Statistical methods

The primary endpoint was to examine 3 year DFS stratified by pathological response (QpCR versus non-QpCR). Secondary endpoints included predictors for QpCR, clinical response, the rate of BCS, and safety.

For the primary efficacy analysis, we assumed that approximately 25% of patients would achieve QpCR and that the 3 year DFS rate in patients with non-QpCR would be 70%. To demonstrate a 20–25% reduction in the hazard of DFS between patients achieving QpCR compared with those without QpCR, we planned to enroll 200 patients. Using the log rank test this would provide $\alpha = 0.05$ and $\beta = 0.2$.

Kaplan–Meier analysis was used to estimate the values of DFS. DFS was compared using a log-rank test stratified for QpCR and non-QpCR. Events for the calculation of DFS include all local, regional, or distant recurrence, all clinically inoperable and residual disease at surgery, all second cancers, contralateral breast cancers, and all deaths.

In the logistic regression analyses, adjustments were made for the stratification variables of menopausal status, tumor size, estrogen receptor status, progesterone receptor status, HER2 status, clinical response to FEC treatment and clinical response to docetaxel following FEC treatment. Analyses were performed with JMP (version 6, SAS Institute Inc.). Analyses of endpoint data reported here are based on information received as of July 2007.

Results

Patient characteristics

Between June 2002 and June 2004, 202 patients were prospectively enrolled. As two patients were ineligible and two patients withdrew consent, 198 patients were assessed for safety. One patient was removed from the study after planned chemotherapy but before surgery because of a protocol violation (non-protocol chemotherapy), four patients elected to not have surgery and withdrew from the study, and two were lost to follow-up, leaving 191 evaluable for clinical, pathologic assessment and DFS.

The median age of the assessable 198 patients was 46 years, and 72% of patients were pre-menopausal. The majority of the patients had T2 tumors (74%), with 20% of the patients having T3 tumors and 6% with T1 tumors (Table 1). Distribution with regard to hormone receptor or HER2 overexpression was representative of that seen in common practice in Japan [15].

Table 1 Patients characteristics ($n = 198$)

	No. of patients	%
Age (years)		
Median	46	
Range	25–60	
Menopausal status		
Pre	142	72
Post	56	28
Tumor stage		
T1	12	6
T2	146	74
T3	40	20
Nodal stage		
N0	80	40
N1	117	59
N2	1	1
Hormone receptor status		
ER		
Positive	133	67
Negative	62	31
Unknown	3	2
PgR		
Positive	100	51
Negative	95	48
Unknown	3	2
HER2 (IHC)		
0	60	30
1+	54	27
2+	42	21
3+	38	19
Unknown	4	2

ER estrogen receptor, *PgR* progesterone receptor, *IHC* immunohistochemistry

Percentages may not add up to 100% because of rounding

Compliance to chemotherapy and toxicity

Dose reduction due to toxicities was made in 18% of the patients during FEC treatment; febrile neutropenia (19), grade 3–4 neutropenia without fever (10), suspicion of febrile neutropenia (4), vomiting, and deterioration in liver function (1 each) and 14% of patients during docetaxel therapy, febrile neutropenia (5), grade 3–4 neutropenia without fever (5), neutropathy (2), deterioration in liver function (2), myalgia (2) allergy (1) previous reduction of FEC (8), and unknown (2).

Six patients (3%) discontinued FEC treatment due to toxicities (3: two patients with febrile neutropenia and one with vomiting), progression of disease (2), and mental disorder (1). Ten (please refer toxicity section) patients (5%) discontinued docetaxel treatment due to toxicity (3:

one patient each with rash, febrile neutropenia, and phototoxicity), progression of disease (3), and patients' requests for early surgery (2) changing hospital (1), patient's request (1).

Percentage of treatment cycles requiring dose reduction for FEC, docetaxel and all were 11.1, 11.6 and 11.3%. Percentage of treatment cycles (FEC, docetaxel and all) including rh G-CSF were 10.5, 8.2 and 9.4%, respectively.

The safety profile is summarized in Table 2. Four patients didn't receive docetaxel treatment at patients' request. For toxicity 198 and 194 patients were evaluable for FEC treatment and docetaxel treatment, respectively. The most common adverse event was grade 3 or 4 neutropenia, which was observed in 44% of patients during FEC treatment and 35% of patients during docetaxel treatment. Fever, including febrile neutropenia, was seen in 20% and 7% during treatment with FEC and docetaxel, respectively. The only grade 3–4 non-hematologic toxicities reported were; nausea (12 patients), vomiting (11) and fatigue (3). No fatal events were observed.

Response to treatment

The overall clinical response was 74% (95% CI, 67–80%) with 22% CR and 52% PR. Thirty-eight (51%) of 75 FEC non-responders had a response to docetaxel treatment. One hundred and six of 118 FEC responders maintained their response or had a continued decrease in tumor size with docetaxel (Table 3). QpCR were seen in 25% of patients (including 16% complete disappearance of invasive carcinoma in the breast). One patient was removed from assessable for BCS because of a protocol violation. BCS was achieved in 85% of all the assessable patients. Ninety-two percent of patients who had original tumor size 3 cm or less underwent BCS; those with larger tumors had an 80% rate of BCS. As of July 11, 2007, with a median follow up of 40 months, the estimated 3-year DFS was 91% for all patients. Patients who achieved QpCR had significantly improved DFS compared to those without QpCR (QpCR (98%) and non-QpCR (89%), log rank test, $P = 0.0333$, Fig. 1). HR 0.38 [95% CI 0.09–0.84], $P = 0.0134$).

Predictive factors of pathological response

A multiple logistic regression analysis was performed to examine which factors among menopausal status, tumor size, estrogen receptor status, progesterone receptor status, HER2 status and clinical response to FEC were associated with QpCR (Table 4). HER2 status and response to the initial FEC treatment and response to docetaxel were independent predictive factors for QpCR. The QpCR rates stratified by HER2 and ER are shown in Fig. 2. QpCR rate was 67, 33, 35 and 13% in HER2 positive/ER negative, HER2 positive/ER positive, HER2 negative/ER negative, HER2 negative/ER positive, respectively.

Table 2 Treatment related toxicities

	FEC (n = 198)		Docetaxel (n = 194)	
	All grades n (%)	Grade 3, 4 n (%)	All grades n (%)	Grade3, 4 n (%)
Non-hematologic toxicities				
Fatigue	83 (42%)	2 (1%)	83 (42%)	1 (1%)
Diarrhea	17 (9%)	1 (1%)	31 (16%)	0
Nausea	162 (82%)	11 (6%)	81 (42%)	1 (1%)
Vomiting	98 (50%)	10 (5%)	38 (20%)	1 (1%)
Neurotoxicity	6 (3%)	0	85 (44%)	2 (1%)
Constipation	67 (34%)	0	50 (26%)	1 (1%)
Arthralgia/myalgia	12 (6%)	0	60 (30%)	1 (1%)
Hematologic toxicities				
Hemoglobin	119 (60%)	1 (1%)	101 (52%)	0
Platelets	26 (13%)	1 (1%)	3 (2%)	1 (1%)
AST/ALT	81 (41%)	3 (2%)	70 (36%)	1 (1%)
Leukocytes	131 (66%)	68 (35%)	92 (47%)	57 (30%)
Neutrophils	137 (69%)	85 (44%)	85 (44%)	67 (35%)
Febrile neutropenia	–	40 (20%)	–	14 (7%)

FEC fluorouracil, epirubicin, cyclophosphamide

Table 3 Clinical response after FEC and after docetaxel following FEC treatment ($n = 194$)

Clinical response, *N* (%)	Overall	
	Responder	Non-responder
FEC		
Responder	106 (90%)	13 (10%)
Non-responder	38 (51%)	37 (49%)

cCR + cPR responder, *cSD + cPD* non-responder, *FEC* fluorouracil, epirubicin, cyclophosphamide, *CI* confidence interval

Discussion

We have presented results from the largest study to date that enrolled Japanese women undergoing preoperative chemotherapy for early stage breast cancer. Our findings demonstrated that four cycles of preoperative FEC followed by four cycles of docetaxel conferred a high rate of BCS, even among patients with primary tumors larger than 3 cm. We found a significant improvement in DFS when QpCR could be achieved, compared to the absence of QpCR. HER2 overexpression, response to FEC and response to docetaxel were significant predictors of QpCR with this regimen.

Regarding toxicity, there were no fatal events and no significant differences in the types and severity of toxicity as compared to other recent studies using similar regimens outside of Japan [6, 8, 9, 16–18]. Compared with overseas studies that also did not allow rh G-CSF the incidence of fever was the same in this study [8, 19]. In another studies which showed lower incidence of febrile neutropenia (13.5%) all patients were treated with rh G-CSF [16].

One of the merits of neoadjuvant chemotherapy for operable breast cancer is to decrease the size of the primary tumor in order to allow for BCS. The study protocol did not provide guidelines for breast conservation; therefore, the BCS rate that we observed reflected the biases that may occur in real-life clinical practice in Japan. Nevertheless, the BCS rate of 80% that we observed was favorable compared with other neoadjuvant studies performed overseas [3, 16].

