Table 3. Comparison of the OSNA assay with the histopathologic examination

Number of lymph nodes	OSNA*	Histopathologic examination †			
		Macrometastasis	Micrometastasis	ITC	Negative ‡
325 from 101 patients	++	34 (34)	0 (0)	0 (0)	0 (0)
	+	6 (5)	3 (1)	0 (0)	4 (0)
	-	0 (0)	2 (2)	13 (11)	263 (0)
81 SLNs from 49 patients	++	11 (11)	0 (0)	0 (0)	0 (0)
	+	1 (0)	2 (1)	0 (0)	1 (0)
	-	0 (0)	2 (2)	3 (2)	61 (0)
144 from 60 pN0 patients	++	0 (0)	0 (0)	0 (0)	0 (0)
	+	0 (0)	0 (0)	0 (0)	0 (0)
	-	0 (0)	0 (0)	3 (3)	141 (0)

*In the OSNA assay, (++), (+), and (-) show >5 × 10^3, 2.5 × 10^2 to 5 × 10^3, and <2.5 × 10^2 copies/μL of CK19 mRNA, respectively.
† Histopathologic examinations with H&E and CK19 immunohistochemistry were carried out in all samples. In cases where metastatic foci were observed in the histopathologic examination by either H&E or CK19 immunohistochemistry, the sample was categorized as macrometastasis, micrometastasis, or ITC. The results of the three-level CK19 immunohistochemistry-based histopathologic examination were determined by the consensus of three third-party pathologists. The number of lymph nodes judged to be positive based on the three-level H&E-based histopathologic examination is shown in parenthesis.
‡ No cancer cells were observed in either the immunohistochemistry- or H&E-based histopathologic examinations.

Discussion

The detection of lymph node metastasis by RT-PCR (37-40) and by QRT-PCR (12, 19-25) has been studied previously. CK19 mRNA has been described as having the highest sensitivity at nearly 90%. However, there are drawbacks using CK19 mRNA due to the concomitant amplification of pseudogenes in genomic DNA that lead to false positive results. For this reason, a combination of two or three markers has been used.

We evaluated 45 potential mRNAs and finally selected CK19 mRNA as the best marker for the OSNA assay. To use CK19 mRNA as a marker, we designed RT-LAMP primers that do not amplify the known CK19 pseudogenes (see Materials and Methods). In addition, the lymph node solubilization step in the OSNA assay was carried out at pH 3.5. At this pH, almost all genomic DNA precipitates out. Even when the sample still contained genomic DNA, DNA amplification is unlikely to occur in the OSNA assay because the RT-LAMP step is carried out at 65°C, a temperature at which genomic DNA typically does not denature. Indeed, purified genomic DNA from metastatic lymph nodes was not amplified in the OSNA assay.

In the present clinical study assessing 325 lymph nodes from 101 patients, an overall concordance rate between the OSNA assay and the CK19 immunohistochemistry-based three-level histopathology was 98.2%. A concordance rate of 96.4% was obtained with 81 SLNs from 49 patients. On the other hand, 1 of 40 macrometastatic cases and 2 of 5 micrometastatic lymph nodes, as defined by CK19 immunohistochemistry-based histopathology, were missed by H&E-based histopathology. Therefore, the sensitivity of three-level H&E-based histopathology was 93.3% based on the three-level CK19 immunohistochemistry-based histopathology. Furthermore, the sensitivity of one- and two-level CK19 immunohistochemistry-based histopathologies is 86.7% and 91.1%, respectively, based on the three-level CK19 immunohistochemistry-based histopathology (Supplementary Table S3). These results indicate that the performance of the OSNA assay is better than that of one- and two-level CK19 immunohistochemistry-based histopathologies and almost equivalent to three-level CK19 immunohistochemistry-based histopathology.

Chu and Wiess (41) reported that 98.2% of primary breast cancer tissues exhibit CK19 protein expression. Two of our authors (Tsujimoto and Tsuda) also examined the CK19 immunohistochemistry-based histopathologic examination of primary breast cancer tissues and found that there was no CK19 protein expression in 20 (2.2%) of 896 cases examined. However, low CK19 mRNA expression in lymph nodes has not been reported.

Table 4. Discordant cases between the OSNA assay and three-level histopathologic examination

Discordant case	CK19 mRNA (copy/μL)	CK19 protein (ng/μL)*	Histopathologic examination †	Nodal status
1	9.6 × 10^2	1.4	Negative	pN2
2	1.5 × 10^3	1.6	Negative	pN1
3	2.3 × 10^3	Not tested	Negative	pN1
4	3.6 × 10^3	Not tested	Negative	pN1
5	ND	0.04	Micrometastasis	pN1
6	ND	Not tested	Micrometastasis	pN1

Abbreviation: ND, not detected.
*Amount of CK19 protein was determined by Western blot analysis (see Materials and Methods).
† Results of CK19 immunohistochemistry-based histopathologic examination of the sections i', ii', and iii' of protocol C (Fig. 3C).

In the present clinical study, CK19 immunohistochemistry-based histopathologic examination of two lymph nodes from one patient revealed metastatic foci smaller than macrometastasis despite the presence of macrometastasis defined by H&E-based histopathologic examination; the histologic type of this primary tumor was neuroendocrine carcinoma. These samples unequivocally had low CK19 expression. The OSNA assay of these samples was positive, indicating that CK19 mRNA was expressed despite the low protein expression found by CK19 immunohistochemistry.

In QRT-PCR studies in which several mRNA markers have been used (12, 19, 24, 25), the ability to quantitatively discriminate macrometastasis from micrometastasis has not been discussed. In the OSNA assay, the solubilization of a lymph node is followed by mRNA amplification. Regardless of the size of the lymph node, a constant portion of lysate is transferred to an RT-LAMP reaction. This indicates that the OSNA assay can, in principle, discriminate macrometastasis from micrometastasis and micrometastasis from nonmetastasis when the cutoff values of CK19 mRNA are properly set. To ensure the quantitative capacity of the OSNA assay, endogenous factors should not interfere with the RT-LAMP reaction. We showed that the presence of a lysate obtained from a lymph node (130-600 mg) did not interfere with the OSNA assay (Fig. 1B). A 600-mg sample of lymph node is equivalent to that having a diameter of about 1 cm. The presence of fat or the reagents that were used to identify SLNs, e.g., radioisotope colloid and blue dyes, did not also interfere with the reaction (data not shown).

