Table III. Univariate and multivariate analysis for survival in breast cancer patients (n=42).

Factors	DFS				OS				DFS	OS	DFS	OS
	Uni-variate p-value	Multi-variate p-value	Multi-variate p-value	Multi-variate p-value	Uni-variate p-value	Multi-variate p-value	Multi-variate p-value	Multi-variate p-value	Multi-variate p-value	Multi-variate p-value	Multi-variate p-value	Multi-variate p-value
Pathological nodal metastasis 0-3 vs 4~	NS	0.0423	0.0016	0.0259	NS	NS	0.0311	0.0340	0.0459	NS	0.0016	0.0311
Estrogen receptor Negative vs positive	0.0185	NS	NS	NS	0.0268	NS	NS	NS	NS	NS	NS	NS
HER2 Negative vs positive	0.0163	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Post NAC tumor size T1, 2 vs T3, 4	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Post M30 ≤35 vs >35	0.0009	0.0035	-	-	0.0023	0.0177	-	-	-	-	-	-
Post MIB-1 ≤140 vs >140	0.0014	NS	-	-	0.0090	NS	-	-	-	-	-	-
Post MIB-1/M30 ratio ≤5 vs >5	0.0003	-	-	0.0005	0.0025	-	-	0.0100	-	-	-	-
Pre-post M30 increase[a] ≤35 vs >35	0.0110	-	NS	-	0.0333	-	NS	-	-	-	-	-
Pre-post MIB-1 decrease[a] ≤20 vs >20	0.0001	-	<0.0001	-	0.0112	-	0.0171	-	-	-	-	-
Pre-post M30 increase/ MIB decrease[b] High/high vs others	0.0092	-	-	-	0.0025	-	-	-	0.0212	NS	-	-
Pre-post M30 increase/ MIB decrease[b] Low/low vs others	<0.0001	-	-	-	0.0112	-	-	-	-	-	<0.0001	0.0171

[a]Pre-post M30 increase and pre-post M30 increase were the differences between before and after pre-operative chemotherapy. [b]M30 increase and MIB-1 decrease were divided into low and high categories at 35, 20 respectively. DFS, disease-free survival; OS, overall survival; NS, not significant.

worse prognosis as compared with those with opposite values. In addition, increase of M30 index by the treatment and decrease of MIB-1 index by the treatment also showed potent prognostic significance (p=0.0110, p=0.0001, respectively, log-rank test). The details of univariate analyses are summarized in Table III. Survival curves by Kaplan-Meier method are shown in Fig. 4. A significant difference was demonstrated between post-treatment M30 index high, MIB-1 index low group and others (p=0.0004, log-rank test). Tumors having <5 post-treatment MIB-1/M30 ratio showed favorable prognosis (p=0.0003, log-rank test). Pre-post increase in M30 and pre-post decrease in MIB-1 index were divided into low and high categories. M30 high and MIB-1 low group showed a significantly favorable prognosis as compared with M30 low and MIB-1 high group (p<0.0001, log-rank test). Neither clinical tumor response nor pathological tumor response grade (<2 vs 2) showed any significant prognostic value (data not shown). No pathological tumor response grade 3 was seen in this 42-patient subgroup.

Multivariate analyses also confirmed that post-treatment M30 index, post-treatment MIB-1/M30 ratio and pre-post MIB-1 decrease, as well as post-treatment histological nodal status had an independent prognostic value (Table III). Similar results were obtained between disease-free survival (DFS) and overall-survival (OS).

Growth kinetics and relapse. Scatter diagrams of pre- and post-treatment M30 index and MIB-1 index are shown in Fig. 5. Eleven patients (Fig. 5, colored symbols) showed a growth kinetics with >35 M30 index and <140 MIB-1 index after pre-CT, although one case showed such growth kinetics before pre-CT (Fig. 5, red circle). A dramatic change in tumor growth kinetics revealed by M30 index and MIB-1 index was observed.