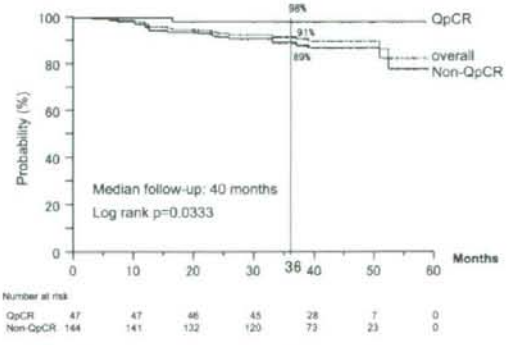

Fig. 1 Relationship of QpCR and non-QpCR to disease free survival

The PACS 01 trial which compared six cycles of adjuvant FEC with a sequential regimen of three cycles of FEC followed by three cycles of docetaxel 100 mg/m^2 (FEC-D) demonstrated an 18% risk reduction in DFS and 27% risk reduction in OS with FEC-D (adjusted $P = 0.017$). This study supports the conclusions that sequential adjuvant chemotherapy with FEC followed by docetaxel significantly improves DFS and OS in node-positive breast cancer patients [9]. In the current study the dose of docetaxel 75 mg/m^2 was selected based on the recommended doses for docetaxel in Japan, and we showed that the actual 3-year DFS rate of 91% was better than expected based on the results of overseas studies [7, 9, 20]. This confirms that the approved doses of 75 mg/m^2 is an appropriate does in Japanese women.

Furthermore a new definition of QpCR was defined for pathological effect in this study. When stratified between QpCR and non-QpCR, patients with QpCR had significantly favorable DFS. Indeed by adding docetaxel to FEC patients with QpCR resulted in improved survival similar to previous studies.

Even without anti-HER2 targeting therapy, a QpCR rate >60% was achievable in ER negative and HER2 positive tumors. A multivariate analysis has indicated the significant value of HER2 overexpression, which seems to suggest the importance of HER2 in the prediction of QpCR with this regimen. In this study both an anthracycline and docetaxel were used, so it is not clear which treatment was more strongly associated with HER2 as a predictive value of QpCR. Data in the metastatic and adjuvants setting suggest that docetaxel regimens may be more active than non docetaxel regimens in HER2 positive tumors [8, 21]. The value of HER2 status as a predictor of response to anthracycline-based chemotherapy is still a matter debate. On the other hand, there are several implicative data showing the predictive value of topoisomerase (Topo)-II for anthracyclines because Topo-II is a molecular target of anthracyclines [22–25]. There is evidence that HER2 amplification and Topo-II amplification usually occur in parallel and it is rare to have Topo-II amplification without HER2 amplification [23, 26]. In this study QpCR rate might clarify the difference between HER2 positive tumors and HER2 negative tumors. No patient has received trastuzumab in the adjuvant setting. Future translational studies will be necessary to explore the significance of Topo-II amplifications as well as HER2 gene amplifications in the prediction of the pathological response of this regimen. This result will be included the information in the future if

Table 4 Predictive variables for QpCR

Variables	Before treatment OR 95% CI (*P*)	After FEC treatment OR 95% CI (*P*)	After docetaxel following FEC treatment OR 95% CI (*P*)
Menopausal status	1.43	1.38	1.37
Pre (versus post)	0.94–2.15 (NS)	0.89–2.14 (NS)	0.87–2.12 (NS)
Tumor size	0.89	0.93	0.87
>3 cm (vs ≤3 cm)	0.61–1.3 (NS)	0.63–1.37 (NS)	0.59–1.28 (NS)
ER	1.4	1.44	1.35
Negative (versus Positive)	0.87–2.27 (NS)	0.88–2.36 (NS)	0.81–2.23 (NS)
PgR	1.61	1.49	1.65
Negative (versus Positive)	0.97–2.67 (NS)	0.89–2.51 (NS)	0.98–2.79 (NS)
HER2	2.02	2.24	2.11
3+ (vs <3+)	1.31–3.11 (0.0014)	1.42–3.53 (0.0005)	1.36–3.3 (0.0009)
Clinical response to FEC treatment	–	1.78	–
Response (versus non-response)	–	1.15–2.76 (0.0096)	–
Clinical response to docetaxel following FEC treatment	–	–	1.99
Response (versus non-response)	–	–	1.14–3.47 (0.0154)

QpCR quasi pathological complete response, *FEC* fluorouracil, epirubicin, cyclophosphamide, *OR* odds ratio, *ER* estrogen receptor, *PgR* progesterone receptor, *CI* confidence interval, *NS* not significant

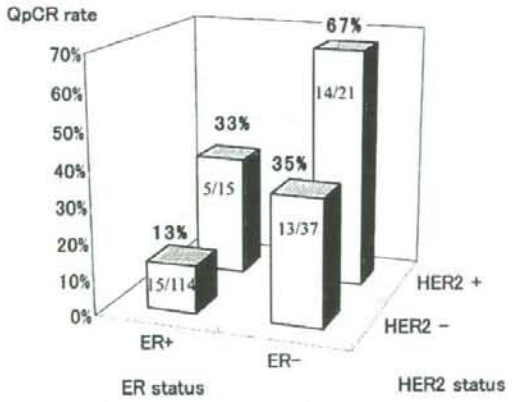

Fig. 2 Relationship between QpCR and HER2/ER status (n=187)

we use anthracycline and trastuzumab for all HER2 positive patients.

In the present study, though a multivariate analysis hasn't indicated the significant value of the status of hormone receptor, QpCR rate was higher in ER negative tumors than ER positive tumors, and QpCR rate in ER negative and HER2 positive tumors was remarkably high compared with ER positive and HER2 negative tumors. This model suggests that ER status is a dependent predictor, for QpCR possibly because it is related to HER2 expression. The sample size was perhaps too small to effectively determine the true impact of ER negative status as a predictor of QpCR. As most patients who are HER2 positive are also ER negative, it is likely that ER status will have some predictive value. However, larger studies are needed to determine this. These results are important for considering individual preoperative systemic therapy. This trend was similar to previous studies using AC followed by paclitaxel regimens, though the therapeutic situations are different [10, 12, 27, 28]. According to recent meta-analyses of post-operative adjuvant therapy, chemotherapy including cyclophosphamide/methotrexate/5FU (CMF)-type regimens, anthracycline-containing regimens and anthracycline followed by paclitaxel are more effective for hormone receptor negative tumors than for hormone receptor positive tumors [10–12, 27–32]. However, while hormone receptor negative tumors may be more responsive to preoperative regimens, a survival benefit can be observed regardless of receptor status [2]. In this study a multivariate analysis hasn't indicated the significant value of the status of hormone receptor. This may be affected by addition of docetaxel. Dose response with anthracycline is also different between hormone receptor positive tumors and hormone receptor negative tumors. For ER negative tumors, higher anthracycline doses may be required for improved prognosis, however, for ER positive tumors it might not be necessary [29].

In this study, most tumors responded to docetaxel even if they did not respond to FEC. However, some tumors showed a response to the initial therapy but a lesser response to the second therapy. This underscores the need to include non-cross resistant treatments in the

management of early stage breast cancer [33]. Various non-cross resistance molecules may be involved in this clinical phenomenon. Recent investigations indicate that initial chemotherapy may change the phenotype of the tumor by inducing pro-survival molecules in tumor cells or stroma [2, 3, 5, 7, 16]. In particular, key mediators such as nuclear factor-kappa B, cyclooxygenase-2 and thymidine phosphorylase are known to be induced by chemotherapy frequently, which may change those tumors relatively anti-apoptotic to the second chemotherapy [34–36]. From the clinical point of view, it would be useful to modify the treatment schedule based on initial response to treatment. Since the types of pro-tumor molecules and the magnitude of induction are different between agents, it might be reasonable to consider a different sequence (taxane followed by anthracycline), if information on the tumor phenotype could be obtained before starting treatment. Various treatment scenarios for non-responders to FEC could be considered. According to recent study results, surgery might be an option for non-responders to initial anthracyclines [37]. In order to enhance the effect of docetaxel, the combination with fluoropyrimidines such as capecitabine may be an option. Obviously for HER2 overexpressing tumors, anti-HER2 containing therapy should be considered. For the ER positive and HER2 negative phenotype, hormone therapy might be an option if tumors are relatively well differentiated. Individual treatment based on ER/HER2 status and the clinical response to the initial anthracyclines may be integrated as future direction [37].

In conclusion, 8-cycle preoperative chemotherapy with non-cross resistant regimens, FEC followed by docetaxel, is safe, feasible, and effective as primary systemic therapy for women with early stage breast cancer. In particular, the regimen allows a majority of Japanese patients to avoid the need for mastectomy. Patients with QpCR demonstrated significantly superior survival results. HER2 over-expression, response to FEC and response to docetaxel were significant predictors for QpCR. Based on our results, preoperative FEC followed by docetaxel should be considered a standard option for the treatment of Japanese women with operable breast cancer.