We observed no false positive result in the OSNA assay from 144 histopathologically negative lymph nodes (60 pN0 patients). In the statistical analysis of the copy numbers of CK19 mRNA in these 144 lymph nodes, the average value of CK19 mRNA expression +3 SD was $<2.5 \times 10^2$ copies/μL, indicating that the probability of negative lymph nodes showing $>2.5 \times 10^2$ copies/μL is low in the OSNA assay. In the OSNA assay, all 13 ITC cases were judged as nonmetastasis (Table 3).

Based on the serial sectioning experiment (Table 1), the average copy numbers equivalent to 0.2^3, 0.3^3, and 0.4^3 mm^3 can be calculated to be 3.9×10^1, 1.3×10^2, and 3.1×10^2 copies/μL, respectively. Therefore, the cutoff value of 2.5×10^2 copies/μL in the OSNA assay can theoretically detect metastatic foci of 0.3^3 to 0.4^3 mm^3.

The OSNA assay identified 34 cases of macrometastasis out of 40 macrometastatic lymph nodes defined by the permanent three-level CK19 immunohistochemistry-based histopathology. The concordance rate was 85.0%. The remaining six cases were identified as micrometastasis. This is the first example of a molecular biological method with the potential to quantify the size of metastatic foci in a lymph node.

Six discordant cases were observed between the OSNA assay and CK19 immunohistochemistry-based histopathologic examination (Table 4). The quantitative Western blot analysis of the discordant cases (samples 1 and 2) clearly showed the presence of an amount of CK19 protein equivalent to micrometastasis. Although the possible presence of benign epithelial cells such as glandular inclusions in the lymph nodes cannot be eliminated, the results may be better explained by the presence of metastatic foci in the lymph nodes in light of the results of the specificity study and the amount of CK19 protein expression. Two other cases (Table 4, *samples 5* and *6*) were negative according to the OSNA assay, but were judged positive for micrometastasis according to three-level histopathology. These two cases showed metastatic foci of 0.3 and 0.4 mm. Therefore, the amount of metastatic foci in blocks a′ and c′ used for the OSNA assay cannot be estimated exactly. Indeed, in sample 5, the quantitative Western blot analysis of CK19 protein showed no expression of CK19 protein (Table 4).

The results of the clinical study indicate that using one-half of a lymph node in the OSNA assay gave nearly the same results as the three-level histopathology. It became clear in the clinical study conducted at six facilities that the OSNA assay is rapid enough to be done during surgery. Furthermore, the assay would be convenient and objective compared with the intraoperative immunohistochemistry-based histopathologic examination, which is usually done by an experienced pathologist (42, 43).

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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

MASAKAZU TOI[1], TADASHI IKEDA[2], FUTOSHI AKIYAMA[3], MASAFUMI KUROSUMI[4], HITOSHI TSUDA[5], GOI SAKAMOTO[6] and OSAHIKO ABE[7]

[1]Department of Surgery (Breast Surgery), Graduate School of Medicine, Kyoto University, Kyoto; [2]Department of Surgery, Teikyo University School of Medicine, [3]Department of Breast Pathology, Cancer Institute of Japanese Foundation for Cancer Research, Tokyo; [4]Department of Pathology, Saitama Cancer Center Hospital, Ina-machi, [5]Department of Pathology II, National Defense Medical College, Tokorozawa, Saitama; [6]Sakamoto Memorial Clinic, Academy of Breast Pathology, [7]St. Luke's Hospital, Tokyo, Japan

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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.

Correspondence to: Dr Masakazu Toi, Department of Surgery (Breast Surgery), Graduate School of Medicine, Kyoto University, 54 Shogoin-Kawaramachi, Sakyo-ku, Kyoto 606-8507, Japan
E-mail: toi@kuhp.kyoto-u.ac.jp

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.

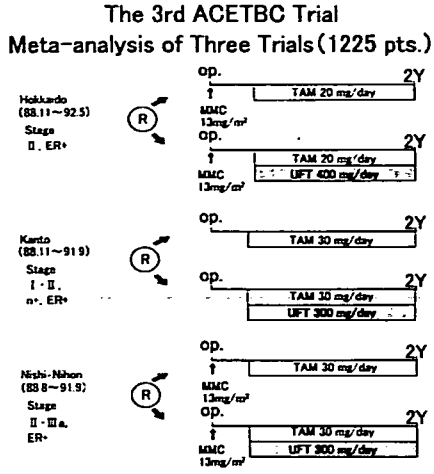

Figure 1. Protocols of the 3rd ACETBC trial.

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 pre-menopausal.

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 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.

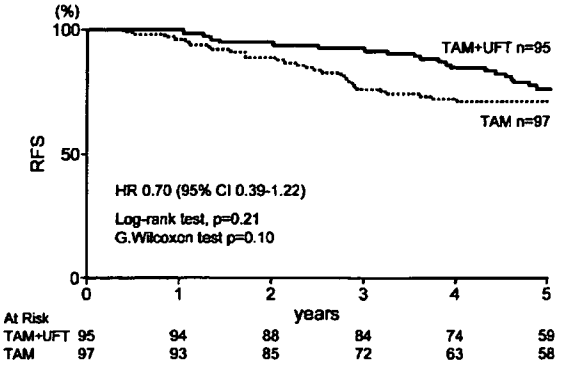

Figure 2. Relapse-free survival (RFS) according to study group (n=192).