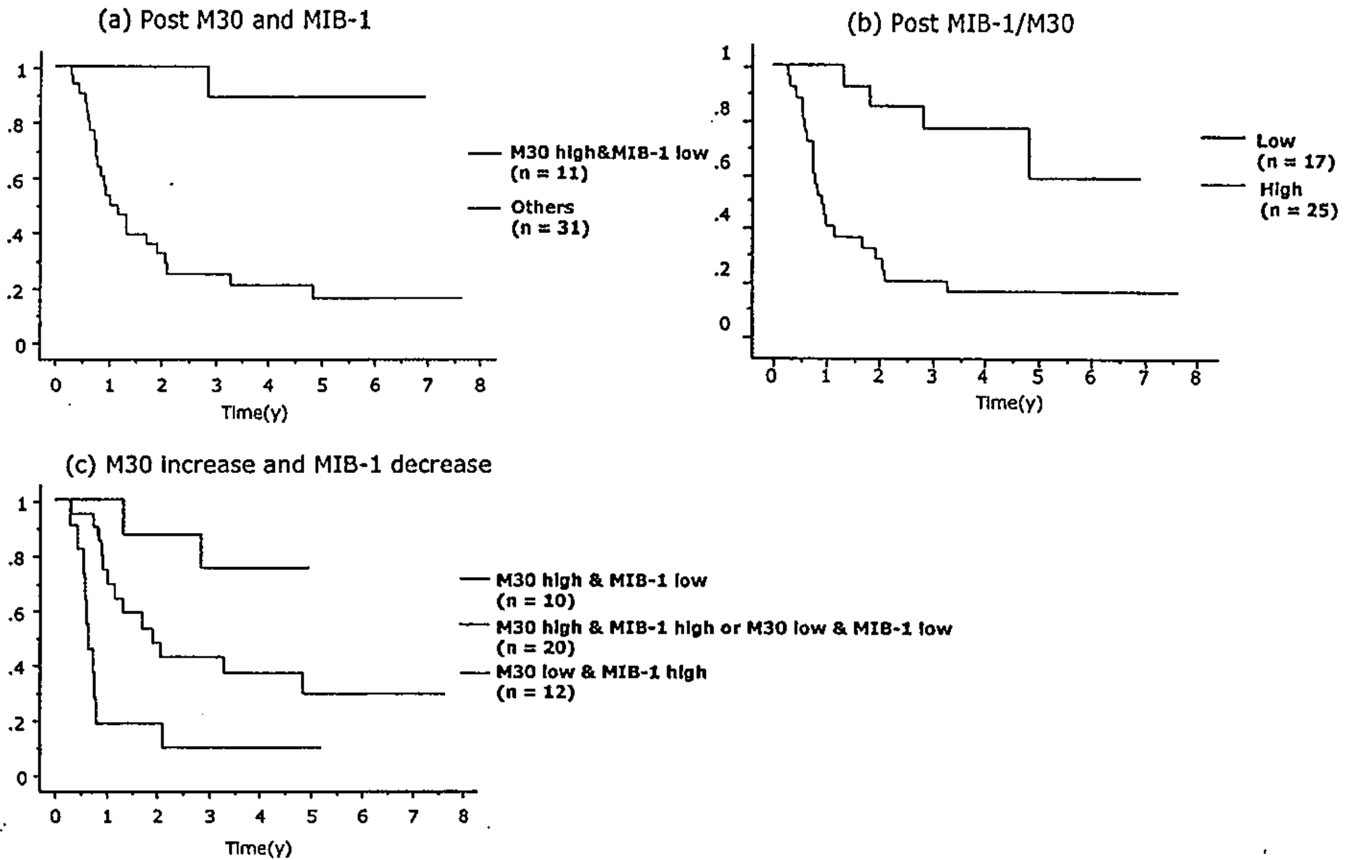

Figure 4. Disease-free survival curves stratified by M30 index and MIB-1 index. (a), Post-treatment M30 index and post-treatment MIB-1index were divided into low and high categories at 35 and 140, respectively. A significant difference was demonstrated between M30 index high, MIB-1 index low and others (p=0.0004, log-rank test). (b), Post-treatment MIB-1/M30 ratio was divided into low and high categories at 5 (p=0.0003, log-rank test). (c), Pre-post increase in M30 index and pre-post decrease in MIB-1 index were divided into low and high categories at 35 and 20, respectively. The survival analyses were conducted in the 3 groups. M30 high and MIB-1 low showed a significantly more favorable prognosis as compared with M30 low and MIB-1 high group (p<0.0001, log-rank test).

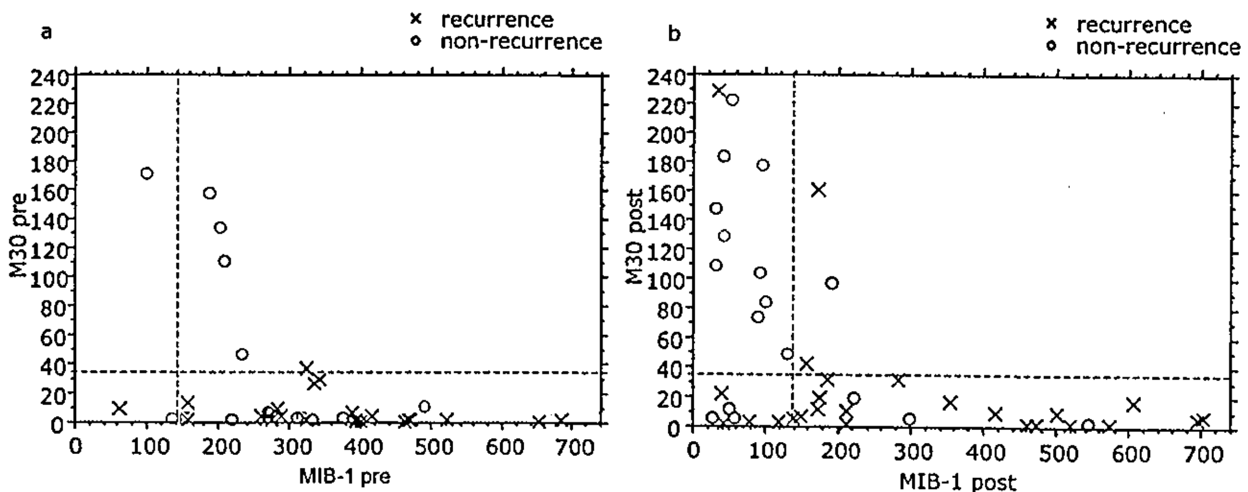

Figure 5. Scatter diagrams of M30 and MIB-1 index. Scatter diagrams of M30 index and MIB-1 index at pre-treatment and post-treatment are shown. Relapse and non-relapse are plotted with different types of marks. Eleven patients (colored legends) showed favorable growth kinetics with >35 M30 index and ≤140 MIB-1 index after pre-CT, although one case showed such growth kinetics before pre-CT (red circle). Difference in colored symbols (red, blue, green and purple) indicates the categories of M30 or MIB-1 index before pre-CT.

Discussion

Several biological markers such as p53 mutation and Her-2 overexpression have been proposed for predicting the efficacy of chemotherapy and for determining the dosages of chemotherapeutic agents, particularly anthracyclines, in adjuvant treatment of primary breast cancer (2,3,14-17) However, controversies still remain for clinical application because of

the cost or for other reasons (18). There is a requirement to develop a novel practical predictive marker for adjuvant chemotherapy of primary breast cancer, especially a surrogate marker for predicting survival benefit from chemotherapy.

Among many apoptosis-related markers, M30 is characterized to detect the relatively early-phase of apoptosis in tumor cells (7-11). According to preliminary analysis comparing M30 immunostainings and the apoptosis assessed by TdT-mediated dUtp-x nick end labeling (TUNEL) method, a heterogeneous concordance in M30 positively stained tumor cells and TUNEL-positive apoptotic cells was found (data not shown). It is suggested that M30 recognizes the different phase of apoptosis from the phase detected by TUNEL analysis. Leers *et al* reported that the generation of CK18NE detected by M30 antibody occurred early in the apoptotic cascade before annexin V reactivity or positive DNA nick end labeling (7). A flow cytometric analysis proved that the majority of M30-positive cells appeared in the sub G1 peak in the cell-cycle. According to these data, an enzyme-linked immunosorbent assay of M30 is utilized for screening of pro-apoptotic drugs against cancer (9). Thereby, it was considered that tumor growth kinetics study using CK18NE, presented as the M30 index, would provide different clinical implication from others (19,20).

Pre-CT with an anthracycline-based regimen changed the M30 index or MIB-1 index dramatically in more than half of the examined cases. In particular, in pathological response grade 2 cases, M30 index increased remarkably and MIB-1 index decreased significantly. Interestingly, there was no significant correlation between the changes in these indexes and clinical tumor response. Since there is an agreement that clinical tumor response to pre-CT does not necessarily correlate with prognosis, but histological response does (5), the growth kinetics displayed by M30 and MIB-1 should be useful for predicting survival.

In the present study, survival analyses were performed in a subgroup of 42 patients who underwent FAC chemotherapy alone. Although the number of examined cases was small, the subgroup was thought to be appropriate to assess the pure effect of chemotherapy on survival, because full-dose of FAC therapy was given for all the patients pre- and post-operatively. Surprisingly, post-treatment M30 index and post-treatment M30/MIB-1 ratio (<5) showed a potent prognostic significance. Very few patient recurred in the group of patients who had a high post-treatment M30 index (>35). The changes in M30 index and MIB-1 index by the treatment also exhibited a significant prognostic value. The patients whose M30 index did not increase and MIB-1 index did not decrease significantly showed an extremely poor prognosis.