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ORIGINAL PAPER

Histopathological assessment of anastrozole and tamoxifen as preoperative (neoadjuvant) treatment in postmenopausal Japanese women with hormone receptor-positive breast cancer in the PROACT trial

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Abstract
Purpose The PReOperative 'Arimidex'® Compared with Tamoxifen (PROACT) trial compared neoadjuvant anastrozole and tamoxifen in postmenopausal women with large, operable or potentially operable, locally advanced hormone receptor-positive breast cancer. Here, we compare objective clinical responses with histopathological tumor responses to therapy in a cohort of 97 Japanese patients, in order to investigate the consistency of assessment methods and the change in estrogen-receptor (ER) and progesterone-receptor (PgR) status.
Methods Histopathological response and the change in ER and PgR status were assessed by comparing pathological specimens collected at baseline (via needle biopsy) with those collected at 3 months (from excised tumors). The response was evaluated using Pathological Response Criteria for Breast Cancer as defined by the Japanese Breast Cancer Society. The patients were randomized to receive anastrozole ($n = 48$) or tamoxifen ($n = 49$).
Results A numerically greater histopathological response rate was observed when neoadjuvant anastrozole compared with neoadjuvant tamoxifen (35.4 and 12.2%, respectively). The histopathological and clinical objective response rates agreed in 63/97 patients. The ER status of 5/40 patients changed from positive at baseline to negative at 3 months in the anastrozole group compared with 20/37 patients in the tamoxifen group. The PgR status of 16/17 patients in the anastrozole group and of 1/11 patients in the tamoxifen group changed from positive to negative.
Conclusions These data support the findings of the main PROACT trial, which confirmed that anastrozole, as compared with tamoxifen, is an effective neoadjuvant endocrine treatment in objective response rates for postmenopausal women with large operable hormone-receptor positive breast cancer. Further follow-up is required to confirm whether histopathological responses to therapy correlate with an overall improvement in survival.

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Keywords Anastrozole · Breast neoplasms · Histology · Neoadjuvant therapy · Pathology · Postmenopause · Tamoxifen

Introduction

The concept of preoperative or neoadjuvant therapy to achieve tumor downstaging prior to surgery is not new. Downstaging may be clinically significant, in that a tumor considered inoperable may become operable, or more conservative surgery may become feasible, where previously mastectomy was the only possible option. Neoadjuvant cytotoxic chemotherapy has already proven effective in downstaging tumors and in increasing the number of operable cancers in the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-18 trial (Wolmark et al. 2001). Analysis of data from the NSABP B-18 trial ($n = 1,523$) confirmed that clinical response to neoadjuvant chemotherapy was significantly correlated with treatment outcome for disease-free survival ($P = 0.0008$), recurrence-free survival ($P = 0.0002$), and overall survival ($P = 0.05$) at 9 years follow-up. Overall survival at 9 years was 78% in patients achieving a clinical complete response (cCR; i.e. the absence of clinical evidence of breast tumor on physical examination). However, pathological response differentiated the patients achieving a cCR in terms of outcome. Patients with a pathological complete response (pCR; i.e. no histological evidence of invasive carcinoma on pathological examination of the surgical specimen) showed improved overall survival at 9 years compared with patients achieving a cCR who had residual invasive cancer on pathological examination (overall survival 85 and 73%, respectively) (Wolmark et al. 2001). Patients with a pCR had a reduction of 50% in risk of death compared with the group as a whole [relative risk 0.50; 95% confidence intervals (CI) 0.32, 0.78]. These data, therefore, suggest that pathological response to neoadjuvant treatment is a significant prognostic factor for survival (Kurosumi 2004).

More recent data indicate an association between the expression of estrogen and progesterone receptors (ER and PgR, respectively) and therapeutic response in terms of event-free survival (Vyzula et al. 2004). Patients with tumors expressing both ER and PgR experienced significantly better event-free survival than those with tumors with other hormone-receptor profiles. It is, therefore, logical to investigate endocrine therapies for neoadjuvant treatment, as they might be expected to be effective in downstaging hormone receptor-positive tumors.

Studies reporting the benefits of neoadjuvant tamoxifen, and more recently aromatase inhibitor treatment, have been published (Dixon et al. 2000; Eiermann et al. 2001; Ellis et al. 2001; Mansi et al. 1989; Preece et al. 1982; Semiglazov et al. 2003), although the effect (including the pathological effect) of neoadjuvant treatment on response to consequential adjuvant treatment has yet to be studied.

In the 'Arimidex', Tamoxifen, Alone or in Combination (ATAC) trial, anastrozole proved to be more effective (in terms of disease-free survival) and better-tolerated than tamoxifen as adjuvant therapy for postmenopausal women with hormone receptor-positive breast cancer (Trialists' Group 2005). It is, therefore, reasonable to compare their relative efficacies in the neoadjuvant setting and, furthermore, non-comparative studies have already reported beneficial outcomes for neoadjuvant treatment with anastrozole (Dixon et al. 2000; Anderson et al. 2002).

The PReOperative 'Arimidex'® Compared with Tamoxifen (PROACT; NCT00232661) trial confirmed that is compared with tamoxifen, anastrozole is an effective preoperative treatment for postmenopausal women with large, operable or potentially operable (T2-4b, N0-2, M0) hormone receptor-positive breast cancer (Cataliotti et al. 2006). Here, we look beyond objective-response data evaluating the histopathological response and the change in ER and PgR status at the end of the preoperative period in the Japanese cohort of the PROACT study. Some of the results were reported by Tsuda et al. (2005) at the American Society of Clinical Oncology annual meeting in 2005.

Patients and methods

Patients

Postmenopausal women with large, operable or potentially operable, locally advanced, ER-positive and/or PgR-positive breast cancer were recruited from 25 study centers in Japan over a period of 25 months.

Eligible patients had to satisfy the following criteria:

1. Have operable (T2 [≥3 cm], T3, N0-2, M0) or potentially operable, locally advanced (T4b, N0-2, M0), measurable breast cancer (≥3 cm in diameter, measured using both ultrasound and caliper methods)
2. Have histologically and cytologically proven ER-positive and/or PgR-positive invasive breast cancer using needle-biopsy specimens (Kurosumi 2003).
3. Be likely to benefit from neoadjuvant endocrine therapy, in the opinion of the investigator. (Patients were not included if the investigator judged that other therapy, e.g. chemotherapy should be administered.)
4. Be considered postmenopausal (defined as patients aged ≥60 years, or aged 45–59 years with amenorrhea for ≥12 months and an intact uterus, or with amenorrhea

for <12 months and postmenopausal levels of follicle-stimulating hormone [including patients who had undergone a hysterectomy or were receiving hormone-replacement therapy], or patients who had undergone bilateral oophorectomy).

Patients were ineligible if they had inoperable tumors, true inflammatory carcinoma, or were unwilling to undergo mastectomy at the end of neoadjuvant treatment.

Patients were not permitted any previous exposure to tamoxifen or other selective ER modulators, or treatment with a non-approved or experimental drug at 3 months prior to randomization.

For those centers that used neoadjuvant chemotherapy, specification of the chemotherapy regimen was performed before any patients were randomized. Only one chemotherapy regimen was allowed for all patients randomized from a single center and was chosen from commonly used worldwide regimens.

The use of chemotherapy and/or radiotherapy in the adjuvant period of the study was allowed. If given, the treatment followed the standard patient-management procedures in use at the investigational site.

Trial design

This was a randomized, double-blind, double-dummy study evaluating the efficacy of anastrozole versus tamoxifen as neoadjuvant therapy for postmenopausal women with large, operable or potentially operable, locally advanced, hormone receptor-positive, invasive breast tumors. Patients were randomized on a 1:1 basis to receive either anastrozole (1 mg daily p.o.) or tamoxifen (20 mg daily p.o.), with or without chemotherapy and/or radiotherapy for 12 weeks before primary surgery, and they were to continue receiving study medication for 5 years or until recurrence, intolerable toxicity, or patient refusal. In the rare event of progression during the neoadjuvant part of the trial, patients were withdrawn from study treatment and further treatment, including surgery, was at the investigator's discretion.

The primary objectives of this study were to assess the histopathological response and consistency of histopathological and clinical tumor response following neoadjuvant study treatment. Secondary objectives were to summarize the changes in ER and PgR status between baseline and surgery. Adverse events, monitored as part of the main PROACT trial, are reported elsewhere (Cataliotti et al. 2006).

This study was conducted in accordance with the principles specified in the Declaration of Helsinki and was consistent with International Conference on Harmonization/Good Clinical Practice requirements. All patients gave written and informed consent.

Assessments

The longest diameter of the tumor was recorded at baseline and at 3 months using ultrasound. In order to avoid operator variability and improve reproducibility, the ultrasound measurements were determined to be performed by the same doctor before and after treatment (i.e. at baseline and 3 months). Although RECIST criteria suggest that ultrasound should not be used to assess tumor response, except in operable and locally advanced breast cancer, it has been shown that ultrasound measurement provides the closest correlations with overall pathological measurements of primary breast tumors (Dixon et al. 1999). Therefore, ultrasound measurements were used in this study.

The objective tumor response at 3 months was defined as a CR (disappearance of the tumor) or partial response ($\geq$30% decrease from baseline in the largest diameter of the tumor), according to the World Health Organization Response Evaluation Criteria in Solid Tumors (RECIST) (World Health Organization 2002).

The histopathological response was assessed by comparing the pathological specimen collected at baseline (via needle biopsy) with that collected at 3 months (from excised tumors). The response was evaluated using the Pathological Response Criteria for Breast Cancer as defined by the Japanese Breast Cancer Society (Table 1) (Kurosumi et al. 2001). Central evaluation was performed by three pathologists, and final judgment was confirmed by consensus. All evaluations of pathological effects were performed blinded to information on clinical response and treatment received.

