patients showed increases in both markers, whereas others only showed increases in total CK18. This result suggests that CEF therapy induces different death modes in different tumors.

Increases in CK18 are associated with clinical response. We examined the association between clinical response and serum CK18 increases in 43 patients receiving neoadjuvant CEF therapy. Patients normally leave the hospital after drug infusion—leading to difficulties to collect blood samples—but a limited number of paired samples is sufficient to achieve high statistical power using matched-pair statistics. Patients with partial clinical response showed significant increases in total CK18 at day 1 after treatment ($P < 0.0001$, Wilcoxon matched pair test; Fig. 5A). In contrast, nonresponding patients did not show significant changes in total CK18 levels ($P = 0.19$; Fig. 5A). Similar results were obtained using matched pair t test.

Patients who received neoadjuvant therapy were stratified according to the ratios of posttreatment to pretreatment values of CK18 and overall survival plots were constructed (Fig. 5B). A cutoff value of 18% increase in CK18 gave the best prognostic significance for survival ($P = 0.035$ by the log-rank test). The Cox proportional hazards model gave a hazard ratio of 7.28 (95% confidence interval, 0.84-62.9).

Discussion

Previous studies have shown that different anticancer agents induce increases in the levels of caspase-cleaved and total serum CK18 in prostate cancer patients and that serum CK18 is derived from tumor cells (11, 13). These results were promising with regard to the use of serum CK18 as a pharmacodynamic biomarker for tumor cell death. Previous studies have not established whether increases in serum CK18 occurring during treatment are associated with clinical responses because response monitoring is inaccurate in patients with hormone refractory prostate cancer (26). We here studied breast cancer patients receiving neoadjuvant treatment for local disease and from which accurate clinical data were available. The results show that increases in serum CK18 levels are associated with clinical response to CEF therapy. Interestingly, CK18 increases were not exclusively observed in patients showing clinical response but also in some patients showing stable disease during treatment (Fig. 4A), suggesting that serum CK18 is a sensitive response biomarker. In patients with stable disease, therapy-induced cell death may be balanced by tumor cell regrowth between treatment cycles (27). Both the sensitivity of the assays and the favorable performance characteristics in terms of antigen stability during storage (14) and during freeze-thawing (Table 3) suggest that CK18 biomarkers will be useful for monitoring treatment effects.

Table 4. Increased levels of CK18 in patient serum during treatment using different agents

Treatment	Increased levels of CK18 during therapy (%)	
	Caspase cleaved*	Total*
Docetaxel (breast)†	19.8 ($P = 0.0089$)	16.5 (NS)
Docetaxel (prostate)‡	18.7 ($P < 0.0001$)	21.4 ($P < 0.0002$)
Vinorelbine (prostate)‡	7.2 ($P < 0.001$)	6.7 ($P < 0.011$)
Estramustine (prostate)‡	-1 (NS)	8.2 ($P < 0.0001$)
CEF (breast)§	12.9 ($P < 0.00001$)	32.7 ($P < 0.00001$)

*Increased median levels of CK18-Asp^{396} and total CK18 (measured by the M30-Apoptosense and M65 ELISA assays).
†Increase over pretherapy levels at 72 h.
‡Increase over pretherapy levels 48 h (prostate data are from ref. 13).
§Increase over pretherapy levels at 24 h.

Apoptosis has received considerable attention as a major cellular outcome of chemotherapy, including DNA-damaging agents (2). Recent studies have implied that necrosis may also be a possible consequence of treatment (28). Doxorubicin has been shown to induce both apoptosis (29) and necrosis *in vitro* (30, 31). Our studies of tumor organ cultures from seven clinical cases of breast carcinoma showed induction of caspase-cleaved CK18 by doxorubicin in all seven cultures, showing apoptosis. Apoptotic responses were also observed using CEF therapy (cyclophosphamide is converted by the liver into active metabolites; acrolein was used for these studies).[6] In contrast, the *in vivo* CK18 response to CEF therapy was heterogeneous, characterized by increases in caspase-cleaved CK18 in the serum of some, but not all, patients with increases in CK18. This heterogeneous response could be due to defects in apoptosis signaling in some tumors. Furthermore, differences in factors such as tumor hypoxia, nutrition, or variations in the drug concentrations reached in different tumors may also be determinants of cell death mode. It has been reported that DNA-alkylating agents induce a rapid necrotic response due to activation of poly(ADP)ribose polymerase, leading to poly(ADP)ribose polymerase-mediated depletion of β-NAD$^+$ (7). Tumor cells, which are dependent on glycolysis for ATP production, undergo rapid ATP depletion and necrotic death. This response by DNA-damaging agents could be speculated to be more pronounced in hypoxic and poorly nourished tumors. That anthracycline-based therapy may induce a necrotic response is supported by the finding that complete pathologic responses to doxorubicin/docetaxel are associated with the presence of tumor necrosis in tissue sections (32). Induction of a necrotic response could explain the efficiency of chemotherapy in tumors with defective apoptotic pathways (discussed in ref. 7), including efficacy in p53-defective breast cancers (33).

Taxanes induce mitotic catastrophe, characterized by the occurrence of aberrant mitosis followed by cell division. Mitotic catastrophe is not a cell death mode, but will trigger cell death, either by apoptosis or by nonapoptotic mechanisms (1, 34-36). The findings in the present and a previous study (13), demonstrating increases in caspase-cleaved CK18 molecules in serum during docetaxel treatment, shows that this agent induces apoptosis *in vivo* (Table 4). It is likely that the efficiency of microtubule-interacting agents does not rely on the presence of an apoptotic machinery in the target cells;

[6] Our unpublished data.