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

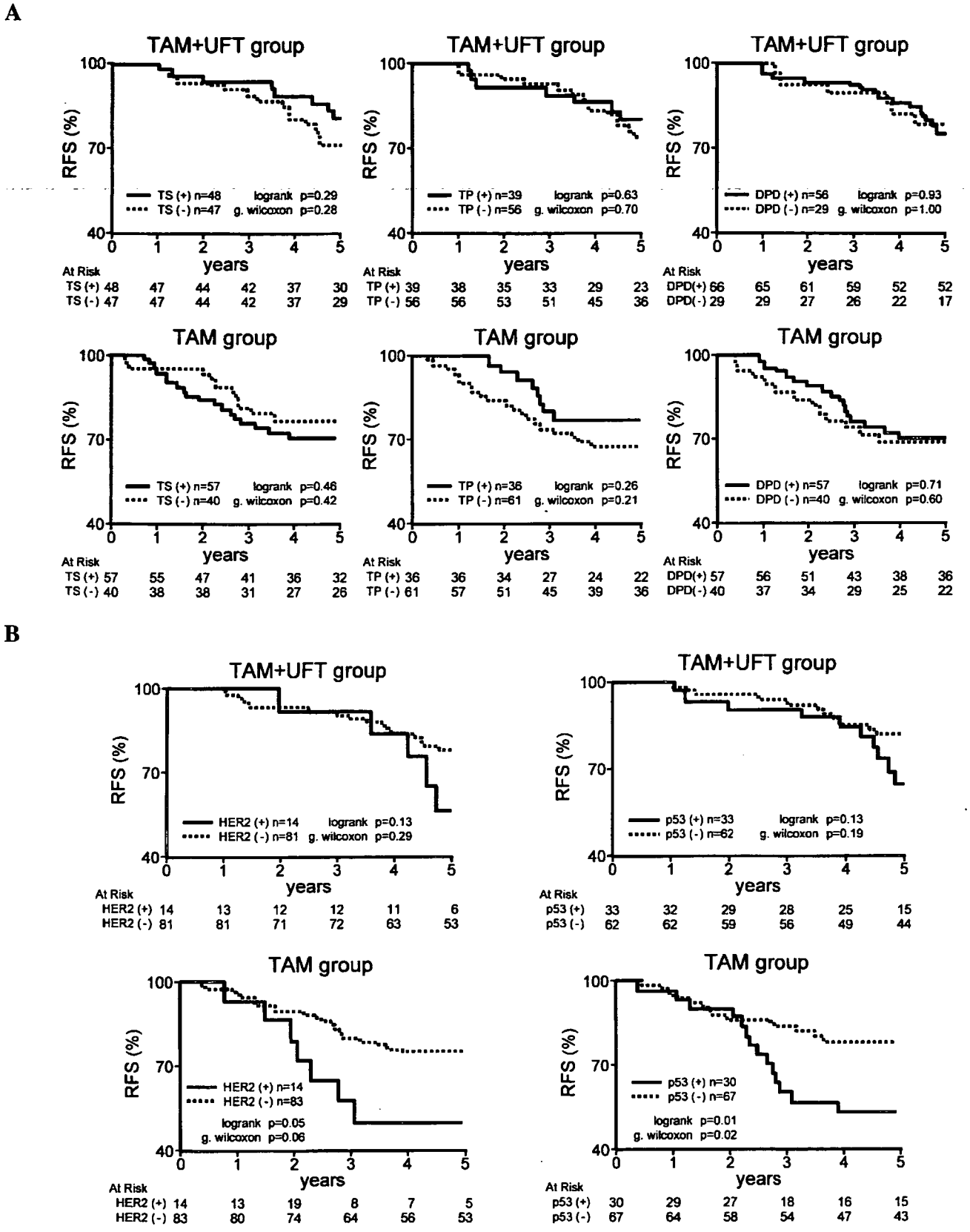

Figure 3. (A) Relation between relapse-free survival (RFS) and tumor expression of thymidylate synthase (TS), thymidine phosphorylase (TP), and dihydropyrimidine dehydrogenase (DPD) according to treatment. (B) Relation between relapse-free survival and tumor expression of HER2 and p53 according to treatment.

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.

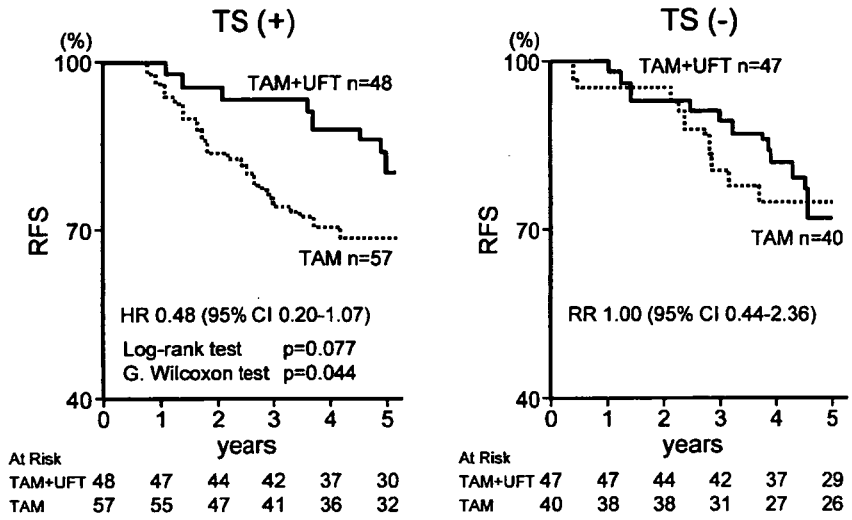

Figure 4. Comparison of relapse-free survival (RFS) between TAM and TAM+UFT treatment according to thymidylate synthase (TS) status.

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.

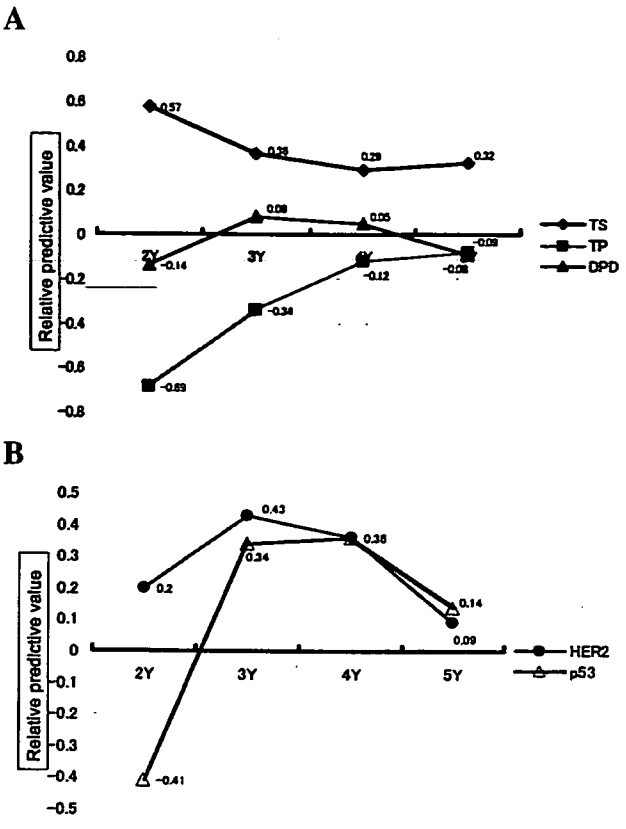

Figure 5. (A) Change in relative predictive values (TS, TP, and DPD). (B) Change in relative predictive values (HER2 and p53). Relative predictive value: log [RR of marker (-)/RR of marker (+)]. RR: risk ratio (TAM vs. TAM+UFT).