ER and PgR status using tissue samples obtained at baseline and at 3 months was assessed using an immunohistochemical method, where a 10% or more staining of the nucleus of a cancer cell is categorized as positive.

Statistical analysis

The intent-to-treat (ITT) population for histopathological assessment included all randomized Japanese patients (living in Japan). The per-protocol population of all major protocol violators and deviators, and patients with negative ER and PgR status excluded from the ITT population at baseline, as assessed by the Central Pathological Review Committee.

The histopathological response rate was calculated with two-sided 95% CI for each treatment group. No formal treatment comparison between anastrozole and tamoxifen was performed.

Table 1 Classification of response criteria

Grade	Response criteria
0	*No response.* Almost no change in cancer cells following treatment
1a	*Mild response.* Mild changes[a] in cancer cells regardless of the area, or marked changes[b] seen in <1/3 cancer cells
1b	*Moderate response.* Marked changes in ≥1/3 but <2/3 tumor cells
2	*Marked response.* Marked changes in ≥2/3 tumor cells
3	*Complete response.* Necrosis or disappearance of all tumor cells. Replacement of all cancer cells by granuloma-like and/or fibrous tissue. In the case of complete disappearance of cancer cells, pretreatment pathological evidence of the presence of cancer was necessary

When response assessment of intraductal components and lymph-node metastases is necessary, the above-mentioned criteria should be used and the assessment result for the intraductal components (d) and lymph-node metastases (n) can be added to the result of assessment of primary lesion response, e.g. Grade 1a + 1b(d) + 2(n)

Biopsy specimens (obtained by needle biopsy or incisional biopsy) should not be used for the final response assessment; but histopathological findings of individual specimens should be described

If the therapeutic response ranges over two grades, the lower grade of response should be selected

For Grade 3 assessment, multiple specimens must be examined

[a] Mild changes include slight degenerative changes in cancer cells not suggestive of the death of cancer cells (including cancer cells with vacuolation of cytoplasm, eosinophilic cytoplasm, and swelling of the nucleus, etc.)

[b] Marked changes include marked degenerative changes in cancer cells, suggesting that the cancer cells should barely survive (including liquefaction, necrosis, and disappearance of the cancer cells)

Results

Patients

A total of 97 patients, enrolled from 25 Japanese centers, were randomized to receive anastrozole ($n = 48$) or tamoxifen ($n = 49$) (Fig. 1). Of these patients, 43 in the anastrozole group and 39 in the tamoxifen group received endocrine therapy alone in the neoadjuvant phase of the study. The remaining 15 patients received chemotherapy as well as endocrine treatment. The recruited patient population was representative of the target population of postmenopausal women with large, operable or potentially operable, locally advanced, ER-positive and/or PgR-positive breast cancer. The two treatment arms were well balanced with respect to patient characteristics and demographics (Table 2).

Histopathological response

In the ITT population, the histopathological response rate following preoperative anastrozole treatment was numerically greater than that of the following preoperative tamoxifen. A total of 17/48 patients receiving preoperative anastrozole treatment had tumors of Grade ≥1b, corresponding to a histopathological response rate of 35.4% (95% CI 22.2, 50.5). This finding compared with 6/49 patients receiving preoperative tamoxifen, a histopathological response rate of 12.2% (95% CI 4.6, 24.8) (Fig. 2). For patients who did not receive any chemotherapy during preoperative anastrozole therapy, 16/43 patients had responses of Grade ≥1b (a histopathological response rate of 37.2%) compared with 3/39 patients treated with preoperative tamoxifen who did not receive chemotherapy (a histopathological response rate of 7.7%; Fig. 3). The endocrine-therapy-only subgroup was not pre-specified and is, therefore, included for descriptive purposes only.

There were no recorded histopathological CRs in either study arm. As in the ITT population, histopathological response rates for the per-protocol population were numerically greater in the anastrozole group than in the tamoxifen group; histopathological response rates of 37.0% (95% CI 23.2, 52.5) and 13.6% (95% CI 5.2, 27.4) were calculated for patients receiving anastrozole and tamoxifen, respectively. For patients in the per-protocol population who did not receive preoperative chemotherapy, 16/43 patients who received anastrozole had a histopathological response of Grade ≥1b (37.2%), compared with 3/36 patients receiving tamoxifen (8.3%).

Consistency of histopathological and clinical objective tumor response

The comparison of histopathological and clinical objective responses for the ITT population is shown in Table 3. Overall, 63/97 patients (64.9%; 11 responders and 52 non-responders) in the ITT population had consistent histopathological and clinical objective responses. The per-protocol population showed a similar level of consistency, with agreement between histopathological and clinical objective response in 57/90 patients (63.3%; 11 responders and 46 non-responders).

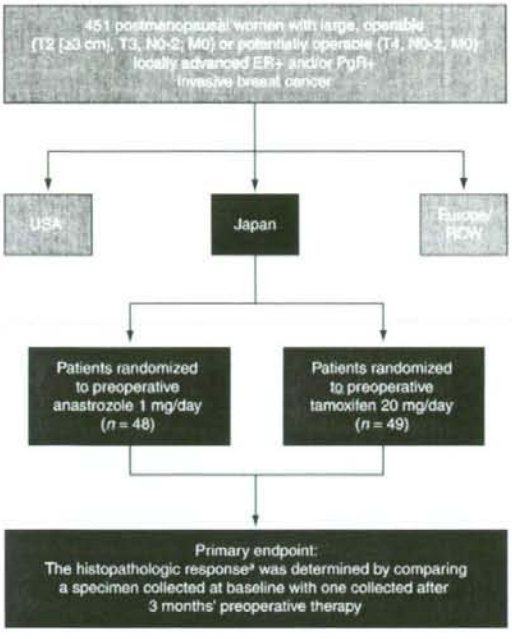

Fig. 1 Patients and study design. [a] The response was evaluated on a 5-grade categorical scale (Grade 0, 1a, 1b, 2, and 3), according to the Japanese Histopathological Criteria for Assessment of Therapeutic Response in Breast Cancer, by the Central Pathological Review Committee. A response was defined as Grade ≥1b (i.e. a degenerative change in ≥1/3 of constituent carcinoma cells). The main outcome was an assessment of the invasion area

Table 2 Patient demographics and baseline disease characteristics

	Anastrozole 1 mg/day ($n = 48$)	Tamoxifen 20 mg/day ($n = 49$)
Median age (range), years	61.5 (51.4–84.7)	61.6 (51.4–81.3)
Median body mass index (range), kg/m^2	23.7 (15.7–32.0)	23.6 (18.9–35.2)
Feasible surgery, *n* (%)		
Breast conserving	2 (4.2)	2 (4.1)
Mastectomy	46 (95.8)	47 (95.9)
Median tumor dimension (range), cm		
By ultrasound	3.6 (1.7–9.0)	3.6 (1.8–6.3)
By caliper	4.2 (3.0–11.5)	4.5 (3.0–11.0)
TNM classification, *n* (%)		
T2	31 (64.6)	26 (53.1)
T3	9 (18.8)	15 (30.6)
T4a	0 (0)	0 (0)
T4b	8 (16.7)	8 (16.3)
N0	32 (66.7)	32 (65.3)
N1	15 (31.3)	14 (28.6)
N2	1 (2.1)	3 (6.1)
M0	48 (100.0)	49 (100.0)
ER and PgR status		
ER+ and PgR+	32 (66.7)	27 (55.1)
ER+ and PgR−	15 (31.3)	21 (42.9)
ER− and PgR+	1 (2.1)	1 (2.0)
Hormonal therapy only	43 (89.6)	39 (79.6)

ER estrogen receptor, *PgR* progesterone receptor, *TNM* Tumor, node, metastases

Change in hormone-receptor status

In the ITT population, a total of 40 patients who received anastrozole and 37 who received tamoxifen had an ER-positive status at baseline (Table 4). Of these patients, the ER status of 5 patients who received anastrozole changed to negative compared with 20 patients who received tamoxifen at 3 months. The ER status of the remaining patients (anastrozole, $n = 35$; tamoxifen, $n = 17$) remained positive at 3 months.

In the ITT population at baseline, 17 patients who received anastrozole and 11 patients who received tamoxifen were PgR-positive (Table 4). At 3 months, the PgR status of 16 patients who received anastrozole changed to negative when compared with only one patient who received tamoxifen.

The change in ER/PgR status in the per-protocol population was very similar to the results of the ITT population in both treatment groups (data not shown).

Discussion

A numerically higher histopathological response rate was observed following preoperative treatment with anastrozole than following preoperative tamoxifen in both the ITT and per-protocol populations. Histopathological response was consistent with the clinical response in approximately two-thirds of cases in both the ITT and per-protocol populations.

The histopathological assessment criteria used in this study were originally designed for the assessment of the efficacy of cytotoxic chemotherapeutic agents. Here, these criteria have been used to describe therapeutic responses of lesser magnitude than pCR with endocrine agents, which are better tolerated than chemotherapy. The histopathological response rate of 35% (37.2% in patients who did not receive preoperative chemotherapy) with anastrozole concurs with the clinical objective response rate (by RECIST criteria) and can, therefore, be considered reliable.