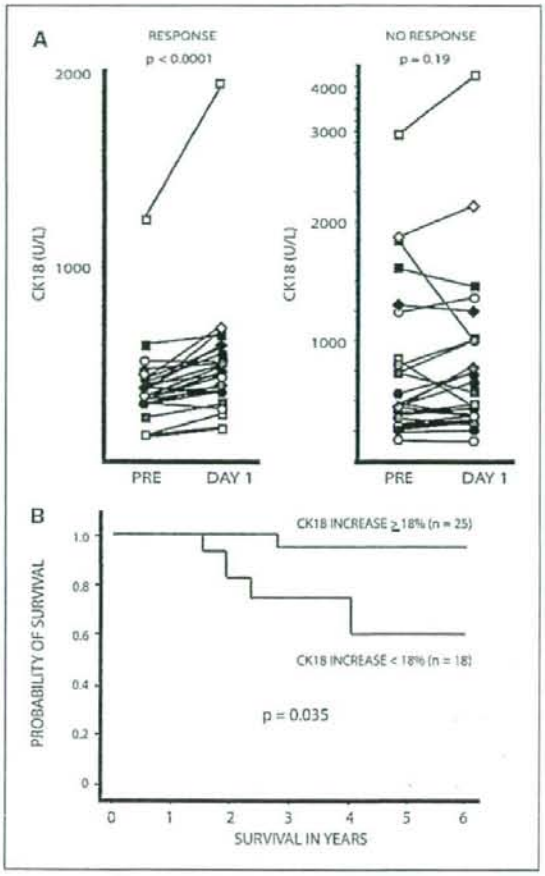

Fig. 5. Association to response. *A*, increases of total CK18 at day 1 of the first CEF treatment cycle are associated with therapy response. Pretherapy and day 1 CK18 levels are shown of patients showing response or no response; *P* values are from Wilcoxon matched pair test. *B*, improved overall survival curves of patients showing >18% increases in CK18 at the first cycle of CEF treatment. Kaplan-Meier plots, *P* value calculated by the log-rank test.

if the machinery is not present, cells are likely to die from other mechanisms. It should be noted that we have observed patients where docetaxel induces increases predominantly in total serum CK18 (11). However, there is a quantitative difference in the response to mitotic inhibitors and CEF, whereas mitotic inhibitors induce similar median increases in caspase-cleaved CK18 and total CK18, CEF induces larger increases in total CK18 reflecting a relatively larger proportion of tumors where necrotic cell death occurs.

Our data show that only a minor fraction of caspase-cleaved CK18 will remain in the insoluble, cytoskeletal fraction. Soluble caspase-cleaved CK18 consisted of molecular weight fragments in the 10 to 20 kDa range, which we presume to be monomeric caspase digestion products, and also of higher molecular weight material (50-100 kDa). Only the higher molecular weight material was present in serum from cancer patients, and we suggest that the smaller fragments are being filtered in the kidney glomerulus. The caspase-cleaved CK18 material present in serum reacted with CK7, CK8, or CK19 antibodies, showing that they at least partly are present in complexes. Although the precise nature of these interactions are unknown or if there are any alterations among the cytokeratin complexes under normal or abnormal physiologic conditions, our data showed that the serum CK18 levels from repeated blood draws of healthy donors were fairly stable without any trending (data not shown). The variations from multiple time points in normal donors are consistent with the data reported from cancer patients (15). The presence of caspase-cleaved CK18 in protein complexes is likely to explain the stability of the cleavage products in the circulation and in blood samples, explaining the excellent performance of serum CK18 with regard to stability and yields after repeated freeze-thaw cycles (refs. 14, 15; Table 3). It is likely that many other caspase-cleaved fragments released from apoptotic cells will not show a similar stability. The adequate assay performance adds to the advantages of serum CK18 as a biomarker for rapid monitoring of clinical response to cancer therapy.

We conclude that serum CK18 measurements may be useful for assessing treatment effects. The data suggesting that the initial cell death response determined by CK18 biomarkers is an important determinant of treatment outcome. The method is robust and samples can be frozen and stored before analysis, making the method suitable for multicenter clinical trials of novel anticancer drugs.

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CLINICAL TRIAL

Phase II study of preoperative sequential FEC and docetaxel predicts of pathological response and disease free survival

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Abstract *Purpose* This multicenter phase II study examined the impact of pathological effect on survival after preoperative chemotherapy in Japanese women with early stage breast cancer. *Patients and methods* Prior to surgery, patients received four cycles of FEC (fluorouracil 500 mg/m^2, epirubicin 100 mg/m^2, cyclophosphamide 500 mg/m^2 q3w) followed by four cycles of docetaxel (75 mg/m^2 q3w). Primary endpoint was 3 year disease free survival (DFS) stratified by the absence or presence of Quasi-pCR (QpCR; absence of invasive tumor or only focal residual tumor cells). Secondary endpoints were predictors for QpCR, clinical response, breast conservation rate, and safety. *Results* Between June 2002 and June 2004, 202 women were enrolled. Among 191 assessable patients, 25% achieved QpCR. With 40 months median follow-up, 3 year DFS was estimated at 91% for all patients. 3 year DFS for patients with QpCR was 98% vs. 89% without QpCR (hazard ratio 0.38 [95% Confidence Interval 0.09–0.84], $P = 0.0134$). HER2 status and response to FEC were independent predictors of QpCR. The overall clinical

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response was 75%; 85% of patients achieved breast conservation. Grade 3/4 neutropenia was the most common adverse event, observed in 44% and 35% of patients during FEC and docetaxel, respectively. Treatment related side effects were manageable; there were no treatment related fatalities. *Conclusion* FEC followed by docetaxel is an active and manageable preoperative regimen for women with early stage breast cancer. QpCR following preoperative chemotherapy predicts favorable DFS. HER2 overexpression and clinical response to FEC predict QpCR.

Keywords Clinical trial · Docetaxel · Early stage breast cancer · FEC · Preoperative chemotherapy · Phase II

Introduction

Preoperative systemic chemotherapy has been widely used for patients with operable breast cancer to increase the chance for breast conservation [1–3]. Furthermore, response to preoperative treatment can provide information on long-term survival outcomes. Pathological complete response (pCR) in the breast and axillary lymph nodes predicts a favorable prognosis, whereas non-pCR of the breast or node-positive status does not, which can facilitate tailoring of subsequent treatment [1, 3]. In addition, correlative studies of tumor samples before and after treatment may provide information on markers that could predict response or resistance to treatment [4].

Results from the National Surgical Adjuvant Breast and Bowel Project (NSABP) study B-18 demonstrated the impact of preoperative chemotherapy in patients with operable early stage breast cancer [5]. The protocol-specified anthracycline-containing regimen of four cycles of doxorubicin and cyclophosphamide (AC), resulted in an increased chance of breast-conserving surgery (BCS) compared to no preoperative chemotherapy. The study established pCR as a prognostic marker for long-term disease-free survival and demonstrated that there was no difference in survival whether chemotherapy was administered before or after surgery. Subsequently, studies such as the Aberdeen trial have demonstrated the benefit of the sequential addition of taxanes to preoperative anthracycline regimens [6, 7]. NSABP Protocol B-27 demonstrated that compared to preoperative AC alone, the addition of sequential docetaxel doubled the pCR rate, increased the clinical complete response (cCR) rate, and increased the proportion of patients with negative axillary nodes [3, 7]. Although NSABP B-27 did not show that the addition of docetaxel to AC significantly improved disease free survival (DFS) and overall survival (OS) compared to AC alone, other studies, mainly of patients with node-positive disease, have shown favorable DFS and OS by including a taxane with an anthracycline, either in sequence or combination [8–12]. Multiple neoadjuvant studies demonstrated that patients with pathological complete response to chemotherapy had a good prognosis [1, 2].