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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Review Article

Issues in the Assessment of the Pathologic Effect of Primary Systemic Therapy for Breast Cancer

Katsumasa Kuroi*[1,2], Masakazu Toi*[1,3], Hitoshi Tsuda*[1,4], Masafumi Kurosumi*[1,5], and Futoshi Akiyama*[1,6]

*[1]*Japan Breast Cancer Research Group,* *[2]*Department of Surgery, Showa University Toyosu Hospital,* *[3]*Division of Clinical Trial and Research, Tokyo Metropolitan Cancer and Infectious Disease Center, Komagome Hospital,* *[4]*Department of Pathology II, National Defense Medical College,* *[5]*Department of Pathology, Saitama Cancer Center,* *[6]*Department of Breast Pathology, Cancer Institute, Japan.*

Background: Emerging evidence suggests that induction of pathologic complete response (pCR) after primary systemic therapy (PST) is, at least to some extent, predictive of survival. However, standards for processing surgical specimens and for histopathologic evaluation of the pathologic response to therapy appear to be lacking.

Methods: To perform a systematic review of representative articles on this topic, a computerized (MEDLINE) search was undertaken followed by a manual search based on the reference lists of the publications identified.

Results: Several classification systems have been used to assess pathologic response to PST, the term pCR has not been applied in a consistent standardized manner, and only limited information is available about the reliability and validity of these classification systems. However, definitions of pCR can be summarized as follows: near pCR, only focal invasive tumor residues in the removed breast; quasi pCR, total or near total disappearance of invasive tumor in the removed breast; pCRinv, only *in situ* tumor residual in the removed breast; comprehensive pCR, no evidence of residual invasive tumor in the removed breast; strict pCR, disappearance of all tumor cells in the removed breast; comprehensive pCR_{br+n}, no evidence of residual invasive tumor in the breast and axillary nodes; strict pCR_{br+n}, no malignant tumor cells in the removed breast and axillary nodes. Comparison of the use of the term "pCR" in various trials reveals that it is not applied equivalently in these studies.

Conclusion: Assessment of pCR needs to be standardized, with verification for reliability and validity. For now, the non-equivalency in the definition of pCR should be taken into account when comparing the results of PST.

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Key words: Primary systemic therapy, Neoadjuvant chemotherapy, Preoperative chemotherapy, Pathologic complete response, Breast cancer

Primary systemic therapy (PST) means the first post-diagnosis systemic treatment that a patient receives, and it is a standard care to attempt to achieve tumor reduction in case of inoperable, locally advanced breast cancer, thus facilitating mastectomy or irradiation[1,2]. Furthermore, the role of PST in the treatment of operable breast cancer is currently under intensive investigation in the hope of allowing greater conservation of the breast[1-6]. Emerging evidence suggests that induction of a pathologic complete response (pCR) is, at least to some extent, predictive of long-term clinical response[7-12]. If this correlation can be confirmed in ongoing randomized controlled trials, pCR could be used as a surrogate end-point to substitute for survival, possibly eliminating the need for the arduous process of large randomized trials. A current problem, however,

Reprint requests to Katsumasa Kuroi, Department of Surgery, Showa University Toyosu Hospital, 4-1-18 Toyosu, Koutou-ku, Tokyo, 135-8577, Japan.
E-mail: kurochan@dd.iij4u.or.jp

Abbreviations:
BCT, Breast conserving therapy; DFS, Disease-free survival; NSABP, National surgical Adjuvant Breast and Bowel Project; PST, Primary systemic therapy; OS, Overall survival; pCR, Pathologic complete response

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appears to be the lack of standards for tumor processing and for evaluating pathologic response. This review highlights several issues in specimen processing and histopathologic evaluation of PST, especially in the setting of preoperative chemotherapy. To allow a systematic review of representative articles on this topic, a computerized (MEDLINE) search was performed, followed by a manual search based on the reference lists of the publications identified.

Histopathologic Changes in Breast Cancer Tissue and Lymph Nodes

Several studies of PST have identified a number of histopathologic changes in the tumor and in adjacent breast tissues[13-20]. For example, PST frequently results in nuclear and cytoplasmic changes in residual tumor cells and atrophy in the surrounding breast tissue. Nuclear changes consist of prominent nucleoli, nuclear membrane irregularities, chromatin clumping including pyknosis, and karyorrhexis. Moreover, enlargement and vacuolization of the cytoplasm, formation of multinuclear bizarre giant cells, and phagocytosis are often seen. In tumors that display regression, the original tumor consists largely of moderately cellular and loosely collagenized connective tissue, whereas tumors without regression lack the characteristic fibrous replacement and cytologic changes. In particular, sites of pCR are characterized by prominent fibrotic scar tissue with patchy lymphocytic infiltration, groups of foam cells, and lack of glandular tissue and increased vascularity, which is quite different in nature from that seen in normal breast tissues[2]. These changes are not specific to particular agents, and cytomorphological changes can be variable among tumors.

On the other hand, invasive breast carcinoma is composed of invasive and noninvasive components in varying proportions, and these two components can show different histopathological responses. For example, Sharkey *et al.*[13] examined pathological specimens from 43 patients enrolled in the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-18 protocol and found unusually prominent intraductal and/or intralymphatic tumor in 40% of patients treated with doxorubicin and cyclophosphamide, suggesting that these tumors may persist, even after the disappearance of an invasive component because of relative treatment resistance. Wu *et al.*[21] also found similar results.