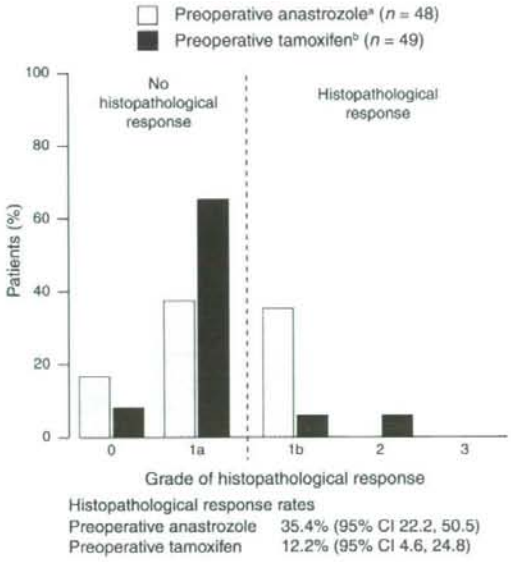

Fig. 2 Histopathological response at 3 months for the intent-to-treat population. [a]Five patients in the anastrozole group discontinued preoperative treatment. [b]Six patients in the tamoxifen group discontinued preoperative treatment, and one patient did not receive any study treatment

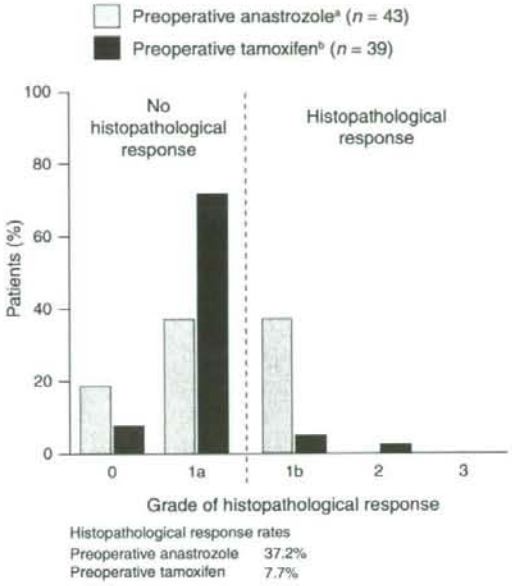

Fig. 3 Histopathological response at 3 months for patients in the intent-to-treat population who did not receive preoperative chemotherapy. [a] Three patients in the anastrozole group discontinued preoperative treatment. [b] Four patients in the tamoxifen group discontinued preoperative treatment, and one patient did not receive any study treatment

In this study, the results of pathological evaluation were consistent with ultrasonographic findings in 63/97 patients (64.9%), and not consistent in 34 (≈35%) patients. It is well known that the clinical response may not be completely consistent with the pathological one (Beresford et al. 2007); however, pCR is used as the endpoint of many preoperative treatments. The degree of therapeutic response evaluated by histological examination can differ from that of based on ultrasonographic findings due to various factors, two examples follow. The therapeutic response based on ultrasonographic findings is lower when cancer cells at the center of the mass disappear and are replaced by fibrous elements (Thomas et al. 2007), but marginal cancer cells still form a ring; in this example the histological response is evaluated higher than the ultrasonographic one. The therapeutic response based on ultrasonographic findings is higher when the treatment is effective on intraductal components of cancer with a significant decrease in the overall volume of the mass, but it is hardly effective on small foci of invasion (Matsuo et al. 2002); in this example the histological response is evaluated lower than the ultrasonographic one. Further follow-up is required to establish an association between clinical or histopathological response and treatment outcome, as it has been established for neoadjuvant chemotherapy.

Table 3 Cross-tabulation of histopathological and objective tumor responses in the intent-to-treat patient population

Histopathological response	Clinical objective tumor response		
	Responder	Non-responder	Total
Responder	11	12	23
Non-responder	21	52	73[a]
Not collected[b]	0	1	1
Total	32	65[a]	97

[a] Eleven patients withdrew before 3 months and are included with the non-responders

[b] Patient did not receive any study treatment

Considering that adjuvant endocrine therapy is typically administered over a period of 5 years, and that endocrine therapy in effect starves tumor cells of estrogenic stimulation rather than directly eliminating them, a period of 12 weeks may be insufficient to gain a pCR with neoadjuvant endocrine treatment. This factor may account for the lack of pCRs in our patients. The prognostic significance of a moderate response (i.e. a cCR or partial response, but not a pCR) has yet to be established for neoadjuvant endocrine therapy, but will be defined with longer follow-up.

Table 4 Change in estrogen/progesterone-receptor status by histopathological assessment in the intent-to-treat patient population

Baseline	Post-observation		
	Positive	Negative	Not evaluated
ER status			
Anastrozole ($n = 48$)			
Positive ($n = 40$)	35 (72.9)	5 (10.4)	0 (0.0)
Negative ($n = 3$)	2 (4.2)	1 (2.1)	0 (0.0)
Not evaluated ($n = 5$)	0 (0.0)	0 (0.0)	5 (10.4)
Tamoxifen ($n = 49$)			
Positive ($n = 37$)	17 (34.7)	20 (40.8)	0 (0.0)
Negative ($n = 5$)	1 (2.0)	4 (8.2)	0 (0.0)
Not evaluated ($n = 7$)[a]	0 (0.0)	0 (0.0)	7 (14.3)[a]
PgR status			
Anastrozole ($n = 48$)			
Positive ($n = 17$)	1 (2.1)	16 (33.3)	0 (0.0)
Negative ($n = 26$)	0 (0.0)	26 (54.2)	0 (0.0)
Not evaluated ($n = 5$)	0 (0.0)	0 (0.0)	5 (10.4)
Tamoxifen ($n = 49$)			
Positive ($n = 11$)	10 (20.4)	1 (2.0)	0 (0.0)
Negative ($n = 31$)	6 (12.2)	25 (51.0)	0 (0.0)
Not evaluated ($n = 7$)[a]	0 (0.0)	0 (0.0)	7 (14.3)[a]

[a] Six patients discontinued the neoadjuvant phase and one patient did not receive any study treatment

These results support the clinical findings from the main PROACT trial, in which preoperative anastrozole produced numerically superior objective response rates compared with preoperative tamoxifen, and highlight the value of preoperative endocrine therapy with aromatase inhibitors for postmenopausal women with large, operable or potentially operable breast tumors (Cataliotti et al. 2006). Significantly higher objective response rates compared with tamoxifen have been obtained with preoperative letrozole (20 and 30% for tamoxifen and letrozole, respectively; $P = 0.0006$), and ongoing preoperative endocrine studies should elucidate receptor cross-talk and endocrine resistance pathways further (Dixon et al. 2002; Smith (2003); Huober et al. (2004).

Preliminary evidence suggests that hormone receptor-positive patients experience weaker responses to neoadjuvant chemotherapy than hormone receptor-negative patients (pCR rate of 5.0 and 20.6%, respectively) (Buzdar et al. 2003). Recently published data indicate that neoadjuvant chemotherapy that includes trastuzumab [a monoclonal antibody for the human epidermal growth-factor receptor (HER) 2 protein], for the treatment of patients with HER2-positive disease, elicits significantly higher rates of pCR than chemotherapy alone (25 and 66.7% for chemotherapy and chemotherapy plus trastuzumab, respectively; $P = 0.02$) (Buzdar et al. 2005). Although based on a small number of patients ($n = 42$), these data illustrate that better responses may be produced by tailoring neoadjuvant therapy to individual tumor type.

Histopathological assessment of changes in ER/PgR status suggests that anastrozole therapy may induce a negative PgR change, whereas, tamoxifen therapy may induce a negative ER change. Although the mechanism of these changes has not been clarified; this result is consistent with other studies with aromatase inhibitors (Miller et al. 2001, (2003). In the first of these studies, for PgR was reduced in 16/17 PgR-positive cancers treated with anastrozole and in all 21 positive cancers treated with the aromatase inhibitor, letrozole (Miller et al. 2001). Likewise, in the second of these studies, PgR reactivity was reduced in 20/21 evaluable cases treated with letrozole, becoming undetectable in 16 patients. Only marginal changes were observed in ER expression following letrozole therapy, but following treatment with tamoxifen, ER expression was markedly reduced in most cases (Miller et al. 2003).

In conclusion, these data support the efficacy and tolerability of neoadjuvant endocrine therapy in patients with hormone receptor-positive disease shown in the PROACT trial. Neoadjuvant anastrozole produced a numerically greater response rate compared with neoadjuvant tamoxifen. The histological response to therapy showed some correlation with the observed clinical response, although further follow-up is required to confirm a subsequent effect on survival.

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Conflict of interest The authors state that they have no conflicts of interest to declare.

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What Causes Discrepancies in HER2 Testing for Breast Cancer?

A Japanese Ring Study in Conjunction With the Global Standard

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Key Words: Breast cancer; HER2 testing; Assessment; Ring study; Multicenter study; Immunohistochemistry; Fluorescence in situ hybridization

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Upon completion of this activity you will be able to:
- apply the ASCO/CAP guidelines for HER2 testing of breast carcinomas.
- define the immunohistochemical reactivity rate for HER2 in breast carcinoma that should trigger additional testing by FISH.
- analyze causes of discrepancy in HER2 testing of breast carcinomas by IHC.
- outline a protocol for single institutional guidelines for combined use of IHC and FISH for HER2 testing in breast carcinomas, incorporating quality assurance considerations.