These data indicate several important points to consider. First of all, the analysis on the alteration of tumor growth kinetics using M30 index might be useful for identifying the particular subgroup that benefits from chemotherapy among non-pCR patients. There is a consensus that pCR patients after pre-CT showed a significantly favorable prognosis as compared with non-pCR patients. However, regarding literature-based research, few reports have focused on the identification of susceptible patients for chemotherapy among non-pCR patients. The method and criteria for assessment used in the present study might be useful for this purpose, although this strategy should be tested and confirmed in a large-scale clinical trial. In particular, there might be a criticism that the subgroup that showed favorable tumor growth kinetics after pre-CT, such as >35 M30 index and <140 MIB-1 index, might be potential candidates of pCR patients, because the dosages of anthracycline we used in this study were lower than the dosages commonly used in the US and Europe, and pre-operative treatment cycles might be insufficient to achieve pCR. However, even so, it is true that the changes in growth kinetics after the exposure of 2 or 4 cycles of chemotherapy result in extremely favorable prognosis. The shift of pattern in the scattered diagram of M30 index and MIB-1 index between pre- and post-treatment strongly suggests that this change is elicited by pre-CT. Secondly, these data raise an issue regarding the switch of the treatment. According to our findings, if the patients do not show favorable growth kinetics after the first pre-CT, the therapy should be switched to other non-cross resistant regimens or different types of treatments, because the prognosis is extremely poor in such cases. This is speculation from the result of this study, however, a recent report has demonstrated clearly that a sequential combination of 4 cycles of anthracycline-based regimen and 4 cycles of docetaxel increased pCR rate in about double and improved the survival as compared with the 8-cycle anthracycline-based regimen alone, even for the responders to the initial treatment of anthracycline (21). Growth kinetics should be taken into consideration in deciding treatment-switch in pre-CT. Thirdly, in common practice, we obtain the tumor sample prior to starting the pre-CT by core-needle biopsy (CNB) rather than open biopsy. In order to assess the pre-treatment tumor growth kinetics precisely, it may remain a problem in cases of CNB because of the heterogeneity issue. We need more sophisticated methods or techniques. Nevertheless, for the purpose of predicting survival, to determine the post-treatment status of tumor growth kinetics might be adequate. Presumably, post-treatment MIB-1 status would represent a combined value of 'original prognostic value' and 'therapeutic prognostic value', however post-treatment M30 status would represent more 'therapeutic prognostic value' (22-27).

There are a number of approaches to analyze and monitor chemotherapy-induced apoptosis. Expressions of Bcl-2 family and those changes in the expression or phosphorylation by chemotherapy have been examined (20,28-31). These approaches have also been utilized to identify the susceptible subgroup to endocrine therapy as well (32). For instance, Parton *et al* (20) focused upon DNA fragmentation factor (DFF)-40 cleaved by caspases and documented that the level of DFF40 that emerges after 24-h exposure to chemotherapy correlated significantly with that of active caspase 3 and caspase 6, indicating that DFF40 is a promising molecule for assessing chemotherapy-induced apoptosis. This direction of research, as well as the research on chemo-resistance and on the selection of chemo-unnecessary cases would be crucial to advance pre-CT (33).

A variety of pre-operative combination chemotherapies are being tested currently in order to achieve further survival advantages. To maximize the efforts for demonstrating the efficacy of new treatment modalities, adjuvant trials are shifted from post-operative to pre-operative, because pCR can be a surrogate marker of long-term survival outcome in

case of pre-CT. This study indicated that the analysis of tumor growth kinetics with CK18NE/M30 index, in conjunction with MIB-1 index, might be useful as a surrogate marker for predicting survival benefits from chemotherapy in non-pCR patients. It is warranted to confirm its clinical usefulness in the larger collaboration studies.

Acknowledgements

The authors would like to thank Ms. Yumiko Takenaka, Ms. Makiko Hirata, and Ms. Taeko Ogawa for their excellent assistance.

References

1. Early Breast Cancer Trialists' Collaborative Group: Multi-agent chemotherapy for early breast cancer. Cochrane Database Syst Rev 1: CD000487, 2002.
2. Aas T, Borresen AL, Geisler S, *et al*: Specific P53 mutations are associated with *de novo* resistance to doxorubicin in breast cancer patients. Nat Med 2: 811-814, 1996.
3. Geisler T, Lonning PE, Aas T, *et al*: Influence of TP53 gene alterations and c-erbB-2 expression on the response to treatment with doxorubicin in locally advanced breast cancer. Cancer Res 61: 2505-2512, 2001.
4. Fisher B, Bryant J, Wolmark N, *et al*: Effect of preoperative chemotherapy on the outcome of women with operable breast cancer. J Clin Oncol 16: 2672-2685, 1998.
5. Wolmark N, Wang J, Mamounas E, Bryant J and Fisher B: Preoperative chemotherapy in patients with operable breast cancer: nine-year results from National Surgical Adjuvant Breast and Bowel Project B-18. J Natl Cancer Inst Monogr 30: 96-102, 2001.
6. Smith IE and Lipton L: Preoperative/neoadjuvant medical therapy for early breast cancer. Lancet Oncol 2: 561-70, 2001.
7. Leers MP, Kolgen W, Bjorklund V, *et al*: Immunocytochemical detection and mapping of a cytokeratin 18 neo-epitope exposed during early apoptosis. J Pathol 187: 567-572, 1999.
8. Mirzaie-Joniani H, Eriksson D, Sheikholvaezin A, *et al*: Apoptosis induced by low-dose and low-dose-rate radiation. Cancer 94: 1210-1214, 2002.
9. Hagg M, Biven K, Ueno T, *et al*: A novel high-through-put assay for screening of pro-apoptotic drugs. Invest New Drugs 20: 253-259, 2002.
10. Mourits MJ, Hollema H, De Vries EG, Ten Hoor KA, Willemse PH and van der Zee AG: Apoptosis and apoptosis-associated parameters in relation to tamoxifen exposure in post-menopausal endometrium. Hum Pathol 33: 341-346, 2002.
11. Ferrandina G, Ranelletti FO, Larocca LM, *et al*: Tamoxifen modulates the expression of Ki-67, apoptosis, and microvessel density in cervical cancer. Clin Cancer Res 7: 2656-2661, 2001.
12. Kataoka A, Sadanaga N, Mimori K, *et al*: Overexpression of HRad17 mRNA in human breast cancer: correlation with lymph node metastasis. Clin Cancer Res 7: 2815-2820, 2001.
13. Toi M, Bando H, Ogawa T, Muta M, Hornig C and Weich HA: Significance of vascular endothelial growth factor (VEGF)/ soluble VEGF receptor-1 relationship in breast cancer. Int J Cancer 98: 14-18, 2002.
14. Kandioler-Eckersberger D, Ludwig C, Rudas M, *et al*: TP53 mutation and p53 overexpression for prediction of response to neoadjuvant treatment in breast cancer patients. Clin Cancer Res 6: 50-56, 2000.
15. Muss HB, Thor AD, Berry DA, *et al*: c-erbB-2 expression and response to adjuvant therapy in women with node-positive early breast cancer. N Engl J Med 330: 1260-1266, 1994.
16. Petit T, Borel C, Ghnassia JP, *et al*: Chemotherapy response of breast cancer depends on HER-2 status and anthracycline dose intensity in the neoadjuvant setting. Clin Cancer Res 7: 1577-1581, 2001.
17. Daidone MG, Veneroni S, Benini E, *et al*: Biological markers as indicators of response to primary and adjuvant chemotherapy in breast cancer. Int J Cancer 84: 580-586, 1999.
18. Sledge GW Jr: Is HER-2/neu a predictor of anthracycline utility? No. J Natl Cancer Inst Monogr 30: 85-87, 2001.
19. Lipponen P, Aaltomaa S, Kosma VM and Syrjanen K: Apoptosis in breast cancer as related to histopathological characteristics and prognosis. Eur J Cancer 30A: 2068-2073, 1994.
20. Parton M, Krajewski S, Smith I, *et al*: Coordinate expression of apoptosis-associated proteins in human breast cancer before and during chemotherapy. Clin Cancer Res 8: 2100-2108, 2002.
21. Smith IC, Heys SD, Hutcheon AW, *et al*: Neoadjuvant chemotherapy in breast cancer: significantly enhanced response with docetaxel. J Clin Oncol 20: 1456-1466, 2002.
22. Hayes DF and Thor AD: c-erbB-2 in breast cancer: development of a clinically useful marker. Semin Oncol 29: 231-245, 2002.
23. Chang J, Ormerod M, Powles TJ, Allred DC, Ashley SE and Dowsett M: Apoptosis and proliferation as predictors of chemotherapy response in patients with breast carcinoma. Cancer 89: 2145-2152, 2000.
24. Cameron DA, Gregory WM, Bowman A, *et al*: Identification of long-term survivors in primary breast cancer by dynamic modelling of tumor response. Br J Cancer 83: 98-103, 2000.
25. Kuerer HM, Newman LA, Smith TL, *et al*: Clinical course of breast cancer patients with complete pathologic primary tumor and axillary lymph node response to doxorubicin-based neoadjuvant chemotherapy. J Clin Oncol 17: 460-469, 1999.
26. Collecchi P, Baldini E, Giannessi P, *et al*: Primary chemotherapy in locally advanced breast cancer (LABC): effects on tumor proliferative activity, bcl-2 expression and the relationship between tumor regression and biological markers. Eur J Cancer 34: 1701-1704, 1998.
27. Shao ZM, Li J, Wu J, *et al*: Neo-adjuvant chemotherapy for operable breast cancer induces apoptosis. Breast Cancer Res Treat 53: 263-269, 1999.
28. Shitashige M, Toi M, Yano T, Shibata M, Matsuo Y and Shibasaki F: Dissociation of Bax from a Bcl-2/Bax heterodimer triggered by phosphorylation of serine 70 of Bcl-2. J Biochem 130: 741-748, 2001.
29. Gasparini G, Barbareschi M, Doglioni C, *et al*: Expression of bcl-2 protein predicts efficacy of adjuvant treatments in operable node-positive breast cancer. Clin Cancer Res 1: 189-198, 1995.
30. Sjostrom J, Blomqvist C, Heikkila P, *et al*: Predictive value of p53, mdm-2, p21, and mib-1 for chemotherapy response in advanced breast cancer. Clin Cancer Res 6: 3103-3110, 2000.
31. Sjostrom J, Blomqvist C, von Boguslawski K, *et al*: The predictive value of bcl-2, bax, bcl-xL, bag-1, fas, and fasL for chemotherapy response in advanced breast cancer. Clin Cancer Res 8: 811-816, 2002.
32. Dowsett M, Harper-Wynne C, Boeddinghaus I, *et al*: HER-2 amplification impedes the antiproliferative effects of hormone therapy in estrogen receptor-positive primary breast cancer. Cancer Res 61: 8452-8458, 2001.
33. Van 't Veer LJ, Dai H, van de Vijver MJ, *et al*: Gene expression profiling predicts clinical outcome of breast cancer. Nature 415: 530-536, 2002.