At the same time, chemotherapy has some effect on non-neoplastic tissues, and in breast tissue the epithelium of the terminal duct-lobular unit is the most severely affected by chemotherapy and radiation[14]. The morphological alterations seen, such as an increase in nuclear-cytoplasmic ratio, nuclear pleomorphism, clumping of chromatin, and the presence of prominent, multiple and irregular nucleoli, may cause confusion with other breast lesions such as lobular cancerization. Therefore, to ensure appropriate examination of the surgical specimen, pathologists should be informed about whether PST was used. The presence of mixed atypical and normal cells, a lack of necrosis, and retention of a continuous layer of myoepithelial cells are useful criteria in avoiding a misdiagnosis of malignancy[14].

In addition, after PST lymph nodes usually display considerable lymphoid depletion and relative expansion of sinusoids, and fibrosis and hyalinization are also prominent in the subcapsular region of axillary lymph nodes[13, 14]. In general, metastatic deposits are commonly found in or around these hyalinized areas, and the cytopathic changes observed in metastatic cells in lymph nodes are similar to those present in residual tumor cells in the breast[14, 20].

Definition of pCR

So far, several classification systems have been used to assess the pathologic response to PST[1, 7, 15, 22-27] (Table 1). However, the term pCR has not always been applied in a consistent, standardized manner[3, 28]. Some systems include references to macroscopic features, extent of residual tumor, and nodal status, or ignore the findings for axillary lymph nodes. Importantly, the absence of a gross lesion does not necessarily indicate a complete response; residual microscopic disease can be found in up to 60% of cases[29]. The fundamental manifestation of the effect of PST is the disappearance of tumor cells, and therefore evaluation of pathologic response should be based on microscopic findings.

Under these circumstances, an international expert panel has defined pCR as follows: pCR, no invasive or *in situ* tumor cells in the removed breast tissues; "pCR inv" (here referred to as pCR_{inv}), only *in situ* tumor residuals in the removed breast tissues; "pCR breast + nodes" (here referred to as pCR_{br+n}), no malignant tumor cells in removed breast and lymph nodes, respec-

Table 1. Classification Systems of Pathologic Response

Shinn's classification[22]		
Grade 0	No effect	
Grade 1	Resorption and tumor sclerosis	
Grade 2	Minimal focally invasive residues of 5 mm or smaller	
Grade 3	Only noninvasive tumor residues	
Grade 4	No viable tumor cell detectable	
Honcoop's classification[23]		
PCR	No residual tumor cells in breast or axillary lymph node	
MPR	Normal macroscopic examination but microscopic evidence of scattered foci of tumor	
MRD	PCR or MPR	
GRD	Tumor observed macroscopically	
Miller and Payne grading system[15]		
Primary site response		
Grade 1	Some alteration to individual malignant cells but no reduction in overall numbers as compared with the pretreatment core biopsy	
Grade 2	A minor loss of invasive tumor cells but overall cellularity still high	
Grade 3	A moderate reduction in tumor cells up to an estimated 90% loss	
Grade 4	A marked disappearance of invasive tumor cells such that only small clusters of widely dispersed cells could be detected	
Grade 5	No invasive tumor, ie, only in-situ disease or tumor stroma remained	
Axillary LN response (proposed)		
N-A	True axillary LN negative	
N-B	Axillary LN positive and no therapeutic effects	
N-C	Axillary LN positive but evidence of partial pathologic response	
N-D	Initially axillary LN positive but converted to LN negative after PST	
Chevallier's classification[24]		
Grade 1	Disappearance of all tumor either on macroscopic or microscopic assessment	
Grade 2	Presence of in situ carcinoma in the breast, invasive tumor and no tumor found in the lymph nodes	
Grade 3	Presence of invasive carcinoma with stromal alteration, such as sclerosis or fibrosis	
Grade 4	No or few modification of the tumoral appearance	
NSABP's classification[7, 38, 39]		
pCR	No histological evidence of invasive tumor cells	
pINV	Histological evidence of invasive disease of any extent	
International consensus panel's classification[1]		
pCR inv	Only in situ tumor residuals in the removed breast tissue	
pCR	No invasive or in situ tumor cells in the removed breast tissue	
pCR breast + nodes	No malignant tumor cells in removed breast and lymph nodes	
Sataloff's classification[25]		
Primary site response		
T-A	Total or near total therapeutic effect	
T-B	Subjectively greater than 50 percent therapeutic effect but less total or near total	
T-C	Less than 50 percent therapeutic effect, but effect evident	
T-D	No therapeutic effect	
Axillary lymph node response		
N-A	Evidence of therapeutic effect, no metastatic disease	
N-B	No nodal metastasis or therapeutic effect	
N-C	Evidence of therapeutic effect but nodal metastasis still present	
N-D	Viable metastatic disease, no therapeutic effect	
Response criteria of Japanese Breast Cancer Society[26, 27]		
Grade 0	No response	Almost no change in cancer cells after treatment
Grade 1	Slight response	
Grade 1a	Mild response	Mild changes in cancer cell regardless of the area, or marked changes in cancer cell seen in less than one third of cancer cells
Grade 1b	Moderate response	Marked changes in one third or more but less two thirds of tumor cells
Grade 2	Marked response	Marked changes in two thirds or more of tumor cells
Grade 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 is necessary

Abbreviations: GRD, gross residual disease; MRD, minimal residual disease; LN, lymph node; pCR, pathologic complete response.

Table 2. Category of pCRs

Category	Definition
Near pCR	Near total disappearance of invasive tumor in the removed breast (only focal invasive tumor residues in the removed breast tissue)
Quasi pCR (QpCR)	Total or near total near total disappearance of invasive tumor in the removed breast
Comprehensive pCR (CpCR)	No evidence of residual invasive tumor in the removed breast
Strict pCR (SpCR)	Disappearance of all tumor cells in the removed breast tissue
pCR_{inv}	Only in situ tumor residuals in the removed breast
Comprehensive pCR_{br+n} ($CpCR_{br+n}$)	No evidence of residual invasive tumor in the breast and axillary nodes
Strict pCR_{br+n} ($SpCR_{br+n}$)	No malignant tumor cells in the removed breast and axillary nodes

tively[1]. Grade 4 in Shinn's classification and grade 3 of the Japanese Breast Cancer Society (JBCS)' classification are equivalent to this "pCR", and this category is here defined as "strict pCR" (SpCR). In this manner, the definition of pCR can be extended as follows: "near pCR", only focal invasive tumor residues in the removed breast tissue; "quasi pCR" (QpCR), total or near total disappearance of invasive tumor in the removed breast; "comprehensive pCR" (CpCR), no evidence of residual invasive tumor in the removed breast; "comprehensive pCR_{br+n}" ($CpCR_{br+n}$), no evidence of residual invasive tumor in the breast and axillary lymph nodes; "strict pCR_{br+n}" ($SpCR_{br+n}$), no malignant tumor cells in the removed breast and axillary lymph nodes (Table 2). By definition, SpCR is the most vigorous response in the breast, and $SpCR_{br+n}$ represents the ultimate response to PST.