Here we conducted a multicenter prospective neoadjuvant trial with four cycles of fluorouracil, epirubicin, and cyclophosphamide (FEC) followed by four cycles of docetaxel in Japanese patients with operable breast cancer to investigate the relationship between pathological effect and survival. The pathological effect was determined using the definitions of Quasi-pCR (QpCR: complete disappearance of invasive carcinoma in the breast or only focal tumor cells remaining in the stroma in the removed breast) [13]. The primary endpoint was to examine 3 year DFS stratified by pathological response (QpCR versus non-QpCR). We also performed a logistic regression analysis to examine which features were associated with QpCR with this regimen. Clinical response, the rate of BCS, and safety were also evaluated.

Methods

Study design and ethics

This multicenter, open-label, single-arm, phase II clinical study was conducted at 13 institutions throughout Japan. This study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice. The protocol was reviewed and approved by the institutional review board of each participating institution and written informed consent was obtained from all patients prior to the study.

Patients

Women aged 20–59 years of age with histologically proven early stage breast cancer (T1c-3 N0 M0/T1-3 N1 M0)

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were enrolled. No prior chemotherapy, radiotherapy, hormonal therapy, or immunotherapy was allowed. Other inclusion criteria were the following: Eastern Cooperative Oncology Group performance status of 0–1; white blood cell count between 4000/mm^3 and 12000/mm^3; neutrophil count ≥2000/mm^3; platelet count ≥100000/mm^3; hemoglobin ≥9.5 g/dl; serum bilirubin <1.25 times upper normal limit (UNL), creatinine <1.5 times UNL, or AST and ALT <1.5 times UNL. Patients with congestive heart failure or left ventricular ejection fraction ≤60% were excluded. Patients were also excluded if they had confirmed infection; serious concomitant illness such as severe cardiovascular disease, uncontrolled diabetes, malignant hypertension and hemorrhagic disease; active concomitant malignancy; brain metastasis; interstitial pneumonia or lung fibrosis confirmed by chest X-ray or computed tomography; pleural or peritoneal effusion that required treatment; pericardial effusion; motor paralysis, peripheral neuropathy or edema history of severe drug allergy; or had previously received long-term corticosteroid therapy. Pregnant or lactating women were also excluded.

Treatment procedures

Four cycles of FEC (fluorouracil 500 mg/m^2, epirubicin 100 mg/m^2, and cyclophosphamide 500 mg/m^2) administered intravenously (i.v.) on day 1 every 21 days were followed by four cycles of docetaxel i.v. (75 mg/m^2) every 21 days, prior to surgery. The doses of docetaxel and epirubicin selected at the time of this study were higher than the approved doses in Japan (60 mg/m^2 each). Premedication consisted of a 5-HT$_3$ antagonist and dexamethasone i.v. on day 1 with oral dexamethasone on days 2 and 3 with each cycle of FEC and dexamethasone i.v. with or without 5-HT$_3$ antagonist on day 1 with each cycle of docetaxel. Administration of recombinant human granulocyte colony-stimulating factor (rh G-CSF) and antibiotics was left to the judgment of each investigator. If patients prematurely discontinued FEC treatment, they were expected to proceed to four cycles of docetaxel.

Treatment could be postponed for a maximum of 2 weeks for severe toxicity. If toxicity did not improve during this period, chemotherapy was discontinued and surgery was recommended. Dose reductions of epirubicin from 100 mg/m^2 to 75 mg/m^2 and for docetaxel from 75 mg/m^2 to 60 mg/m^2 were permitted in case of febrile neutropenia and grade 3 or 4 non-hematological toxicities except for nausea, vomiting, and fatigue. Following chemotherapy and clinical assessment of response, patients underwent surgery. If the tumor was too large or invasive for breast-conserving surgery, modified radical mastectomy was recommended. Sentinel lymph node biopsy (SNB) was performed to confirm disease stage. Most patients with negative biopsies did not undergo surgical clearance of axillary nodes. Autologous or heterologous reconstructive surgery was performed as needed. All patients who underwent breast-conserving surgery were given standard radiotherapy to the remaining ipsilateral breast tissue after surgical recovery. For patients with node-negative status in the sentinel nodes not requiring axillary dissection, radiotherapy to the axilla was allowed but not required. No recommendations were made for post-study hormone therapy in the protocol.

Assessment

Hormone receptor and HER2 overexpression

Estrogen receptor (ER) status and progesterone receptor (PgR) status were determined by immunohistochemistry at each institute. In general, tumors with >10% positively stained tumor cells were classified positive for ER and PgR. HER2 status was also determined at each institute by immunohistochemistry or by fluorescence in situ hybridization (FISH) analysis. HER2 positive tumors were defined as 3+ on immunohistochemistry staining or as positive by FISH.

Central pathological assessment

Haematoxylin and eosin (H&E) and keratin stained slides were prepared as 5 mm tissue sections from the primary tumor. Pathological breast tumor response was assessed by a central review committee consisting of three pathologists using modified criteria of the Japanese Breast Cancer Society [14]. A blinded central review committee evaluated the pathologic response independently to the local pathologists. In this study, the response of stromal invasion and intraductal component was assessed separately. Cytokeratin immunostaining was performed to confirm residual cancer cells in required cases.

Toxicity and clinical assessment

Toxicity was evaluated according to the National Cancer Institute Common Toxicity Criteria (version 2). Tumor response was assessed using the Response Evaluation Criteria in Solid Tumors (RECIST) guidelines in patients who had measurable lesions. Tumor and toxicity assessments were performed within 4 weeks prior to FEC treatment, after completion of FEC treatment, and before surgery.

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].