DFU, a selective COX-2 inhibitor, suppresses MCF-7 xenograft tumor growth in mice

GAKU MATSUMOTO, MOHAMMAD ATIQUR RAHMAN, MARIKO MUTA, TADAHIKO NAKAMURA, HIROKO BANDO, SHIGEHIRA SAJI, KOJI TSURUTA, ATSUTAKE OKAMOTO and MASAKAZU TOI

Department of Surgery, Tokyo Metropolitan Komagome Hospital 3-18-22, Honkomagome, Bunkyo-ku, Tokyo 113-8677, Japan

Received February 19, 2004; Accepted April 20, 2004

Abstract. Cyclooxygenase-2 (COX-2) inhibitors are regarded as potentially important in strategies for cancer treatment. However, the precise mechanisms of these anti-inflammatory drugs as anti-cancer therapy are still unknown. In this study, we examined the effect of DFU both *in vitro* on MCF-7 cell growth, as well as *in vivo* on tumor growth produced by MCF-7 cell injection in mice. DFU has growth inhibitory effects on tumor growth in mice compared to the control group. We examined the tumor tissues for apoptosis and angiogenesis by immunostaining. Apoptosis was detected only in the treatment group. DFU treatment also resulted in the inhibition of angiogenesis, as well as decreased COX-2 expression. Results of this study suggest that inhibitory effects of DFU might be COX-2 dependent.

Introduction

Breast cancer is one of the most rapidly increasing cancers among women worldwide. Not only is the incidence rate increasing, the death rate is increasing as well. Despite enthusiastic efforts in early diagnosis, aggressive surgical treatment and application of additional non-operative modalities, the prognosis is still dismal. The precise implications of etiological factors in the genetic pathway of breast cancer development are not yet fully understood. Accordingly, understanding the mechanisms that control breast cancer cell growth behavior is of great importance to prevent, and to more efficiently control its genesis. The fundamental understanding of tumor biological and molecular behavior is of high validity in the evolution of effective new therapy.

Cyclooxygenases-1 and -2 (COX-1 and COX-2) are the rate-limiting enzymes for production of prostanoids (prostaglandin and thromboxanes) from arachidonic acid. The function of prostaglandins and cyclooxygenase in cancer pathogenesis is unclear. COX-2 has recently been categorized as an immediate early gene associated with inflammation, cellular growth, differentiation, prevention of apoptosis and tumorigenesis (1-3). It has also been reported that COX-2 induces angiogenesis, which is essential for tumor growth (4). COX-2 overexpression has been observed in colon, head and neck, lung, prostate and breast cancer (5-9). Increased prostaglandin production and enhanced release of angiogenic growth factors by COX-2 may induce neovascularization (1). Recently, it has been shown that COX-2 expression is associated with angiogenesis, lymph node metastasis and apoptosis in human breast cancer (9).

Non-steroidal anti-inflammatory drugs (NSAIDs) can efficiently block cyclooxygenase activity and have beneficial properties in colorectal cancer prevention. Recent studies suggest the anti-proliferative as well as anti-apoptotic effect of NSAIDs in different carcinomas. Instead of COX inhibition, NSAIDs also act through a variety of pathways including prostaglandin receptors and peroxisome proliferator-activated receptor-delta (PPAR-δ). Recently, DFU, a selective NSAID has been developed which has COX-2 specificity (10).

DFU is a structurally related analogue to rofecoxib containing a 5,5-dimethyl furanone ring. It is a highly selective and orally active inhibitor of COX-2, with no sign of gastrointestinal ulceration at >200 times the dose for anti-inflammatory, analgesic and anti-pyretic effectiveness (10). In this study we investigate the effect of DFU on xenograft tumor development with human breast cancer MCF-7 cell lines in male SCID mice. DFU demonstrated anti-tumor activity *in vivo* by inducing apoptosis and inhibiting angiogenesis, in a COX-2 dependent manner.

Materials and methods

Cell line and cell culture. The human breast cancer cell line MCF-7 was obtained from the National Cancer Institute (Bethesda, MD, USA). Pancreatic cancer cell line, PK-8 was provided by Dr M. Kobari (Sendai Open Hospital, Sendai, Japan). Cells were maintained in DMEM (Gibco BRL Co. Ltd., NY, USA) supplemented with 10% fetal bovine serum (Gibco BRL), L-glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 μg/ml) in an atmosphere of 5% CO_2. The MCF-7 cell line was COX-2 negative as detected by Western blot analysis.