Although all of these categories have been grouped together as pCR, a comparison of trials shows that SpCR rates are usually lower than CpCR rates, and that QpCR rates are the highest whereas $SpCR_{br+n}$ rates are the lowest (Table 3). The order of pCR rates is as follows: QpCR > CpCR > SpCR > pCR_{inv}, CpCR > $CpCR_{br+n}$, SpCR > $SpCR_{br+n}$, $CpCR_{br+n}$ > $SpCR_{br+n}$. Thus, the term "pCR" is not equivalent among studies, and when comparing the results of PST we should take into account not only the regimen used and the target populations or tumors, but also the equivalency of the definition of pathologic response. On the other hand, not surprisingly, inclusion of intraductal carcinoma in a trial would not only lead to over-treatment but also result in an artificial increase in pCR_{inv}, CpCR or $CpCR_{br+n}$ rates. Therefore, a diagnosis of invasive cancer should be established before PST is considered.

Handling Methods for Surgical Specimens

An additional issue appears to be lack of standardization for handling surgical specimens. For example, in the study by Matsuo *et al.*[30], the surgical specimen was sliced into tissues of 5 to 6 mm width, at least 30 sections were made for each case, and three pathologists independently examined all the sections and reached a consensus on the pathological evaluation. Similarly, Kuerer *et al.*[9] performed complete sectioning of the breast when mastectomy was performed, and an average of 50 breast sections were examined per mastectomy specimen in the initial 100 patients. As an alternative to complete sectioning, Chevallier *et al.*[24] assessed at least six sections per mastectomy specimen, including at least one from each quadrant, one from the nipple, and one from the areolar and retroareolar area. Similarly, Sataloff *et al.*[25] took multiple sections from the biopsy site and additional random sections of the nipple and subareolar area and the four quadrants. On the other hand, in the study of Semiglazov *et al.*[31], the tumor area was determined according to mammographic data and from the marking of the mastectomy specimen performed by the surgeon during the macroscopic examination. Similarly, in the study of Smith *et al.*[10] sections were taken from the area of fibrosis, targeted by radiologic and clinical information of the original palpable site when no lesion was obvious macroscopically.

Conceptually, an evaluation of pCR based on complete sectioning of the surgical specimen is of a different quality from an evaluation derived from a few sections taken from the representative site (Fig 1). Moreover, if the tumor shrinks to form a honeycomb-like pattern[32], it is possible that the pCR rate will decrease if a greater number of cut sections is examined. Therefore, the international expert panel has recently recommended system-

Table 3. Summary of pCR Rates after PST (Studies Involving 100 or more Patients)

Author, year (Group)	No. of patients	Tumor, node	Preoperative treatment	cCR (%)	cPR (%)	pCR (%)					
						QpCR	CpCR	SpCR	pCR_{inv}	$CpCR_{br+n}$	$SpCR_{br+n}$
Randomized controlled trials comparing PST vs AST											
Fisher[7,39] 1997, 1998 (NSABP B-18)	760	T1-3, N0-1	AC × 4	36	43	–	13*	9*	4	–	–
Makris[48] 1998 (Royal Marsden)	157	T0-3, N0-1	3MT, 2MT × 4	22	61	–	13.4	6.7	6.7	–	–
van der Hage[49] 2001 (EORTC10902)	350	T1c-4d, N0-1	FEC × 4	6.6	42.3	–	4	–	–	–	–
Randomized controlled trials comparing different regimens											
Semiglazov[31] 1994	271	Stage IIB-IIIA	TMF × 1-2 + Radiation to breast and axilla	12.4	56.9	–	–	–	–	–	29.1
			Radiation to breast and axilla	5.9	51.4	–	–	–	–	–	19.4
Buzdar[50] 1999	174	T1-3, N0-1	FAC × 4	24	55	–	17	12	5	–	–
			TXL × 4	27	53	–	8	1	7	–	–
von Minckwitz[51] 2001 (Gepardo)	250	T2-3, N0-2	dose dense A + TXT + G-CSF × 4 (ADoc)	28.9	52.4	25.9	12.1	9.7	2.4	–	–
			dose dense A + TXT + G-CSF × 4 + TAM (ADocT)	28.9	52.4	25.9	12.1	9.7	2.4	–	–
Jackisch[52] 2002 (GeparDUO)	378	T2-3, N0-2	dose dense A + TXT × 4 + G-CSF (dose dense AT)	–	–	–	–	14.8	–	–	–
			AC × 4 → TXT × 4 (AC-DOC)	–	–	–	–	14.8	–	–	–
Smith[10] 2002 (Aberdeen)	104	T2-4, N0-2	CVAP × 8 (responders†)	33	31	–	15.4	–	–	–	–
			CVAP × 4 → TXT × 4 (responders†)	56	29	–	30.8	–	–	–	–
			CVAP × 4 → TXT × 4 (nonresponders†)	11	36	–	1.8	–	–	–	–
Bear[38] 2003 (NSABP B-27)	2411	T1-3, N0-1	AC × 4 (Group I)	40.2	45.4	–	12.9	9.2	3.7	–	–
			AC × 4 → TXT_{100} q3w × 4 (Group II)	63.6	27.1	–	26.1	18.9	7.2	21.8	–
			AC × 4 + TXT as AST (Group III)	40	45.4	–	14.5	10.1	4.4	4.1	–
–			AC × 4 (Group I + III)	40.1	45.4	–	13.7	9.6	4.1	11.5	–
Diéras[12] 2004	200	T2-3, N0-1	ATXL × 4	15	74	(23‡)	–	–	–	16 (8‡)	12
			ACq3w × 4	7	63	(9‡)	–	–	–	10 (6‡)	6
Prospective studies											
Schwartz[53] 1994	189	Stage IIB, III	CMF × 3 → CMF/CAF	–	–	–	17	10	7	–	–
Bonadonna[54] 1998	536	T ≧ 2.5 cm	CMF, Anthracycline × 3, 4	16	60	–	–	2.6	–	–	2.4
Kuerer[55] 1998	156	Stage IIA (T ≧ 4 cm), IIB-IV§	FAC × 4	12	63	–	–	19	–	–	12
Kuerer[8] 1998	165	Stage IIA (T ≧ 4 cm), IIB-IV§	FAC × 4	12	–	–	–	20	–	–	–
Machiavelli[43] 1998	140	Stage IIIA, IIIB	FAC × 3	8	63	18	9	6	–	–	–
Kuerer[9] 1999	372	Stage II (T ≧ 4 cm)-IV§	FAC × 4	–	–	–	16	–	12	–	–
Rouzier[44] 2002	152	T1-3, node positive	FAC × 3, 4	–	–	19.1	–	–	–	(9.2**, 2.6††) 5.9	–
Barni[56] 2004	100	T ≧ 3 cm	E × 3	7	54	10	–	–	2	–	–
Ezzat[57] 2004	126	T2 (> 4 cm), T3, 4, N0-3	TXL + cisplatin × 3, 4	28	63	31	24	–	–	–	–