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Abstract

We assessed interinstitutional and interobserver consistency of human epidermal growth factor receptor type-2 (HER2) testing using immunohistochemical analysis and fluorescence in situ hybridization (FISH) in a set of 20 breast cancer samples among 10 institutions in Japan and a Herceptin adjuvant study participating laboratory in Germany and identified factors that may lead to discordant results.

We found a good agreement in immunohistochemical HER2 scoring between the coordinating institution and 10 participating laboratories ($\kappa = 0.718$) and excellent agreement for FISH ($\kappa = 0.900$). The results of a comparison between 10 Japanese laboratories and the German laboratory was good for immunohistochemical studies ($\kappa = 0.713$) and excellent for FISH ($\kappa = 0.887$). FISH retesting of equivocal samples (2+ immunohistochemically) improved agreement. Discrepancies between results were attributed to the evaluation process in 33.0% of the samples, staining procedures in 25.0%, and a combination of the two in 41.7%. Evaluation of samples according to the American Society of Clinical Oncology/College of American Pathologists guideline increased the number of 2+ immunohistochemical scores. By performing FISH retesting for these samples, consistency among multiple institutions could be archived. The quality of the staining procedures performed and the consistency of evaluations require regular assessment.

The human epidermal growth factor receptor type-2 (*HER2*) gene encodes a protein (185 kDa) that is a cell surface receptor with tyrosine kinase activity.[1] Amplification of the *HER2* gene and/or overexpression of its protein product has been shown in 25% to 30% of breast cancers.[2,3] Moreover, HER2 status is an important factor in predicting prognosis[2,4] and selection of systemic therapies for treatment.[5-9] Overall, *HER2* gene amplification is associated with a poor clinical outcome,[2,4] and, accordingly, HER2 status has been added to the risk category of the St Gallen consensus recommendation.[10] Overexpression of HER2 protein is also associated with resistance to endocrine therapy that may be specific to selective estrogen receptor modulator therapies, such as tamoxifen, but not to aromatase inhibitors.[5,6,11] In contrast, HER2 overexpression correlates with a response to treatment with anthracyclines and taxanes.[7-9]

Accumulating evidence indicates that trastuzumab (Herceptin) is effective not only for the treatment of metastatic breast cancer but also for early breast cancer with HER2 overexpression. International clinical trials[12-16] have revealed that trastuzumab treatment for primary breast cancer in the adjuvant setting reduced the risk of recurrence and mortality. Based on the results of these trials, trastuzumab has been included in the National Comprehensive Cancer Network guidelines and the St Gallen consensus recommendations. In 2006, the European Medicine Agency and the US Food and Drug Administration approved trastuzumab for primary breast cancer in the adjuvant setting. With these approvals, an increased number of patients may be able to receive treatment with trastuzumab based on HER2 testing results.

Misdiagnosis of HER2 overexpression can result in the loss of opportunity for patients to receive the benefits of trastuzumab treatment or in patients being overtreated. Therefore, accuracy in HER2 testing is of significant clinical benefit.

It has been reported that the efficacy of trastuzumab depends on the extent of HER2 overexpression: A tumor with a 2+ immunohistochemical score has a response rate of 0%, whereas a tumor with a 3+ immunohistochemical score has a response rate of 35%.[17] Although the significance of accurate HER2 testing has been emphasized, HER2 testing is not subject to external quality assurance in all countries, despite the fact that evaluation of HER2 serves as a major conclusive factor in the decision to treat with trastuzumab.

HER2 gene amplification was first examined by using Southern blotting in the early phase of a clinical study by Slamon et al.[2] An alternative method for HER2 detection is fluorescence in situ hybridization (FISH). In the early phase of a validation study, HER2 protein overexpression was examined immunohistochemically using anti-HER2 monoclonal antibodies 4D5 and CB11 (denoted the Clinical Trial Assay).[16] HER2 detection using a polyclonal antibody can be more sensitive but is less specific than using a monoclonal antibody. Although immunohistochemical analysis is now relatively inexpensive and universally available in research laboratories, it does not produce results as reliably consistent as those observed with FISH. In 3 clinical studies, the population of patients with tumors categorized as 2+ immunohistochemically varied from 12.7% to 39.5%, and the rate of *HER2* gene amplification in tumors scored 2+ immunohistochemically varied from 17.9% to 48.1%.[16,18,19] Although these data may represent a significant diversity in breast cancer tissue samples with regard to HER2, it is important to consider the sensitivity and specificity of immunohistochemical and FISH analyses.

No assessment system for the standardization of immunohistochemical or FISH analysis of HER2 has been established in Japan thus far. However, in other countries, a standardization process is in place, ie, the Nordic Immunohistochemical Quality Control (http://www.nordiqc.org/news.htm), United Kingdom National External Quality Assessment Service (http://www.ukneqas.org.uk/), the College of American Pathologists (CAP; http://www.cap.org/apps/cap.portal), and the Royal College of Pathologists of Australia Quality Assurance Program (http://www.rcpaqapa.netcore.com.au/index.html).

To investigate the consistency of HER2 testing in Europe, Dowsett et al[20] conducted an international ring study with 5 pathologists, each from a different country, applying immunohistochemical analysis and FISH to 20 slide sets. We conducted a Japanese ring study with 10 participating laboratories, responsible for diagnosing approximately 80% of breast cancer samples in Japan, and 1 laboratory in Germany that participated in the Herceptin adjuvant (HERA) trial.

Materials and Methods

Participants

To compare and assess immunohistochemical and FISH analyses of HER2 expression by different laboratories, 7 institutions in Japan (Tokai University School of Medicine, Isehara; The Cancer Institute Japanese Foundation for Cancer Research, Koto; Niigata Cancer Center Hospital, Niigata; the Saitama Cancer Center, Kita-adachi; Tohoku University School of Medicine, Sendai; Kitakyushu Municipal Medical Center, Kitakyushu; and the National Defense Medical College, Tokorozawa), 3 commercial laboratories (SRL, Tachikawa; BML, Kawagoe; and Mitsubishi Chemical Medience, Itabashi), and 1 laboratory that was a site for a HERA trial (Institut für Pathologie, Klinikum Kassel, Targos Molecular Pathology, Kassel, Germany) participated in this ring study **Figure 1**. The study was coordinated by the Tokai University School of Medicine and approved by the institutional review board of Tokai University.

Sample Selection and Distribution

We selected 20 cases of invasive breast cancer from the surgical pathology files of Tokai University Hospital. The breast cancer tissue samples had previously been tested and were selected to represent a relatively higher proportion of equivocal cases for the purpose of assessment. All of the specimens had been fixed with formalin (12-48 hours) and embedded in paraffin blocks. Tissue sections,

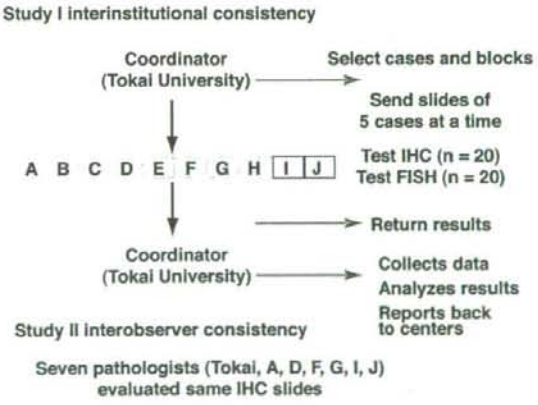

Figure 1 Study designs for studies I and II. Study I examined interinstitutional consistency, and study II examined interobserver consistency of HER2 testing. Institutions A-H participated in immunohistochemical analysis (IHC) and fluorescence in situ hybridization (FISH) analysis, and institutions I and J evaluated IHC results.

4 to 6 μm thick, were mounted on silane-coated slides. A set of 5 cases, with 2 unstained slides for each case, was sent every 2 weeks to participants for immunohistochemical and FISH analyses. The slides for FISH analysis were sent after receiving the results of the immunohistochemical analysis to avoid bias from the FISH results. In this way, an identical series of 20 cases was evaluated independently for immunohistochemical and FISH detection of HER2 expression. Of the 10 participating, 8 institutions performed HER2 testing by immunohistochemical analysis (HercepTest, DakoCytomation, Carpinteria, CA) and FISH (PathVysion, Vysis, Downers Grove, IL) analyses; 2 participants performed immunohistochemical analysis only.

Study Design and Data Analysis

This study was designed to examine interinstitutional consistency (study I) and interobserver consistency (study II) in the analysis of tissue samples. Sample selection and distribution of sections for study I was described in the preceding section, with the evaluated results analyzed by the study coordinator. For study II, the goal was to examine interobserver consistency. Seven pathologists each evaluated one set of 20 cases that were stained at the Tokai University School of Medicine, the same set of 20 cases evaluated in study I. To evaluate the significance of the assessment system proposed by the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines for interobserver consistency in immunohistochemical analysis, evaluation systems described in the manufacturer's protocol (study IIA) and those of the ASCO/CAP guideline were compared (study IIB).