Correspondence to: Dr Gaku Matsumoto, Department of Surgery, Tokyo Metropolitan Komagome Hospital, 3-18-22, Honkomagome, Bunkyo-ku, Tokyo 113-8677, Japan
E-mail: gaku0508@ruby.ocn.ne.jp

Key words: cyclooxygenase-2, DFU, breast cancer

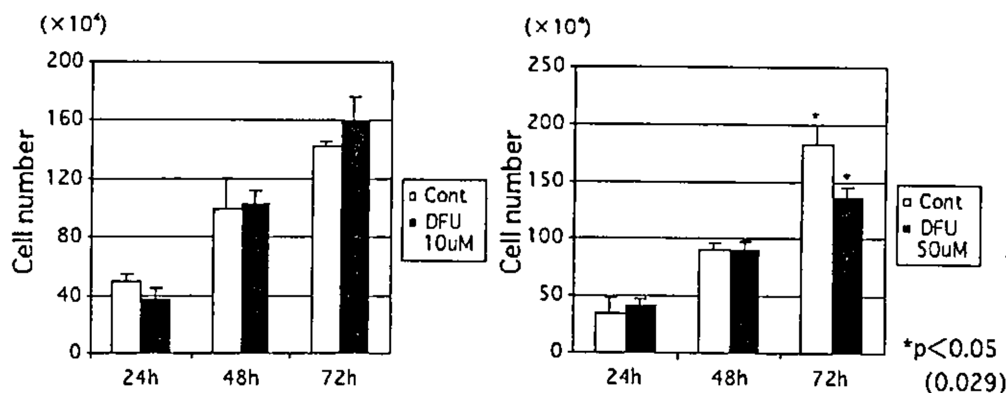

Figure 1. *In vitro* effect of the DFU on cell proliferation of MCF-7 cells. MCF-7 cells cultured for 24, 48 and 72 h in DFU containing medium at 10 and 50 μM concentrations, or DMSO containing medium of the same concentrations were counted in triplicate. Only when MCF-7 cells were cultured for a long period at high drug concentrations, was significant growth inhibition shown. *p=0.029.

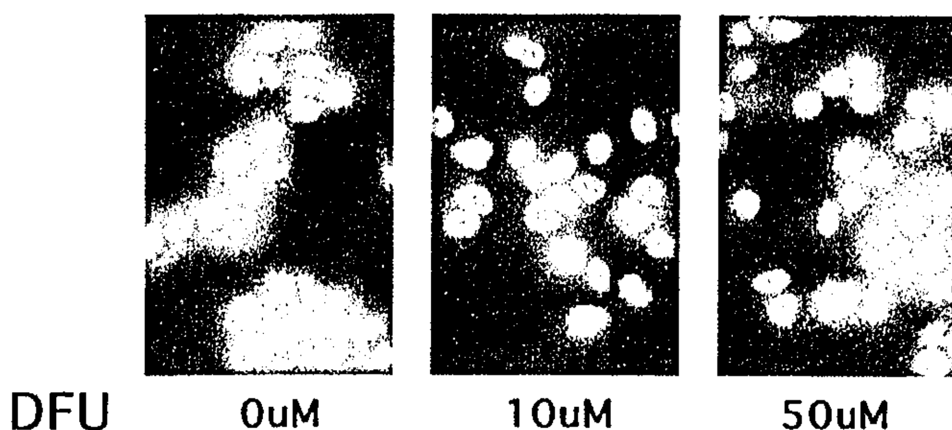

Figure 2. There was no sign of the morphologic markers of apoptosis in different concentrations of DFU treatment as stained by Hochest 33342.

Animals and DFU. Male SCID mice, 5-7 weeks-old were purchased from Nippon Crea, Co. Ltd. (Tokyo, Japan). All animal experiments were approved by the Institutional Animal Care and Use Committee. DFU was dissolved in DMSO (100 mM) as a stock solution for *in vitro* study. For *in vivo* study, DFU was dissolved in 0.5% methylcellulose solution (1 mg/ml).

In vitro assay. The *in vitro* effect of the DFU was assessed by counting the cell number. Approximately 1×10^5 cells/well were cultured in 6-well plates for 24 h. The supernatants were then removed and replaced by the 5 ml medium containing 10 or 50 μM DFU or medium containing DMSO at the same concentrations, in triplicate. After 24, 48 and 72 h culture, cells were removed by trypsin/EDTA and cell numbers were counted manually using trypan blue dye exclusion. Detection of apoptosis in MCF-7 cells by DFU treatment was done by staining with Hochest 33342 (Calbiochem, La Jolla, CA, USA). MCF-7 cells were plated on 6-well plates and cultured overnight. The supernatants were then removed and replaced by the 5 ml medium containing 10 or 50 μM DFU or medium containing DMSO at the same concentrations. After 6 h in culture, the cells were removed from the plate and fixed with 1% glutaraldehyde for 3 h at room temperature. After washing

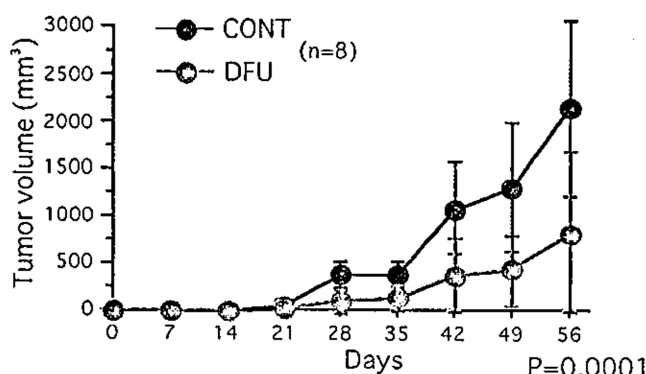

Figure 3. Inhibition of MCF-7 tumor growth by DFU. After subcutaneous inoculation of 1×10^6 of MCF-7 cells (day 0), mice were orally administered 100 μl of 0.5% methylcellulose containing 5 mg/kg DFU (DFU group, n=8), or 100 μl of 0.5% methylcellulose (CONT group, n=8), every day from day 1. The DFU group showed significant growth inhibition of MCF-7 tumors (p=0.0001).

with PBS, cells were suspended in 300 μl of PBS. Two μl of Hochest 33342 solution (1 mM) was added to 10 μl of cell suspension, and this stained cell suspension was plated on the

slide to view with a fluorescence microscope (Nikon, Tokyo). Chromatin condensation, nuclear shrinkage and nucleosomal fragmentation were considered morphologic markers of apoptosis. COX-2 detection was performed by Western blot analysis. The total protein of MCF-7 and PK-8 cells cultured by normal medium or 50 ng/ml TNF-α (Sigma) containing medium for 6 h were prepared, respectively. Briefly, cells were washed with PBS- and lysed with lysis buffer [0.1% SDS, 1% Nonidet P-40, 5 mM EDTA, 0.5% deoxycholate, 150 mM NaCl, 50 mM Tris HCl (pH 8.0), 2 mM DTT, 1 mM $NaVO_3$, protease inhibitor cocktail tablets (Complete mini, Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol followed by incubation at 4˚C for 30 min, and centrifugation at 12000 rpm at 4˚C for 10 min. Supernatants were separated and used as whole cell extracts. Total protein concentrations were determined using albumin as a standard (BCA protein assay kit, Pierce, Rockfold, IL, USA). Denatured samples containing equal amounts of protein (20 µg) were subjected to electrophoresis on an SDS polyacrylamide gel and transferred to a nitrocellulose filter. Placed at 4˚C overnight and then incubated for 1 h with 1:1000 dilution of anti-COX-2 mouse monoclonal antibody (Cayman, Ann Arbor, MI, USA) or 1:10000 dilution of anti-ß-actin mouse antibody (Oncogene, Boston, MA, USA). The filters were then incubated for 1 h with anti-rabbit or anti-mouse secondary antibody (Dako A/G, Glostrup, Denmark), and reactivity was detected by enhanced chemiluminescence system (Amersham Life Science Inc., Little Chalfont, UK).