Abbreviations: 2MT, mitoxantrone, methotrexate and tamoxifen; 3MT, 2MT and mitomycin; A, doxorubicin; AST, adjuvant systemic therapy; C, cyclophosphamide; cPR, clinical partial response; cCR, clinical complete response; E, epirubicin; F, 5-fluorouracil; G-CSF, granulocyte colony stimulating factor; M, methotrexate; P, predonisolone; TXL, paclitaxel; TXT, docetaxel, V, vincristine; TMF, thiotepa, methotrexate and F.

* pCR was assessed in patients who achieved clinical CR.

† Response to first four cycle of CVAP.

‡ pCR rate assessed by central review committee.

§ Stege IV with ipsilateral supraclavicular lymph node involvement only was included.

** QpCR without lymph node metastasis.

†† pCR_{inv} without lymph node metastasis.

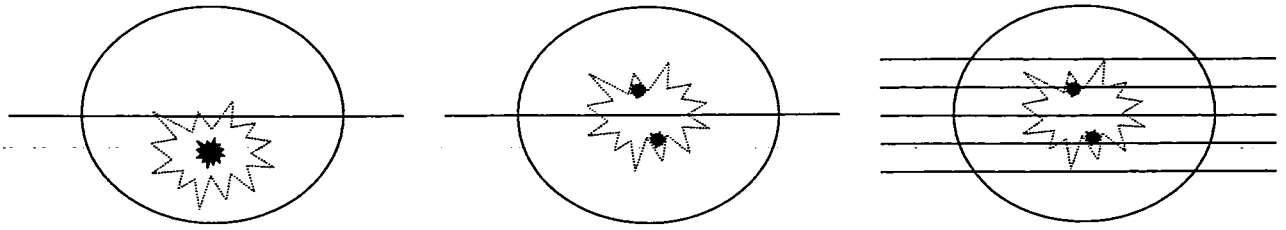

Fig 1. Pitfalls of handling methods of surgical specimens and pCR evaluation. There are two patterns of shrinkage after chemotherapy; concentric and honeycomb-like. If the tumor-bed deviates from the center of the breast specimen, a sections taken from representative site may fail to contain residual tumor foci (a), pathologic examination at representative section may also miss the scattered residual tumor foci even if the tumor-bed is properly resected (b), whereas examination of multiple specimens helps ensure accurate assessment of pathologic response to therapy (c).
Tumor-bed Residual tumor — Section

atic sectioning perpendicular to the long axis of the specimen, instead of random sections or the so-called orange-peel technique[1]. Similarly, the committee for histopathological criteria of the JBCS has recommended the examination of multiple specimens, especially for assessment of a grade 3 (SpCR) response[26, 27]. As the main aim of PST is total eradication of tumor in both breast and axillary lymph nodes and at sites of micrometastasis, it is mandatory for the pathologist to make every effort to find residual tumor. If it is not found on initial sampling, more extensive examination should be performed to confirm pCR. Importantly, pathological evidence of a tumor bed (pre-PST tumor site) characterized by abnormal fibroblastic breast stroma that is devoid of normal lobular units and contains foamy macrophages and a moderate number of fibroblasts and other mononuclear inflammatory cells, as described above, is mandatory for assessment of pCR. The presence of nondescript collagenized lobules or breast fibrous tissue is not considered satisfactory evidence that the tumor site has been adequately sampled[10].

Tumor Localization for Adequate Resection and Evaluation of the Response

As most tumors respond well to PST, accurate determination of the surgical margin becomes difficult, especially in breast-conserving therapy (BCT). As the aim of surgery after PST is to remove all residual foci that are clinically evident and achieve negative margins, precise documentation of tumor localization with a sketch or photographs before initiation of PST is recommended[1]. It is also useful to mark the localization of the tumor with an externally visible or radio-opaque marker. In addition, preoperative breast imaging methods such as high-resolution MRI have an important role in assessing the extent of the residual tumor and its shrinkage pattern[33, 34]. If the resected area in BCT and the sections from the specimen are not appropriate, pathologic examination of the response is itself irrelevant, and there appears to be some doubt about the safety of BCT.

On the other hand, in a situation where true pCR is achieved by PST, it might be possible to avoid definitive surgery. Currently, however, it is imperative to resect any potentially cancerous tissue to confirm the pathologic response as the reliability of imaging techniques for evaluating the pathologic response is still limited[20, 35], and the absence of malignant cells from preoperative core needle biopsy samples does not necessarily indicate true pCR. Further studies should be performed to develop reliable markers to predict pathologic response and to enhance the diagnostic accuracy of imaging. Whether a subgroup of patients could be optimally treated with less invasive modalities such as radiation or ablation therapy without surgery remains to be demonstrated by further studies.

Reliability and Accuracy of Classification Systems

So far, there is little information on the reliability of the pathologic evaluation of response after PST[25, 30, 36]. In this respect, it is interesting to note that Matsuo *et al.*[30] have evaluated the pathologic response using several classification systems, including those of the JBCS's, Chevallier, and Honkoop, and so confirmed the accuracy of the JBCS classification. This finding is in line with another study showing comparable results in pathologic responses assessed by Chevallier's and Sataloff's classifications[37]. However, no study has

so far provided in-depth data about inter- or intra-observer variability. To our best knowledge, only the retrospective study of Sataloff *et al.*[25] has described excellent inter-observer concordance between two independent pathologists. In some studies of PST, bias was avoided by the evaluation of pathologic responses by independent pathologists without knowledge of the clinical outcome[11, 36, 37]. In contrast, evaluation of pathologic response was performed by the institutional pathologists in NSABP-B18 and B-27[7, 38, 39].