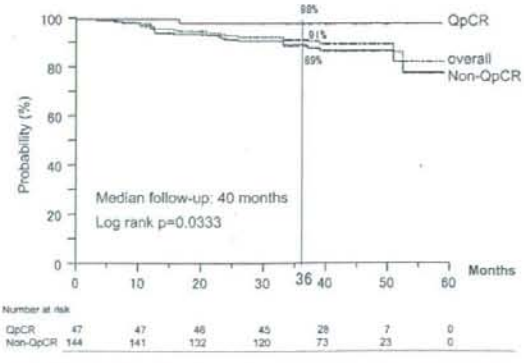

Fig. 1 Relationship of QpCR and non-QpCR to disease free survival

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

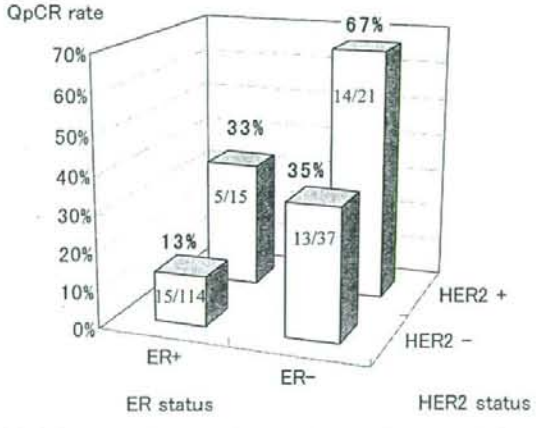

Fig. 2 Relationship between QpCR and HER2/ER status (n=187)

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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Predictive implications of nucleoside metabolizing enzymes in premenopausal women with node-positive primary breast cancer who were randomly assigned to receive tamoxifen alone or tamoxifen plus tegafur-uracil as adjuvant therapy

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Abstract. Recent studies have demonstrated that tegafur-uracil (UFT) is useful for the adjuvant treatment of various types of cancers. To determine whether nucleoside metabolizing enzymes could be used to predict the response to UFT treatment in women with primary breast cancer, we retrospectively analyzed archived tumor tissue samples obtained from the 3rd Adjuvant Chemo-Endocrine Therapy for Breast Cancer (ACETBC) study, in which adjuvant treatment with tamoxifen (TAM) plus UFT for 2 years was compared with TAM alone for 2 years. Samples of tumor tissue were obtained from 192 premenopausal women with node-positive invasive breast cancer. The tissue samples were examined immunohistochemically to study the expression of thymidylate synthase (TS), thymidine phosphorylase (TP), and dihydropyrimidine dehydrogenase (DPD), as well as the expression of HER2 and p53. In patients with TS-positive tumors, the risk of relapse was significantly lower in the tamoxifen plus UFT group than in the tamoxifen alone group. After 2 years, however, there was a trend towards a decrease in the relative predictive value (RPV) of TS with time. No relationship to outcome was detected for TP or DPD. Expression of HER2 or p53 was a significant prognostic indicator in the tamoxifen alone group. TS, but not TP or DPD, may be a useful predictor of response to UFT therapy. After 2 years, the RPV of TS decreased with time, suggesting that 2 years of treatment with oral fluorouracil derivatives may be inadequate. Further studies are required to investigate this possibility.

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Key words: tegafur-uracil, predictive factor, thymidylate synthase, thymidine phosphorylase, dihydropyrimidine dehydrogenase, breast cancer

Introduction

UFT is an oral formulation combining tegafur, a prodrug of 5-fluorouracil, with uracil, an inhibitor of dihydropyrimidine dehydrogenase (DPD), the rate-limiting enzyme governing the metabolism of 5-fluorouracil. Recently, many studies have demonstrated that adjuvant treatment with tegafur-uracil (UFT) is effective against lung cancer and other types of solid tumors (1-4). In breast cancer, the therapeutic usefulness of adjuvant chemotherapy with tegafur preparations has been studied in Japan and other countries for more than 20 years (5,6). Recently, Noguchi *et al* (7) reported the results of a pooled analysis of 6 randomized clinical trials in women with node-negative breast cancer. Their analysis demonstrated that survival was significantly longer in patients who received UFT than in those who did not. In addition, the effects of combined treatment with UFT and tamoxifen were found to be additive. These findings suggested that UFT may be useful for the management of primary breast cancer, although controlled studies with commonly used regimens for polychemotherapy, such as anthracycline plus cyclophosphamide (AC) and cyclophosphamide plus methotrexate plus fluorouracil (CMF), have yet to be reported.

Recent studies have shown that S-1, a combination of tegafur and 5-chloro-2,4-dihydropyrimidine (CDHP), a more potent inhibitor of DPD than uracil, has high antitumor activity against metastatic breast cancer (8). Other studies with 5-fluorouracil derivatives have demonstrated that combined treatment with capecitabine and docetaxel significantly prolongs survival among women with anthracycline-resistant breast cancer, as compared with docetaxel alone (9). Various

trials are now being performed in preoperative or postoperative settings (10). These drugs will most likely play an important role in the future treatment of breast cancer. The benefits of oral 5-fluorouracil derivatives would be further enhanced by the ability to predict response, thereby identifying patients most likely to benefit from treatment and increasing the benefit-risk ratio.

Various approaches have been proposed to predict the response to oral 5-fluorouracil derivatives. Experimental and clinical evidence has suggested that tumor levels of enzymes involved in nucleoside metabolism, such as thymidylate synthase (TS), thymidine phosphorylase (TP), and dihydropyrimidine dehydrogenase (DPD), may be useful for predicting the response to oral 5-fluorouracil derivatives. Predictive accuracy may be further enhanced by using these enzymes in conjunction with other molecular markers.

We retrospectively examined whether the expression of the 3 enzymes TS, DPD, and TP and that of the oncogene HER2 and the tumor-suppressor gene p53 in breast cancer tissue could be used to predict the response to treatment with tamoxifen plus UFT. Resected tissue specimens were obtained from women with breast cancer who were enrolled in the 3rd Adjuvant Chemo-Endocrine Therapy for Breast Cancer (ACETBC) trials, randomized controlled studies comparing tamoxifen alone with tamoxifen plus UFT after surgery.

Patients and methods

Combined analysis of three randomized trials. A meta-analysis of 5 randomized controlled trials (n=1987) performed by the ACETBC study group in Japan has shown that the reduction in the risk of recurrence after treatment with UFT was 21±11% (P=0.06) in women with stage I to IIIA breast cancer who underwent mastectomy (5).

Three of these trials examined the effect of adding UFT (300-400 mg/day) to tamoxifen (20-30 mg/day) in women with estrogen-receptor (ER)-positive tumors who postoperatively received adjuvant chemotherapy for 2 years. ER status was determined at each center. Either biochemical (enzyme immunoassay) or immunohistochemical techniques were used. In 2 of these trials, mitomycin C (10 mg/m^2) was given intravenously on the day of surgery. Combined analysis of these 3 trials (n=1225; median follow up, 5.7 years) was performed according to the method of Peto (Fig. 1). The reduction in the risk of recurrence after treatment with UFT plus tamoxifen was found to be 26±12% (p=0.037). Subset analyses of pooled data in the 3 trials showed that UFT was most effective in premenopausal women with metastases to the axillary lymph nodes (reduction in odds of recurrence, 35±17%).We retrospectively studied the predictive values of biomarkers of response in this patient subset.