In vivo tumor growth assay. After 1 week from subcutaneous inoculation of the E_2 pellet, SCID mice were subcutaneously inoculated with approximately $1x10^6$ MCF-7 cells in the back portion of the right flank (day 0). In the DFU group, mice were orally administered 100 µl of 0.5% methylcellulose containing 5 mg/kg DFU every day from day 1 (n=8). In the control group, mice were orally administered 100 µl of 0.5% methylcellulose every day from day 1 (n=8). In both groups, the treatment of oral administration was performed for 8 weeks. Tumor size was determined at the longest and shortest diameters each week. Tumor volume (mm^3) was calculated as described previously (11).

Immunostain detection of COX-2, apoptosis and angiogenesis. MCF-7 tumors with or without DFU treatment of 5 mg/kg/day were resected at the end of 8 weeks treatment. Tumors were immediately frozen by liquid nitrogen, and stored at -80˚C. Frozen samples in OCT compound were sectioned by cryostat to 7-µm thick and mounted on silane-coated glass. The mouse monoclonal antibody M30 cyto-Death (Roche, Basel, Switzerland) against caspase-cleaved neo-epitope of cytokeratin 18, was used to recognize apoptotic cancer cells (12). Tissue sections were treated with M30 for apoptosis (1:200 dilution for 30 min), CD31 (13) for angiogenesis (1:200 dilution for 30 min) and anti-COX-2 (1:300 dilution for 60 min) and incubated at room temperature. Slides were processed using the commercial Elite ABC kit (Vectastain, Vector Laboratories, Burlingame, CA, USA) directed against mouse IgG. Diaminobenzidine was used as the final chromogen, and Meyer's hematoxylin was used for counterstain.

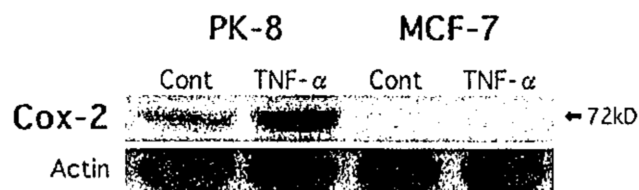

Figure 4. COX-2 protein detection in PK-8 and MCF-7 cell lines by Western blot analysis. The total proteins of MCF-7 and PK-8 cells with or without the stimulation of 50 ng/ml TNF-α for 6 h were prepared, respectively. By using denatured samples containing equal amounts of protein (20 µg), anti-COX-2 mouse monoclonal antibody (1:1000 dilution), and anti-ß-actin mouse antibody (1:10000 dilution), Western blots were performed. COX-2 protein was not detected in MCF-7 cells.

Statistical evaluation. The results of the cell survival assay and VEGF ELISA *in vitro* were analyzed using the Student's t-test. The result of the inhibition potential of DFU for *in vivo* tumor growth was analyzed using Scheffe-F test. Statistical tests were performed using the Stat View software package (Abacus Concepts Inc.), and findings were considered significant when $p<0.05$.

Results

In vitro effect of DFU on MCF-7 cells. DFU did not inhibit the proliferation of MCF-7 cells during 72 h at low drug concentration. DFU significantly inhibited growth when MCF-7 cells were cultured for a long period at a high drug concentration (Fig. 1). Six-hour culture of MCF-7 cells in both low and high concentrations of DFU did not induce apoptosis (Fig. 2).

In vivo effect of DFU on MCF-7 tumors. To evaluate the effect of DFU, xenograft models of human breast cancer were prepared in SDIC mice, and these were randomized to either no DFU treatment (control), or a group treated with DFU. The effect of DFU on tumors was followed every alternate day. In pilot experiments with MCF-7 based xenograft, the mice receiving DFU had a significant decrease in tumor size as compared to the control mice (Fig. 3). Neither vehicle nor DFU treatment produced any gross or histopathological changes in liver, kidney, stomach, intestine or lung attributable to toxicity.

DFU treatment inhibited the expression of COX-2 in the treatment group only. It has been reported, and we also found, that MCF-7 breast cancer cell is COX-2 negative at the protein level (Fig. 4). However, the MCF xenograft expressed that COX-2 and DFU treatment inhibited the expression of COX-2 as compare to the control group (Fig. 5).

DFU treatment induced apoptosis in xenograft tumors. To determine whether the decreased effectiveness of DFU was attributable to programmed cell death, a frozen section of tumor tissue was prepared for immunostaining for apoptosis detection. Apoptosis was then evaluated by staining with anti-M30, a new specific and sensitive marker of apoptosis in epithelial cells. The expression of apoptosis in the DFU treatment group

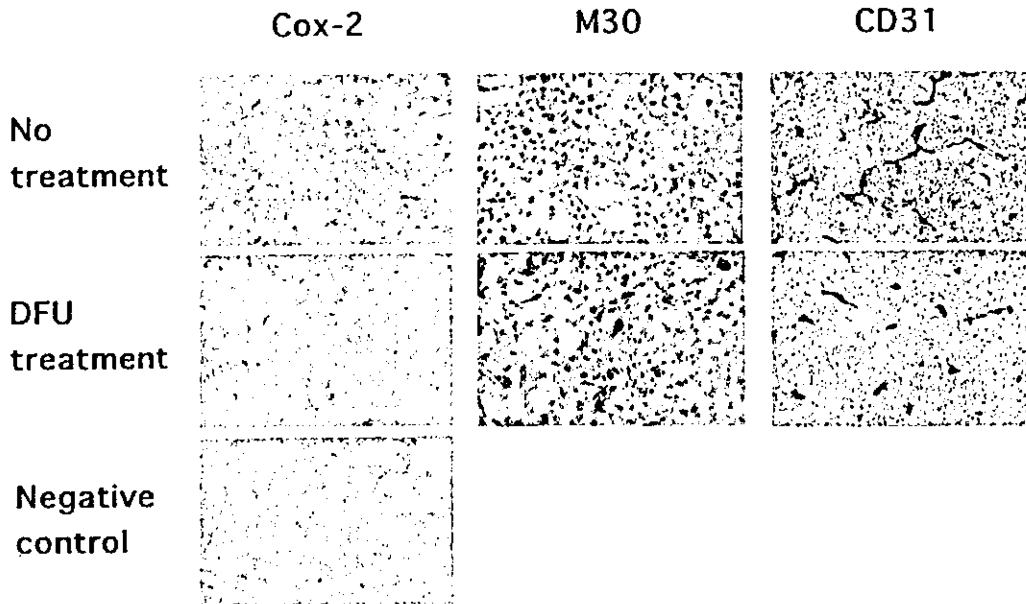

Figure 5. Immunohistochemical evaluation of apoptosis, tumor vessels and COX-2 expression. Immunostaining of frozen tumor sections of both the control and treatment group by M30, CD 31 and anti-COX-2 antibody.

was higher compared to the control group. Many apoptotic cells were observed in the tumors with DFU treatment (Fig. 5).