The results were estimated by concordance rate and κ value. A κ value of more than 0.75 represented excellent agreement, values from 0.4 to 0.75 represented fair to good agreement, and values less than 0.4 represented poor agreement beyond chance. Concordance rates between the coordinating laboratory and participating Japanese laboratories and between the HERA laboratory and the Japanese laboratories were evaluated.

Immunohistochemical Analysis and FISH

Immunohistochemical detection kits for HER2 containing the same lot of polyclonal antibody (HercepTest) were distributed to all participating laboratories. Sections were stained according to the manufacturer's protocols. Staining results were evaluated using the criteria 0, 1+, 2+, and 3+ according to the HercepTest kit instructions, which were the standardized criteria at the time, for studies I and IIA **Image 1**. In study IIB, the ASCO/CAP guidelines were used as the staining criteria.[21]

For FISH detection of HER2, HER2/CEP17 probe kits (Vysis) were distributed and used according to the manufacturer's protocol. Signal numbers for the *HER2* gene (labeled with SpectrumOrange, Vysis) and the *CEP17* gene (labeled with SpectrumGreen, Vysis) were counted in more than 20 tumor cells from each site, and the ratio of the HER2/CEP17 signal numbers was calculated. The results were interpreted as positive when the signal ratio of HER2/CEP17 was equal or greater than 2.0 and negative when it was less than 2.0 according to the manufacturer's protocol.

Results

Interinstitutional Consistency

The results of study I are shown in **Table 1** and **Table 2**. Of 20 samples analyzed immunohistochemically, 14 (70%) tumors were grouped in the same category when the results were categorized into 2 groups as 0, 1+/2+ and 3+; and 8 (40%) of 20 scores were consistent when the results were categorized as negative (0, 1+), equivocal (2+), and positive (3+) (Table 1). The recorded immunohistochemical results were in good agreement between the participating Japanese laboratories and the coordinating laboratory (κ = 0.718), and agreement was also good between participants and the HERA laboratory (institution E in Table 1) (κ = 0.713). For FISH analyses, results for 17 (85%) of 20 samples were consistent for all participants (Table 2). Discrepancies in results were mainly observed for samples with a HER2/CEP17 signal ratio close to 2.0. The FISH results were in excellent agreement between participants and the coordinator (κ = 0.900) and between participants and the HERA laboratory (institution E in Table 2) (κ = 0.887).

FISH Retesting for Cases Immunohistochemically Scored as 2+ Increased Agreement

To verify the algorithm for "Indication of Trastuzumab and HER2 Testing,"[22] simulation analyses were performed. Initially, analysis of study I was conducted to determine whether retesting of FISH for cases scored immunohistochemically as 2+ would improve agreement. There were 11 cases that were determined to be 2+ by at least 1 institution. When these cases were retested by FISH, agreement in scoring improved in 8 of the 11 cases. In the remaining 3 cases, scores were lowered or unchanged **Table 3**. A second analysis was used to validate whether the 2 trees of algorithms are acceptable from the perspective of clinical benefit. According to the distribution of results organized into the FISH tree **Figure 2**, 46.7% of cases had indications for trastuzumab treatment. Based on the distribution of results into the immunohistochemical analysis tree (Figure 2), 45.6% had indications for trastuzumab treatment. Retesting samples by FISH closed the gap further, demonstrating that

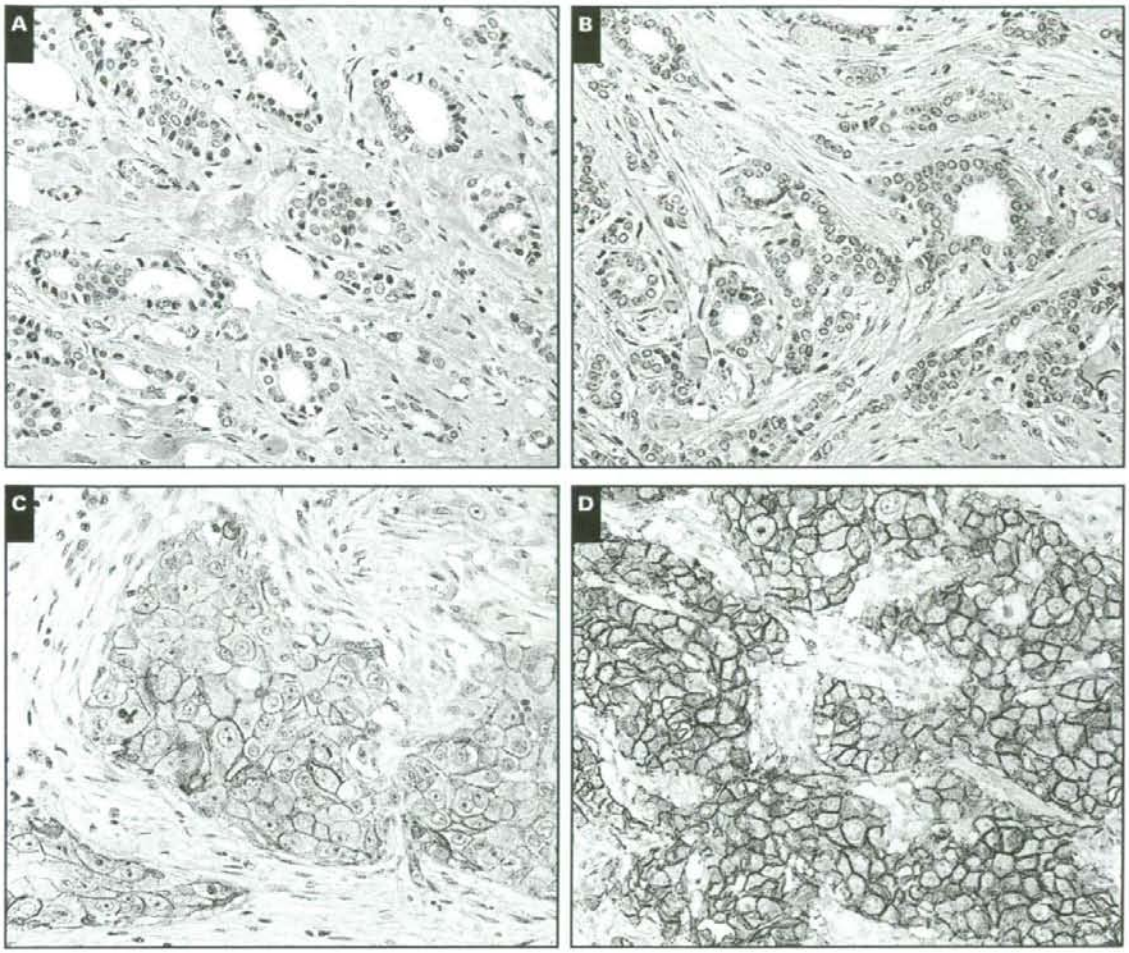

Image 1 Scoring criteria for immunohistochemical examination. **A**, Carcinoma cells lack positive reactivity for HER2 protein (score 0; ×100). **B**, A weakly positive reaction is found, but they are not completely bound to the cell membrane (score 1+; ×100). **C**, Weak to moderate, complete membrane staining is detected in >10% of tumor cells (score 2+; ×100). **D**, Strong, complete membrane staining is observed in >10% of tumor cells (score 3+ in studies I and IIA) and in >30% of tumor cells (score 3+ in study IIB) (×100).

retesting by using FISH can minimize the prescription of treatment that will have no effect or too much of an effect, depending on the type of cancer present.

Interobserver Discrepancies and Their Causes

To analyze the cause of discrepancies that occur in evaluating the pathology of tissue samples, interobserver consistency was examined in study II. By using the evaluations provided by each of the 7 pathologists on the Japanese Pathology Board for Optimal Use of Trastuzumab for 1 set of the 20 cases, in which the variable factor of staining procedures had been excluded, we examined interobserver discrepancy. Nine of the evaluations were inconsistent, with discrepancies between immunohistochemical scores of 2+ and 3+ in 2 samples (cases 4 and 6) **Image 2** and between immunohistochemical scores of 2+ and 1+ in 7 samples (cases 3, 5, 7, 9, 10, 18, and 15; study IIA) **Table 4**. The analysis of discrepancies from study I suggested that a complexity of factors, including interobserver diversity and staining procedures (Table 4, study I), accounted for the differences. However, of the 12 samples in which the results were not consistent in study I, 3 (25%) showed complete agreement in study IIA, and interinstitutional concordance was lower in study I. We hypothesize that

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Table 1
Analysis of Interinstitutional Concordance by Immunohistochemical Scoring Results*

Case No.	Coordinator	Institution										Concordance (%)
		A	B	C	D	E	F	G	H	I	J	
11	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	100
12	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	100
13	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	100
19	3+	3+	3+	3+	3+	3+	2+	3+	3+	3+	3+	91
20	3+	3+	3+	3+	3+	3+	2+	3+	3+	3+	3+	91
4	3+	2+	3+	2+	3+	2+	2+	3+	2+	2+	2+	64
8	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	2+	91
6	2+	3+	3+	3+	2+	3+	2+	2+	2+	2+	2+	64
5	2+	2+	2+	3+	3+	2+	2+	2+	2+	2+	2+	82
7	2+	2+	1+	2+	2+	2+	2+	1+	2+	2+	1+	73
9	2+	2+	2+	2+	2+	2+	2+	1+	2+	2+	1+	82
10	2+	1+	1+	2+	0	2+	1+	1+	2+	1+	1+	64
18	1+	2+	1+	2+	2+	2+	1+	1+	1+	2+	1+	55
15	1+	1+	1+	2+	1+	1+	1+	1+	2+	2+	1+	73
3	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	0	100
16	1+	1+	1+	2+	0	1+	0	1+	1+	1+	0	91
2	1+	1+	1+	0	1+	1+	0	1+	1+	0	0	100
14	1+	1+	1+	1+	0	1+	0	0	0	0	0	100
1	0	0	1+	0	0	0	1+	1+	0	0	0	100
17	0	0	0	0	0	1+	0	0	0	0	0	100

* 0 and 1+ were considered negative; 2+, equivocal; and 3+, positive.