Immunohistochemically studied biomarkers

Collection of tumor samples. A list of subjects was submitted to centers that had agreed to participate in this biomarker study and had registered at least 5 patients to the 3rd ACETBC study. All available paraffin-embedded samples were sent from the centers to the operational office by mail. The samples were stored at room temperature until predictive markers were evaluated.

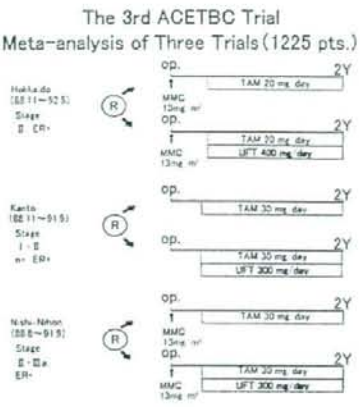

Figure 1. Protocols of the 3rd ACETBC trial.

Immunohistochemical labeling

Antibodies. TS polyclonal antibody RTSSA (dilution, 1:100; Taiho Pharmaceutical Co., Ltd., Tokyo, Japan), TP monoclonal antibody TMA-1 (dilution, 1:100; Taiho Pharmaceutical Co., Ltd.), DPD polyclonal antibody RDPDPA (dilution, 1:100; Taiho Pharmaceutical Co., Ltd.), HER2 polyclonal antibody A0485 (Dako, Carpinteria, CA, USA; dilution, 1:100), and p53 (DO7) monoclonal antibody (Novo-castra, Newcastle, UK; dilution, 1:40) were used for immunohistochemical analyses.

Immunohistochemical analyses. Immunohistochemical analyses were performed at a single central laboratory using the antibodies described above and mouse IgG (Dako) as negative control. An indirect avidin-biotin-peroxidase method was used. Briefly, deparaffinized tissue sections were treated with 0.3% hydrogen peroxide in methanol to block endogenous peroxidase activity. After washing with phosphate buffered-saline (PBS) containing 0.05% Tween-20, the sections were treated with 1.5% normal horse serum in PBS and incubated with each of the antibodies or with mouse IgG for 1 h at room temperature. The sections were washed again with PBS, incubated with biotinylated anti-mouse IgG (Dako) for 30 min, washed again with Tween-20-PBS, incubated with an elite ABC kit (Vector, Burlingame, CA, USA) for 30 min, and visualized with the use of 3,3'-diaminobenzidine tetrahydrochloride-hydrogen peroxide as chromogen. The sections were then counterstained with hematoxylin, dehydrated, and mounted.

Evaluation of staining. The slides were evaluated independently by 3 experienced pathologists (A.F., K.M., T.H.) blinded with regard to treatment group and outcome. Each pathologist evaluated TS, TP, and DPD on the basis of staining intensity of the cytoplasm, scored according to a 4-grade scale (0 to 3), and staining rate, also scored according to a 4-grade scale (≤25%, 0; >25% to ≤50%, 1; >50% to ≤75%, 2; and >75%, 3). The scores agreed on by 2 or more of the pathologists were adopted. Concordance rates of the evaluations among 2 or more pathologists were as follows: TS, staining intensity 95%, staining rate 80%; TP, staining intensity 92%, staining rate

Table I. Patients' characteristics in the biomarker study.

	TAM group (n=97)	UFT group (n=95)	p-value
Age			
≤50	89	89	0.78
>51	8	6	
Number of nodes involved			
1-3	65	73	0.15
≥4	32	22	
Tumor size			
<2 cm	23	24	0.87
≥2 cm	74	71	
TS expression			
Positive	57	48	0.31
Negative	40	47	
TP expression			
Positive	36	39	0.86
Negative	61	56	
DPD expression			
Positive	57	66	0.13
Negative	40	29	
HER2 expression			
Positive	14	14	1.00
Negative	83	81	
p53 expression			
Positive	30	33	0.85
Negative	67	62	

All patients had estrogen receptor-positive tumors and were premenopausal.

87%; and DPD, staining intensity 94%, staining rate 89%. The median score was adopted if all 3 pathologists disagreed on the score. Cases were considered positive if the staining intensity was ≥2, and the staining rate was 3 (staining rate, >75%).

HER2 was evaluated on the basis of staining of the membrane, and p53 was evaluated on the basis of staining of nuclei. The results were considered positive if the staining rate was ≥1%. The evaluation agreed on by 2 or more pathologists was adopted (concordance rates among the evaluations of the 3 pathologists were as follows: HER2, 89%; and p53, 72%).

Statistical analysis. Relapse-free survival was the outcome used to assess treatment efficacy and was defined as the interval elapsed between the date of surgery and the date of documented disease relapse or death. Relapse-free survival was calculated by the Kaplan-Meier method. Differences between groups in Kaplan-Meier estimates of relapse-free survival were evaluated with the log-rank test and generalized Wilcoxon test. Risk

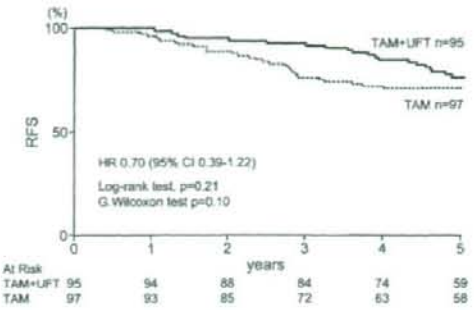

Figure 2. Relapse-free survival (RFS) according to study group (n=192).

ratios (RR) were estimated from Cox proportional-hazards regression models. No overall survival analysis was performed in the subgroups of patients identified by the evaluated biological markers because of the small numbers of events in each treatment group. Cox proportional-hazards regression models were also used to test for interactions between biomarkers and treatment.

Relative predictive values (RPV) were determined with use of the following equation, modified from the method described by Hayes (11): RPV for events in the tamoxifen + UFT group was compared with those in the tamoxifen alone group = Log (RR when tumors stained negatively for biomarkers/RR when tumors stained positively for biomarkers). Differences in distributions between groups were compared with the use of the χ^2 test. Differences were considered statistically significant when p-values were <0.05, and all reported p-values are two-tailed. All analyses were carried out with SAS software (version 6.12).

Results

Collection of samples. Samples collected from 192 (97 given tamoxifen and 95 given tamoxifen plus UFT) of the 204 women at the centers were assessable. There were no significant differences between the groups in demographic characteristics (age, tumor size, number of lymph node metastases) (Table I). The hazard ratio of the effect of adding UFT to tamoxifen was 0.70 (95% confidence interval, 0.39 to 1.22) (log-rank test, p=0.21; Wilcoxon test, p=0.10) (Fig. 2).