DFU treatment inhibited angiogenesis in xenograft tumors. DFU treatment also inhibited tumor vessel formation. Tumor vessel was determined by staining frozen sections of tumor tissue with CD31, a new specific and sensitive marker of angiogenesis. There was a large reduction in CD 31 positive vessels in the tumor of DFU treated mice, as compared to the tumors of the control mice (Fig. 5).

Discussion

Masferrer *et al* (14) classified COX-2 inhibitors as a new class of anti-angiogenic agents, since several studies suggest that tumor derived growth factors promote angiogenesis by inducing the production of COX-2 derived PGE_2, and COX-2 specific inhibitors consistently and effectively inhibited tumor growth and angiogenesis (15). COX-2 has been implicated in the carcinogenic process of several human tumors, and its up-regulation becomes an independent index of prognosis among cancer patients. Recent clinical studies have indicated that the presence of COX-2 in human lung and colon cancers is associated with poor prognosis (16,17). Subsequently COX-2 inhibition has become a field of special interest concerning tumor development, prevention and regression.

Recent evidence indicates that COX-2 modulates angiogenesis either by augmenting the release of angiogenic peptides (vascular endothelial growth factor, basic fibroblast growth factor and nitric oxide) by tumor cells or by directly increasing the production of prostaglandin. Several mechanisms have been supported as to why COX-2 expression in neoplastic tissues enhances tumor growth. There is evidence for amplification of tumor cell proliferation by PGE_2 and inhibition of tumor cell apoptosis, enhancement of stromal cell angiogenesis and decreased immune surveillance of tumor cells. Both COX-2 selective and non-selective drugs suppressed the activity of angiogenic factors (18).

In the present study, we examined the effect of DFU treatment on the growth of MCF-7 xenograft tumor progression in a mouse model. DFU is a structurally related analogue of rofecoxib. In a microsomal COX-1 assay, DFU was 100 times less potent than other COX-2 specific inhibitors. The COX-1/COX-2 selectivity ratio is >1000. DFU is also less ulcerogenic and had no detectable ulceration even at doses of 200 mg/kg/day (10). Although MCF-7 cells do not express COX-2, the MCF-7 xenograft tumor expressed COX-2 protein as detected by immunohistochemistry. This induction of COX-2 *in vivo* is a new finding, and the cause of induction remains unclear. The MCF-7 cell line is TNF-receptor sensitive (19). Therefore, TNF-α might be responsible for the COX-2 induction *in vivo* (20). Additionally, mice were implanted with an estrogen pellet, and sustained release of estrogen may have caused COX-2 induction in the xenograft (Huang JC, *et al*: Am Soc Reproduct Med 5: Abst, 1996). The interaction between the host-tumor immune system and NF-κB are also important. Further studies are necessary to clarify the induction of COX-2 within the tumor *in vivo*.

Breast carcinomas are highly angiogenic and it has been shown that COX-2 induces angiogenesis, which in turn aids tumor growth, invasion and metastasis (22-25). It was reported that COX-2 overexpression in gastric cancer samples was associated with enhanced angiogenesis. Recently, it has been reported that COX-2 expression correlates with tumor neovascularization in human colorectal carcinoma and hepatitis C virus positive hepatocellular carcinoma (17,26). Alternatively, COX-2 inhibitors significantly reduce angiogenesis. It has been reported that COX-2 inhibitors inhibited angiogenesis both in a COX-2-dependent and -independent mechanism. In our experiments, DFU inhibits tumor vessel formation in tumor tissue. It has been shown in primary tumors of the mice that rofecoxib, a specific COX-2

inhibitor, significantly increased IL-12 and appears to function as a tumor-suppressing cytokine (27). We also found that DFU treatment significantly reduces tumor growth in mice. A similar effect may have been produced due to DFU treatment. DFU also inhibits the expression of COX-2 in the xenograft. A growing body of evidence suggests that both COX-2 specific and non-specific NSAIDs induce apoptosis in various tumor cell lines and inhibit chemically-induced colonic (28) and mammary (29) carcinogenesis. It may be possible that prostaglandin produced by the mouse contributes to the growth proliferation of xenograft tumors, and that DFU inhibits the prostaglandin-induced tumor growth.