Table 2
Analysis of Interinstitutional Concordance by Fluorescence In Situ Hybridization Results*

Case No.	Coordinator	Institution								Concordance (%)
		A	B	C	D	E	F	G	H	
11	7.1	7.4	3.8	18.7	8.3	4.9	10.0	6.2	7.3	100
12	4.6	9.4	4.5	13.9	4.4	4.6	7.8	4.2	6.8	100
13	7.3	9.8	9.6	14.1	8.4	7.4	10.3	4.2	5.2	100
19	6.7	6.3	8.1	11.5	3.9	5.5	8.0	13.3	6.2	100
20	6.6	7.7	5.9	7.4	5.4	5.6	6.7	4.5	4.5	100
4	6.1	8.9	4.7	10.7	5.6	5.2	7.9	3.9	5.3	100
8	3.2	4.9	2.7	12.5	2.4	5.0	4.4	5.4	2.7	100
6	6.5	4.4	4.4	20.9	5.4	3.9	4.6	3.9	2.7	100
5	2.5	4.1	3.5	24.7	2.5	2.6	1.6	1.6	1.9	67
7	2.5	2.2	2.1	1.8	1.9	1.3	2.6	3.0	1.8	56
9	1.3	1.2	1.3	1.5	1.5	1.0	1.5	1.2	1.5	100
10	1.6	1.2	1.4	2.0	1.2	1.0	1.0	1.4	1.3	89
18	1.2	1.2	1.2	1.0	1.2	1.2	1.7	1.0	1.1	100
15	1.1	1.3	1.3	1.0	1.0	0.9	1.4	1.4	1.0	100
3	1.3	0.9	1.2	1.0	1.3	1.0	1.4	1.5	1.2	100
16	1.4	1.2	1.0	1.1	1.2	1.1	1.2	1.5	1.1	100
2	1.8	1.3	1.0	1.0	1.5	1.1	1.1	1.0	1.2	100
14	1.9	1.4	1.1	1.1	1.0	1.2	1.3	1.1	1.1	100
1	1.2	1.4	1.2	1.0	1.4	1.1	1.9	1.2	0.9	100
17	1.1	1.2	1.1	1.0	1.0	1.1	1.1	1.1	0.9	100

* Data are given as the HER2/CEP17 ratio. A ratio <2.0 was considered negative, and a ratio ≥2.0, positive.

staining procedures were the cause. In 4 of 12 cases, the interobserver discrepancy present in study IIA was identical to the concordance found in study I. For these cases, interobserver discrepancy was considered to be the cause. In the other 5 of 12 cases, both interobserver and interinstitutional discrepancies suggest that staining procedures and interobserver discrepancies are possible causes.

Significance of ASCO/CAP Guideline

To study the significance of the ASCO/CAP interpretive criteria, we conducted study IIB. The variable factor is the only evaluation system in study II; therefore, we can analyze the significance of the proposed criteria in comparison with previous criteria and the ASCO/CAP guidelines while excluding the influences of staining procedures. Revised

Table 3
Analysis of FISH Retesting for Samples With Immunohistochemical Scores of 2+*

			Institution											
	Coordinator		A		B		C		D		E		F	
Case No.	IHC	FISH	IHC	FISH	IHC	FISH	IHC	FISH	IHC	FISH	IHC	FISH	IHC	FISH
11	3+	7.1	3+	7.4	3+	3.8	3+	18.7	3+	8.3	3+	4.9	3+	10.0
12	3+	4.6	3+	9.4	3+	4.5	3+	13.9	3+	4.4	3+	4.6	3+	7.8
13	3+	7.3	3+	9.8	3+	9.6	3+	14.1	3+	8.4	3+	7.4	3+	10.3
19	3+	6.7	3+	6.3	3+	8.1	3+	11.5	3+	3.9	3+	5.5	2+	8.0
20	3+	6.6	3+	7.7	3+	5.9	3+	7.4	3+	5.4	3+	5.6	2+	6.7
4	3+	6.1	2+	8.9	3+	4.7	2+	10.7	3+	5.6	2+	5.2	2+	7.9
8	3+	3.2	3+	4.9	3+	2.7	3+	12.5	3+	2.4	3+	5.0	3+	4.4
6	2+	6.5	3+	4.4	3+	4.4	3+	20.9	2+	5.4	3+	3.9	2+	4.6
5	2+	2.5	2+	4.1	2+	3.5	3+	24.7	3+	2.5	2+	2.6	2+	1.6
7	2+	2.5	2+	2.2	1+	2.1	2+	1.8	2+	1.9	2+	1.3	2+	2.6
9	2+	1.3	2+	1.2	2+	1.3	2+	1.5	2+	1.5	2+	1.0	2+	1.5
10	2+	1.6	1+	1.2	1+	1.4	2+	2.0	0	1.2	2+	1.0	1+	1.0
18	1+	1.2	2+	1.2	1+	1.2	2+	1.0	2+	1.2	2+	1.2	1+	1.7
15	1+	1.1	1+	1.3	1+	1.3	2+	1.0	1+	1.0	1+	0.9	1+	1.4
3	1+	1.3	1+	0.9	1+	1.2	1+	1.0	1+	1.3	1+	1.0	1+	1.4
16	1+	1.4	1+	1.2	1+	1.0	2+	1.1	0	1.2	1+	1.1	0	1.2
2	1+	1.8	1+	1.3	1+	1.0	0	1.0	1+	1.5	1+	1.1	0	1.1
14	1+	1.9	1+	1.4	1+	1.1	1+	1.1	0	1.0	1+	1.2	0	1.3
1	0	1.2	0	1.4	1+	1.2	0	1.0	0	1.4	0	1.1	1+	1.9
17	0	1.1	0	1.2	0	1.1	0	1.0	0	1.0	1+	1.1	0	1.1

FISH, fluorescence in situ hybridization; IHC, immunohistochemical analysis.
* Data are given for IHC as immunohistochemical scores (0 and 1+, negative; 2+, equivocal; 3+, positive) and for FISH as the HER2/CEP17 ratio (<2.0, negative; ≥2.0, positive).

assessments by at least 1 pathologist were detected in 3 cases **Table 5**. As a result, concordance between sample designations increased, decreased, and remained unchanged for the 3 cases, respectively. It is noteworthy that most of the changes were from an immunohistochemical score of 3+ to 2+.

The FISH results were reviewed according to the ASCO/CAP criteria, and interpretations of equivocal (1.8-2.2) were frequently identified in discrepant cases according to manufacturer's criteria (cases 5 and 7).

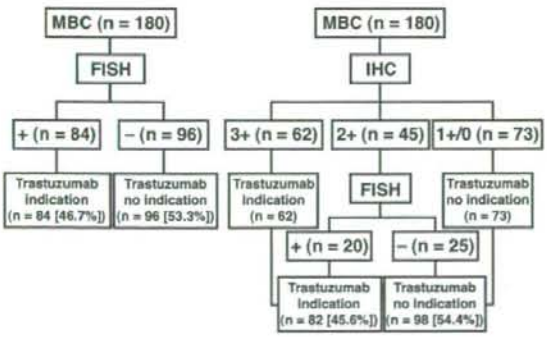

Figure 2 Simulation analysis for retesting fluorescence in situ hybridization (FISH) samples for immunohistochemical analysis (IHC) scores of 2+ according to the HER2 testing algorithm. MBC, metastatic breast cancer.

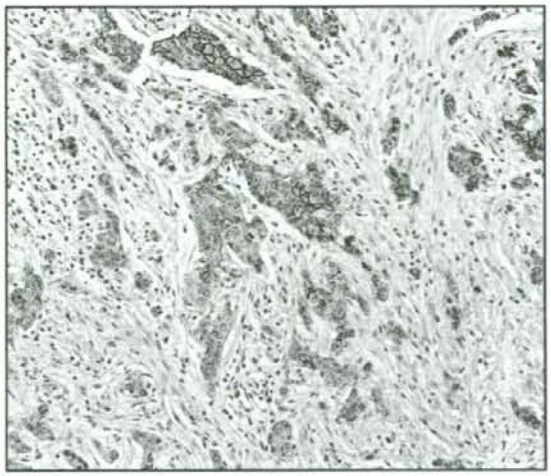

Image 2 (Case 4) Immunohistochemical features. Tumor cells with varied intensity for HER2 protein are heterogeneously distributed (×100).

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