Expression of biomarkers. The rates of positive staining were as follows: TS, 55% (105/192); TP, 39% (75/192); DPD, 64% (123/192); HER2, 15% (28/192); and p53, 33% (63/192). The expression rates of these biomarkers were similar in the tamoxifen group and the tamoxifen plus UFT group (Table I).

Relation between relapse-free survival and expression of biomarkers in tumors. Demographic characteristics were similar in women whose tumors stained positively for each biomarker (TS, TP, or DPD) and those whose tumors stained negatively for each biomarker. Univariate analyses showed no significant differences in relapse-free survival between women whose tumors stained positively for TS, TP, or DPD and those whose tumors stained negatively for these 3

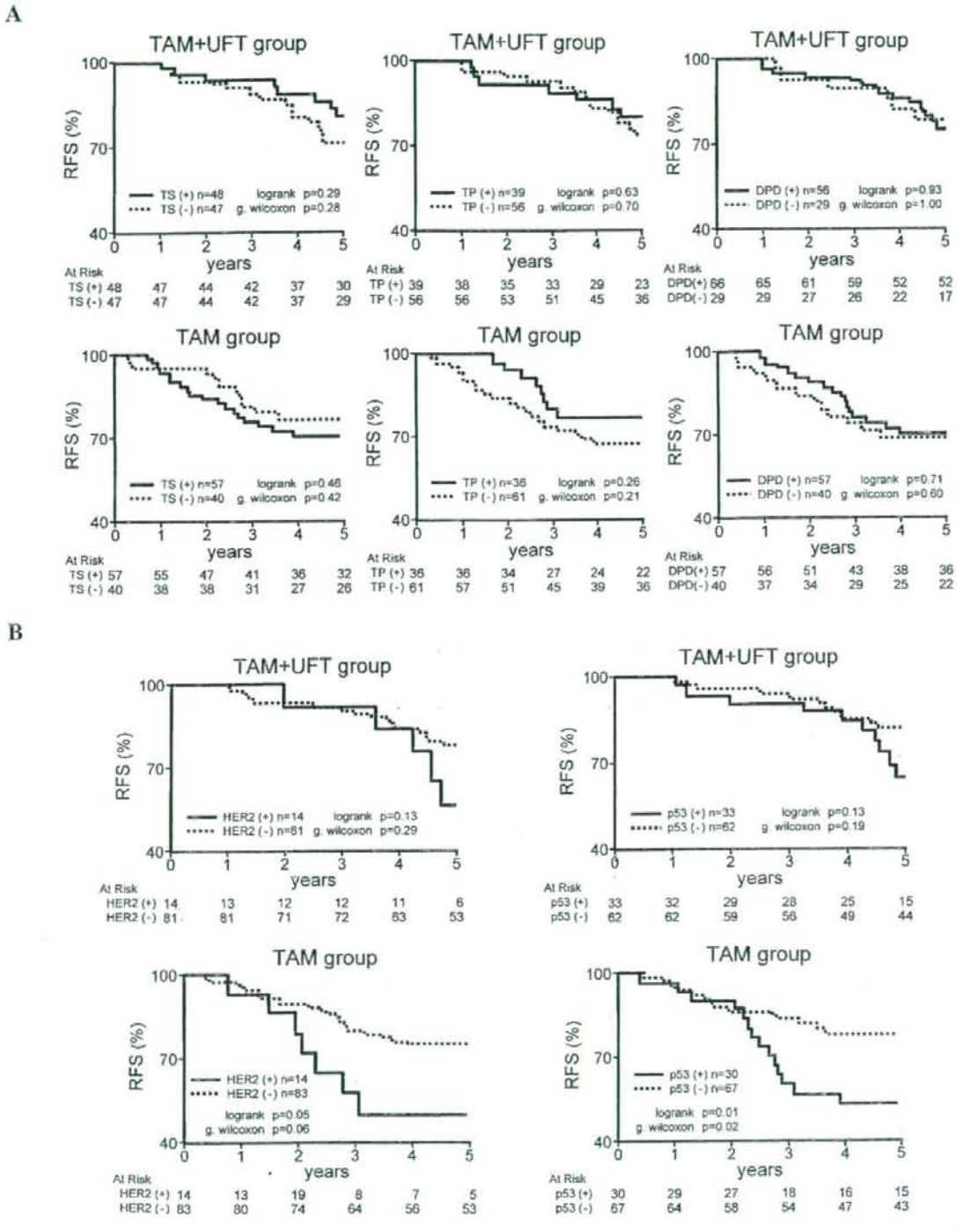

Figure 3. (A) Relation between relapse-free survival (RFS) and tumor expression of thymidylate synthase (TS), thymidine phosphorylase (TP), and dihydropyrimidine dehydrogenase (DPD) according to treatment. (B) Relation between relapse-free survival and tumor expression of HER2 and p53 according to treatment.

biomarkers in either treatment group. Women whose tumors stained positively for HER2 or p53 in the tamoxifen alone group had significantly poorer outcomes than those whose tumors stained negatively for these biomarkers. HER2 and p53 were not significant prognostic factors in the tamoxifen plus UFT group (Fig. 3).

Table II. Relative risk (TAM+UFT vs. TAM) according to biomarker expression.

Biomarker	Biomarker positive			Biomarker negative			Interaction
	RR	95% CI	p-value (G. Wilcoxon test)	RR	95% CI	p-value (G. Wilcoxon test)	p-value
TS	0.48	0.20-1.07	0.04	1.00	0.44-2.36	1.00	0.22
TP	0.80	0.28-2.23	0.60	0.66	0.33-1.30	0.124	0.76
DPD	0.75	0.37-1.52	0.29	0.61	0.21-1.56	0.222	0.73
HER2	0.59	0.17-1.86	0.19	0.72	0.37-1.37	0.220	0.77
p53	0.57	0.25-1.28	0.09	0.78	0.35-1.72	0.418	0.59

RR, relative risk by addition of UFT to TAM; TS, thymidylate synthase; TP, thymidine phosphorylase; DPD, dihydropyrimidine dehydrogenase.

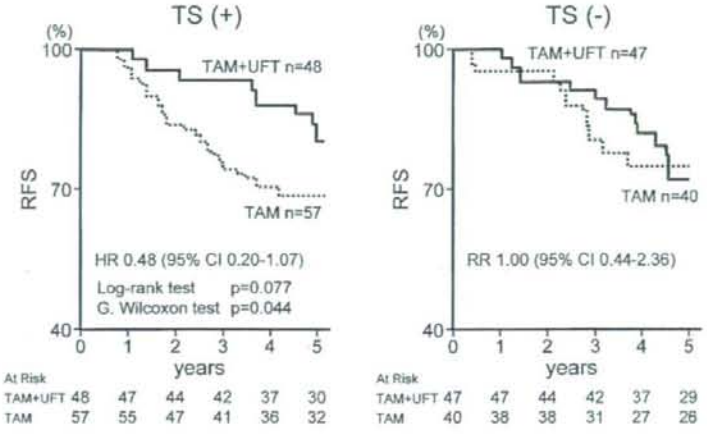

Figure 4. Comparison of relapse-free survival (RFS) between TAM and TAM+UFT treatment according to thymidylate synthase (TS) status.