To address the issues of inter-observer variability or reproducibility between different centers when assessing pathologic response after PST, it appears reasonable to establish a central pathology review committee consisting of experienced breast pathologists. In this respect, it is of particular interest to note that in the study by Dieras *et al.*[12] pCR rates based on central review were lower than those based on institutional evaluation. This observation underscores the importance of verification of the reliability of the pathologic evaluation of response after PST concurrently with validation, in a fashion similar to histologic grading[40, 41].

Validation of Classification Systems

The observation that there is a close correlation between pathologic response of the primary tumor and long-term survival would suggest that the effect of PST on the primary tumor would parallel the effect of PST on distant micrometastases. So far, most studies have adopted CpCR as their definition of pCR, as there are no data to indicate that *in situ* tumor residuals confer a higher risk of distant metastasis or a different long-term outcome than total absence of tumor cells[2], and thus the findings from the axillary lymph nodes have usually been ignored. In this respect, it is interesting to note that in the study of Kuerer *et al.*[9], patients with a $CpCR_{br+n}$ had significantly improved overall survival (OS) and disease-free survival (DFS) compared to those with less than a pCR. In that study, however, patients with $CpCR_{br+n}$ did not have a significant advantage over patients with a pCR in breast or axillary lymph nodes, and thus the investigators concluded that elimination of invasive disease from the breast, the axillary lymph nodes, or both after PST would confer a survival advantage. In some ways, this is in line with the other studies demonstrating that patients without axillary lymph node metastases after PST had improved survival[23, 42-45]. However, as $CpCR_{br+n}$ and $SpCR_{br+n}$ are theoretically more rigorous definitions of pathologic response than QpCR, CpCR or SpCR, further studies are needed to clarify whether pCRs including lymph nodes confer a different long-term outcome compared with pCRs in breast only. Also, it is important to establish the clinical significance of micrometastases or isolated tumor cells in axillary lymph nodes before and after PST in the era of sentinel lymph node biopsy.

On the other hand, there appears to be some concern as to whether or not pCR alone can provide information on prognosis. For example, in the study of Tomczykowski *et al.*[11], pathologic response after PST was subdivided into 4 levels: complete, considerable, slight and no cancer cell damage. Both the complete and considerable response groups showed higher OS and DFS than did the other groups, and distant metastases were detected only in patients with slight or no cancer cell damage, whereas local recurrence was observed regardless of the degree of cell damage. Similarly, Honkoop *et al.*[23] found no significant difference in OS and DFS for patients with SpCR compared with those who had QpCR. In contrast, Amat *et al.*[46] found that both pCR rates and recurrence rates varied according to classification system when using Chevallier's classification restricted to breast and Sataloff's classification. In that study, among patients who presented with recurrences 18.8% had pCR according to the restricted Chevallier's classification (SpCR), whereas 43.8% had pCR according to Satalof's classification (QpCR).

Similarly, Smith *et al.*[15] have reported that grading pathologic response on a 5-point scale using Miller and Payne's classification, rather than simply as complete or incomplete, provides additional prognostic information, suggesting that classification systems employing fewer categories may lose information of prognostic value. In this respect, the JBCS classification employs a 5-grade scale, and residual disease is graded according to the size of the tumor and the extent of the cytotoxic effect of chemotherapy. Also, intraductal components and lymph node metastasis can be evaluated and recorded separately using the same criteria[26, 27].

Moreover, the clinical relevance of the cellular changes observed in residual tumor cells after PST is so far uncertain, and pathologists are not sure whether those cells have the capability to

replicate or the malignant potential to grow and metastasize[20]. Therefore, there appears to be scope for the development of new histologic and molecular approaches to provide information on differential response for tumors in which less than pCR is achieved. Such information could bring us closer to the goal of tailoring therapy to the individual patient.

In addition, at present, no grading classification is designed to evaluate the distribution pattern of residual tumors in resected breast. As the primary tumor can respond in a variety of ways[19,20,32,47], it is important to investigate whether or not the distribution pattern might have prognostic significance. For this, accurate histologic mapping of residual tumors to the macroscopic and radiologic appearance of the tumor bed is necessary. In association with this, Rajan *et al.*[19] have underscored the importance of the assessment of cellularity, and indeed recommend assessment of the product of pathologic size and cellularity for the evaluation of pathologic response to PST. Thus, further studies should investigate whether degree and extent of cell damage as well as the presence of *in situ* tumor cells or minimal focally invasive residues might have additional prognostic significance, or perhaps might predict a different long-term outcome, including loco-regional recurrence and ipsilateral breast tumor recurrence after BCT. It is also important to determine whether increase in pCR can prolong DFS and OS, and whether the clinical correlation of pathologic response with long-term outcome demonstrated for PST holds true for the pathologic response achieved by preoperative radiotherapy with or without PST.

Conclusion

Use of divergent classifications without verification of reliability and validity, and lack of a standardized handling method for surgical specimens, may cause considerable variation between trials, making interpretation and generalization of the results difficult. As appropriate investigation and analysis of pathologic response as an indicator of treatment outcome is extremely important, processing of surgical specimens and histopathologic evaluation need to be standardized and verified for reliability and validity. In most current studies, involvement of the pathologists in the study setting is considered as the norm.

However, in daily practice it may be difficult to fully examine every breast sample in view of pathology laboratory workload and cost. In such a case, examination of a few sections from representative sites appears to be an alternative method. For this, sections should be taken from the obvious lesion, or from a site of fibrosis chosen with care when no obvious lesion is visible. Targeting by radiology or clinical information will help this. At least, the pathologic evidence of tumor-bed is mandatory for assessment of pCR, and more extensive examination should be performed to confirm pCR if residual tumor is not found on initial sampling. Therefore, specimens should be processed systematically to obtain the largest cross section containing the center of the lesion after treatment.

Given the present circumstances, when comparing the results of PST we should take into account several aspects, including the regimen used, the target population, and tumors, and the equivalency of the definition of pCR.

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