-tubulin and β-tubulin, goat anti-mouse IgG (Immun-Star Goat Anti Mouse-AP, 1/3000; Bio-Rad) for anti-acetylated α-tubulin. An Immun-Star™ chemiluminescent Protein Detection System (Bio-Rad) was used to detect the secondary probes. The image was captured on an LAS-4000 with accompanying Image Reader (Fujifilm, Tokyo Japan). Protein expression was analyzed using Multi Gauge v3.2 software (Fujifilm).

Real-time quantitative RT-PCR

RNA was extracted from whole cells using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. cDNAs were synthesized from 1 μg of total RNA using a TaKaRa RNA PCR Kit Ver.3.0 (Takara Bio, Shiga, Japan). Real-time quantitative RT-PCR was performed using the LightCycler 2.0 (Roche Diagnostics) with LightCycler FastStart DNA Masterplus SYBR Green 1 kit (Roche Diagnostics). Target gene expression was normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression level. For PCR, the following ERα primer sequences were used: forward, 5′-CTCCCACATC AGGCACAT-3′; reverse, 5′-CTCCAGCAGCAGGCTA TA-3′. The HDAC6 primers were as follows: forward, 5′-GTCTACTGTGGTCGTTACATC-3′; reverse, 5′-GGC CTGACAGTAACAC-3′.

Patients received NAC at Juntendo University Hospital

All patients who received NAC had tumors over 3 cm in diameter and/or positive axillary nodes, but no distant metastasis prior to NAC. Determinations of invasive cancer, hormone receptor status, and HER2 status were based on pre-NAC core needle biopsy results.

The patients received four cycles of fluorouracil (500 mg/m^2), epirubicin (75 or 100 mg/m^2), and cyclophosphamide (500 mg/m^2) (FEC), followed by taxanes, namely, 12 weekly cycles of paclitaxel (80 mg/m^2) or docetaxel (150 mg/m^2) every 3 weeks for four cycles. Sonography plus MRI was carried out at diagnosis, then again prior to the administration of taxanes, and before surgery. A clinical complete response (cCR) was disappearance of the tumor on these images. A clinical partial response (cPR) was more than 30% shrinkage of the tumor with NAC, and clinical progressive disease (cPD) was a more 20% increase in the tumor. Any response other than these was categorized as clinical stable disease (cSD). A pathological CR (pCR) involved no evidence of intraductal components or metastatic lesions.

We first analyzed 190 patients with advanced breast cancer who received NAC at the Breast Center of Juntendo University Hospital (Tokyo, Japan) between July 2006 and January 2008. To assess the relationships between ER activity in breast cancer cells and the clinical response to NAC, we analyzed 31 breast cancer tissues, obtained by core needle biopsy and/or surgical resection at Juntendo University Hospital from June 2009 to March 2010. All tumor specimens were obtained after informed consent from patients. The Juntendo University Hospital Ethics Committee approved this study.

Immunohistochemistry (IHC)

ER status was determined using IHC methods. Slides were stained and evaluated using the Ventana I-VIEW Breast Panel (Roche). Immunoreactivity greater than 10% was considered to indicate receptor-positive status. HER2 protein status, as assessed using the Herceptest (Ventana, Switzerland), was scored on a scale of 0 to 3+ according to the Dako scoring system. HER2/neu-positive status was defined as HER2 protein 3+ or 2+ and a fluorescence in situ hybridization (FISH) ratio of more than 2.2.

Assay of ERE activity in primary tumor cells (adenovirus ERE–GFP method)

To assess ERE activation in primary tumor cells, we used Ad-ERE-tk-GFP [20, 21]. Cancer tissue specimens were minced to $\sim$1 mm^3 after rinsing with PBS and digested with collagenase solution for 20–30 min at 37°C. The cells, including tumor cells, were washed several times with PBS, and incubated in 24-well plates using 400 μl of phenol-red-free RPMI 1640 supplemented with 10% heat- and charcoal-treated fetal calf serum (DCC-FCS). The cells were then infected, either immediately or 1 day later, with 2×10^9 PFU (plaque-forming units) (in 293A cells) of Ad-ERE-tk-GFP, and incubated for 3 days. GFP-expressing cells were counted by fluorescence microscopy after incubation. To examine the infectivity of the adenovirus in primary tumor cells, the cells were infected with 2×10^9 PFU of Ad-CMV-DsRed, and at least 95% of cells were confirmed to be infected [21]. When more than 10% of cancer cells expressed GFP, the specimen was considered to have high ER transcriptional activity.