Relation between expression of biomarkers in tumors and effect of adding UFT to tamoxifen

TS. In women with TS-positive tumors, the risk ratio of the effect of adding UFT to tamoxifen was 0.48 (95% confidence interval, 0.20 to 1.07), and response differed significantly between women given tamoxifen alone and those given tamoxifen plus UFT (p=0.04 by the generalized Wilcoxon test, p=0.08 by the log-rank test). In women with TS-negative tumors, however, there was no significant difference in response (hazard ratio, 1.00; 95% confidence interval, 0.44-2.36). Interaction testing showed that the expression of TS was not significantly related to the effect of UFT (p=0.22) (Fig. 3, Table II).

TP. The risk ratio of the effect of adding UFT to tamoxifen was 0.80 (95% confidence interval, 0.28-2.23) in women with TP-positive tumors and 0.66 (95% confidence interval, 0.33-1.30) in women with TP-negative tumors. There were no significant differences in response between the treatment groups. Interaction testing showed no significant relation between the expression of TP and the effect of UFT (p=0.76) (Table II).

DPD. The risk ratio of the effect of adding UFT to tamoxifen was 0.75 (95% confidence interval, 0.37-1.52) in women with DPD-positive tumors and 0.61 (95% confidence interval, 0.21-1.56) in those with DPD-negative tumors. There were no significant differences between the treatment groups. Interaction testing showed that the expression of DPD was not significantly related to the effect of UFT (p=0.73) (Table II).

HER2. The risk ratio of the effect of adding UFT to tamoxifen was 0.59 (95% confidence interval, 0.17-1.86) in women with HER2-positive tumors and 0.72 (95% confidence interval, 0.37-1.37) in those with HER2-negative tumors. There were no significant differences between the treatment groups. Interaction testing showed that the expression of HER2 was not significantly related to the effect of UFT (p=0.77) (Table II).

p53. The hazard ratio of the effect of adding UFT to tamoxifen was 0.57 (95% confidence interval, 0.25-1.28) in women with p53-positive tumors and 0.78 (95% confidence interval, 0.35-1.72) in women with p53-negative tumors. There were no significant differences between the treatment groups.

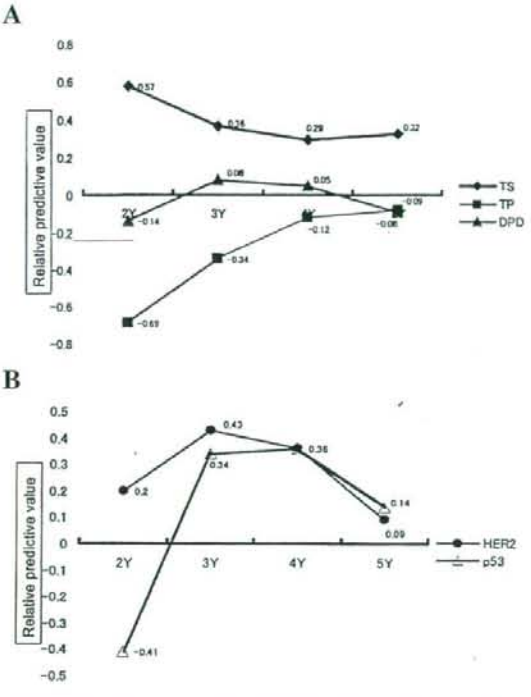

Figure 5. (A) Change in relative predictive values (TS, TP, and DPD). (B) Change in relative predictive values (HER2 and p53). Relative predictive value: log [RR of marker (-)/RR of marker (+)]. RR: risk ratio (TAM vs. TAM+UFT).

Interaction testing demonstrated no relation between the expression of p53 and the effect of UFT (p=0.58) (Table II).

Changes in RPV. Changes in the RPV of each biomarker over time are shown in Fig. 5. (The RPV at 1 year could not be determined because some subgroups of patients had no events at 1 year.) The RPV of TS gradually decreased with time for up to 4 years (0.57 at 2 years, 0.34 at 3 years, and 0.29 at 4 years), and was 0.32 at 5 years. The absolute value for the RPV of TP gradually decreased over time (-0.67 at 2 years, -0.34 at 3 years, -0.12 at 4 years, and -0.08 at 5 years). The RPV of DPD was approximately 0 for up to 5 years (-0.14 at 2 years, 0.08 at 3 years, 0.05 at 4 years, and -0.09 at 5 years). The RPV of HER2 was 0.20 at 2 years, 0.43 at 3 years, 0.36 at 4 years, and 0.09 at 5 years. The RPV of p53 was -0.41 at 2 years, 0.34 at 3 years, 0.36 at 4 years, and 0.14 at 5 years.

Discussion

We immunohistochemically studied whether the biomarkers TS, TP, DPD, HER2, and p53 could be used to predict the effect of adding UFT to tamoxifen in women with breast cancer who underwent mastectomy. In women with TS-positive tumors, relapse-free survival was significantly better in the tamoxifen plus UFT group than in the tamoxifen group, whereas there was no significant difference between the treatment groups in women with TS-negative tumors. These results suggest that TS can be used to predict the response to UFT plus tamoxifen, although interaction testing showed no significant interaction between TS expression and treatment response.

Several studies have reported that TS can be used to predict the response to 5-fluorouracil-based adjuvant chemotherapy in patients with colorectal cancer (12-15). These studies consistently found that 5-fluorouracil-based chemotherapy was ineffective for patients with TS-negative tumors, but effective for patients with TS-positive tumors. Pestalozzi *et al* (16) examined whether TS could be used to predict treatment response in women with breast cancer who were enrolled in a randomized controlled trial (the International Breast Cancer Study Group-V) comparing 1 course of CMF given perioperatively with 6 courses of CMF given postoperatively. Their results showed that suppression of recurrence after 6 courses of postoperative CMF was superior to that after 1 course of perioperative CMF only among women who had TS-positive tumors. Our results are in accordance with their findings. TS, an enzyme involved in DNA synthesis, catalyzes the methylation of deoxyuridine monophosphate to produce deoxythymidine monophosphate. TS is targeted by 5-fluorouracil.

Most experimental studies using cell lines and studies of metastatic cancers (17) have shown that high TS expression is associated with a low antitumor response to 5-fluorouracil, a finding that conflicts with the results of studies in an adjuvant setting. Recent experimental studies by Rahman *et al* (18) have reported that TS has oncogene-like properties. Overexpression of TS under the condition of serum deprivation was clearly demonstrated to induce apoptosis. Therefore, overexpression of TS due to tumor-related or environmental factors may alter the response to 5-fluorouracil-based chemotherapy. In addition, a recent investigation found that tamoxifen up-regulates TS (19). This phenomenon may have a part in the enhanced response to adjuvant chemotherapy with tamoxifen plus UFT.

TP expression was not significantly related to the effect of adding UFT to tamoxifen. TP is an enzyme involved in nucleoside metabolism, antiapoptosis activity, and the promotion of neovascularization. It also converts capecitabine, a prodrug of 5-fluorouracil, and 5'-deoxy-5-fluorouridine (5'-DFUR), an intermediate metabolite of capecitabine, to 5-fluorouracil. Many basic and clinical trials have reported the relation between TP expression and the effects of capecitabine and 5'-DFUR (10). Tominaga *et al* (20) immunohistochemically studied the relation between TP expression and the response to 5'-DFUR in women with early breast cancer who were enrolled in a randomized controlled trial comparing surgery alone with postoperative adjuvant chemotherapy with 5'-DFUR. They concluded that TP expression can be used to predict the response to 5'-DFUR. UFT is a prodrug of 5-fluorouracil, combining tegafur with uracil. Tegafur is converted to 5-fluorouracil principally by liver cytochrome CYP2A6 (21). This mechanism may account for the lack of a relation between TP expression and the effect of adding UFT to tamoxifen in this study.

DPD expression in tumors was also not significantly related to the effect of adding UFT to tamoxifen. DPD, present mainly in the liver, is a rate-limiting enzyme that inactivates 5-fluorouracil. DPD activity in tumors is related

to sensitivity to 5-fluorouracil. Tumors with high DPD expression are thought to respond poorly to 5-fluorouracil derivatives. Indeed, some studies have reported that sensitivity to capecitabine or doxifluridine is governed by DPD (22-24). UFT contains uracil, an inhibitor of DPD, and may be effective against tumors with high expression levels of DPD (25). This characteristic may account for the fact that the effect of adding UFT to tamoxifen was unrelated to tumor DPD expression.

The expression of HER2 and of p53 was also unrelated to the effect of adding UFT to tamoxifen. Previous studies have reported that the expression of HER2 and p53 is related to the response to anthracycline-based chemotherapy (26,27). However, our study suggests that these factors do not influence the response to UFT. HER2 and p53 were significant prognostic factors in the tamoxifen alone group. Because we did not evaluate these factors in the groups not given tamoxifen, we cannot be certain, but our results suggest that HER2 and p53 are predictive markers of the response to treatment with tamoxifen alone. This notion is supported by the findings of Carlomagno *et al* (28), who reported that overexpression of HER2 is related to the response to tamoxifen in women with breast cancer.

Hayes described a method for quantifying the pure predictive values of biomarkers for forecasting treatment response (11). He used risk ratio (RR) in a treated group relative to that in a control group for subgroups of patients whose tumors were positive or negative for a given biomarker. The RR was used in the following equation to derive the RPV of the biomarker: RPV = [1 - RR (biomarker-positive tumors)]/[1 - RR (biomarker-negative tumors)]. Because RR was often >1 for patients with either biomarker-positive or -negative tumors, we modified Hayes' method and used the following equation: RPV = log [RR (biomarker-negative tumors)]/[RR (biomarker-positive tumors)]. The RPV scores were calculated and plotted over time to examine the time course of the RPV (Fig. 4). The RPV was positive if the treatment response was greater when tumors were biomarker positive. Conversely, the RPV was negative if the treatment response was greater when tumors were biomarker negative.

The higher the absolute value of the RPV, the stronger was the power to predict treatment response. Because the natural logarithm was used, the predictive power can be considered weak if the absolute value was <0.3 and strong if the absolute value was ≥0.5. The RPV of TS was 0.57 at 2 years and was then gradually decreased with time, but remained at >0.3 at 5 years. These data suggest that TS is a pure predictive factor of the response to UFT.

A likely explanation for the reduction in the RPV of TS with time is that the magnitude of the effect of adding UFT to tamoxifen decreased from year 2 onward. A recent overview of randomized trials of adjuvant therapy compiled by the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) (29) showed that the response to poly-chemotherapy, including regimens such as AC and CMF, diminishes with time, suggesting that this phenomenon is commonly associated with chemotherapy. In the studies analyzed, both tamoxifen and UFT were given for 2 years. Treatment response may persist if UFT is continued for more than 2 years. However, these data should be interpreted with caution because specific subgroups of patients were studied retrospectively.

The RPV of TP was -0.69 at 2 years, and the absolute values were low at 4 and 5 years (-0.12 and -0.08, respectively). As mentioned previously, TP was not a statistically significant predictive factor in our study, but there was a trend toward a higher additive effect of UFT when TP was negative. The RPV of DPD consistently remained at approximately 0, suggesting that the value of DPD for predicting the response to UFT was low.

The RPVs of HER2 and p53 were >0.3 at 3 and 4 years, but neither of these biomarkers were significant predictive factors in our study. This is attributed to the fact that positive rates for HER2 and p53 were low in our study, thereby diminishing statistical power. Interestingly, the time courses of the RPVs of these markers differed from those of TS and TP.

Our results suggested that the expression of TP and DPD, factors related to the response to capecitabine, do not influence the response to UFT. Therefore, different types of oral fluorouracil derivatives may be most effective in distinct subgroups of patients. In the future, expression of TS, DPD, and TP might be useful for selecting patients most likely to respond to tegafur-based oral fluorouracil derivatives, such as UFT and S-1, and those more likely to respond to capecitabine.

At present, however, breast cancer is often treated by a multidisciplinary approach. Care should be exercised when using oral fluorouracil derivatives in combination with other anticancer drugs because the latter may modify nucleoside-metabolizing enzymes, thereby affecting the metabolism of fluorouracil (30). The measurement of biomarkers before and after treatment may also have an important role in the selection of preoperative chemotherapy.

An important limitation of our study was the retrospective design and the inclusion of only a subset of patients (node-positive premenopausal women) who were enrolled in randomized controlled trials. Our results must therefore be verified in prospective randomized controlled studies in which women with breast cancer are assigned to adjuvant treatment on the basis of the prior determination of biomarker levels.

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