References

1. McAdam BF, Mardini IA, Habib A, Burke A, Lawson JA, Kapoor S and Fitzgerald GA: Effect of regulated expression of human cyclooxygenase isoforms on eicosanoid and isoeicosanoid production in inflammation. J Clin Invest 105: 1473-1482, 2000.
2. Williams CS, Tsujii M, Reese J, Dey SK and DuBois RN: Host cyclooxygenase-2 modulates carcinoma growth. J Clin Invest 105: 1589-1594, 2000.
3. McGinty A, Chang YW, Sorokin A, Bokemeyer D and Dunn MJ: Cyclooxygenase-2 expression inhibits trophic withdrawal apoptosis in nerve growth factor-differentiated PC12 cells. J Biol Chem 275: 12095-12101, 2000.
4. Chapple KS, Scott N, Guillou PJ, Coletta PL and Hull MA: Interstitial cell cyclooxygenase-2 expression is associated with increased angiogenesis in human sporadic colorectal adenomas. J Pathol 198: 435-441, 2002.
5. Sun Y, Tang XM, Half E, Kuo MT and Sinicrope FA: Cyclooxygenase-2 overexpression reduces apoptotic susceptibility by inhibiting the cytochrome c-dependent apoptotic pathway in human colon cancer cells. Cancer Res 62: 6323-6328, 2002.
6. Zweifel BS, Davis TW, Ornberg RL and Masferrer JL: Direct evidence for a role of cyclooxygenase 2-derived prostaglandin E(2) in human head and neck xenograft tumors. Cancer Res 62: 6706-6711, 2002.
7. Hasturk S, Kemp B, Kalapurakal SK, Kurie JM, Hong WK and Lee JS: Expression of cyclooxygenase-1 and cyclooxygenase-2 in bronchial epithelium and non-small cell lung carcinoma. Cancer 94: 1023-1031, 2002.
8. Gupta S, Srivastava M, Ahmad N, Bostwick DG and Mukhtar H: Over-expression of cyclooxygenase-2 in human prostate adenocarcinoma. Prostate 42: 73-78, 2000.
9. Costa C, Soares R, Reis-Filho JS, Leitao D, Amendoeira I and Schmitt FC: Cyclooxygenase 2 expression is associated with angiogenesis and lymph node metastasis in human breast cancer. J Clin Pathol 55: 429-434, 2002.
10. Riendeau D, Percival MD, Boyce S, Brideau C, Charleson S, Cromlish W, Ethier D, Evans J, Falgueyret JP, Ford-Hutchinson AW, Gordon R, Greig G, Gresser M, Guay J, Kargman S, Leger S, Mancini JA, O'Neill G, Ouellet M, Rodger IW, Therien M, Wang Z, Webb JK, Wong E, Xu L, Young RN, Zamboni R, Prasit P and Chan CC: Biochemical and pharmacological profile of a tetrasubstituted furanone as a highly selective COX-2 inhibitor. Br J Pharmacol 121: 105-117, 1997.
11. Blumenthal RD, Waskewich C, Goldenberg DM, Lew W, Flefleh C and Burton J: Chronotherapy and chronotoxicity of the cyclooxygenase-2 inhibitor, celecoxib, in athymic mice bearing human breast cancer xenografts. Clin Cancer Res 7: 3178-3185, 2001.
12. Leers MP, Kolgen W, Bjorklund V, Bergman T, Tribbick G, Persson B, Bjorklund P, Ramaekers FC, Bjorklund B, Nap M, Jornvall H and Schutte B: Immunocytochemical detection and mapping of a cytokeratin 18 neo-epitope exposed during early apoptosis. J Pathol 187: 567-572, 1999.
13. Ferrandina G, Ranelletti FO, Larocca LM, Maggiano N, Fruscella E, Legge F, Santeusanio G, Bombonati A, Mancuso S and Scambia G: Tamoxifen modulates the expression of Ki67, apoptosis, and microvessel density in cervical cancer. Clin Cancer Res 7: 2656-2661, 2001.
14. Masferrer JL, Koki A and Seibert K: COX-2 inhibitors. A new class of antiangiogenic agents. Ann N Y Acad Sci 889: 84-86, 1999.
15. Yoshida S, Amano H, Hayashi I, Kitasato H, Kamata M, Inukai M, Yoshimura H and Majima M: COX-2/VEGF-dependent facilitation of tumor-associated angiogenesis and tumor growth *in vivo*. Lab Invest 83: 1385-1394, 2003.
16. Achiwa H, Yatabe Y, Hida T, Kuroishi T, Kozaki K, Nakamura S, Ogawa M, Sugiura T, Mitsudomi T and Takahashi T: Prognostic significance of elevated cyclooxygenase 2 expression in primary, resected lung adenocarcinomas. Clin Cancer Res 5: 1001-1005, 1999.
17. Masunaga R, Kohno H, Dhar DK, Ohno S, Shibakita M, Kinugasa S, Yoshimura H, Tachibana M, Kubota H and Nagasue N: Cyclooxygenase-2 expression correlates with tumor neovascularization and prognosis in human colorectal carcinoma patients. Clin Cancer Res 6: 4064-4068, 2000.
18. Ostrowski J, Wocial T, Skurzak H and Bartnik W: Do altering in ornithine decarboxylase activity and gene expression contribute to antiproliferative properties of COX inhibitors? Br J Cancer 88: 1143-1151, 2003.
19. Mathiasen IS, Hansen CM, Foghsgaard L and Jaattela M: Sensitization to TNF-induced apoptosis by 1,25-dihydroxy vitamin D(3) involves up-regulation of the TNF receptor 1 and cathepsin B. Int J Cancer 93: 224-231, 2001.
20. Huang WC, Chen JJ, Inoue H and Chen CC: Tyrosine phosphorylation of I-kappa B kinase alpha/beta by protein kinase C-dependent c-Src activation is involved in TNF-alpha-induced cyclooxygenase-2 expression. J Immunol 170: 4767-4775, 2003.
21. Harris AL, Fox S, Leek R, Zhang H, Scott P, Bicknell R and Gatter K: Breast cancer angiogenesis: therapy target and prognostic factor. Eur J Cancer 31A: 831-832, 1995.
22. Ristimaki A, Sivula A, Lundin J, Lundin M, Salminen T, Haglund C, Joensuu H and Isola J: Prognostic significance of elevated cyclooxygenase-2 expression in breast cancer. Cancer Res 62: 632-635, 2002.
23. Subbaramaiah K, Telang N, Ramonetti JT, Araki R, DeVito B, Weksler BB and Dannenberg AJ: Transcription of cyclooxygenase-2 is enhanced in transformed mammary epithelial cells. Cancer Res 56: 4424-4429, 1996.
24. Davies G, Salter J, Hills M, Martin LA, Sacks N and Dowsett M: Correlation between cyclooxygenase-2 expression and angiogenesis in human breast cancer. Clin Cancer Res 9: 2651-2656, 2003.
25. Rahman MA, Dhar DK, Yamaguchi E, Maruyama S, Sato T, Hayashi H, Ono T, Yamanoi A, Kohno H and Nagasue N: Coexpression of inducible nitric oxide synthase and COX-2 in hepatocellular carcinoma and surrounding liver: possible involvement of COX-2 in the angiogenesis of hepatitis C virus-positive cases. Clin Cancer Res 7: 1325-1332, 2001.
26. Yao M, Kargman S, Lam EC, Kelly CR, Zheng Y, Luk P, Kwong E, Evans JF and Wolfe MM: Inhibition of cyclooxygenase-2 by rofecoxib attenuates the growth and metastatic potential of colorectal carcinoma in mice. Cancer Res 63: 586-592, 2003.
27. Tardieu D, Jaeg JP, Deloly A, Corpet DE, Cadet J and Petit CR: The COX-2 inhibitor nimesulide suppresses superoxide and 8-hydroxy-deoxyguanosine formation, and stimulates apoptosis in mucosa during early colonic inflammation in rats. Carcinogenesis 21: 973-976, 2000.
28. Han EK, Arber N, Yamamoto H, Lim JT, Delohery T, Pamukcu R, Piazza GA, Xing WQ and Weinstein IB: Effects of sulindac and its metabolites on growth and apoptosis in human mammary epithelial and breast carcinoma cell lines. Breast Cancer Res Treat 48: 195-203, 1998.

British Journal of Cancer (2004) 90, 2338–2343
© 2004 Cancer Research UK All rights reserved 0007–0920/04 $30.00
www.bjcancer.com

Modulation of thymidine phosphorylase by neoadjuvant chemotherapy in primary breast cancer

M Toi*,[1], H Bando[1], S Horiguchi[4], M Takada[1], A Kataoka[2], T Ueno[3], S Saji[1], M Muta[4], N Funata[4] and S Ohno[2]

[1]*Department of Surgery, Tokyo Metropolitan Komagome Hospital, 3-18-22, Honkomagome, Bunkyo-ku, Tokyo 113-8677, Japan;* [2]*Department of Breast Surgery, National Kyushu Cancer Center, 3-1-1, Notame, Minami-ku, Fukuoka 811-1395, Japan;* [3]*Cancer Center Karolinska, Department of Oncology and Pathology, Karolinska Institute and Hospital, Stockholm S-171 76, Sweden;* [4]*Department of Pathology, Tokyo Metropolitan Komagome Hospital, 3-18-22, Honkomagome, Bunkyo-ku, Tokyo 113-8677, Japan;*

The combination effect of docetaxel and capecitabine on tumour response rate and survival was demonstrated recently in metastatic breast cancer patients. This combination was based on an experimental hypothesis that taxane can increase tumour sensitivity to the effect of capecitabine through the upregulation of thymidine phosphorylase (TP), which is responsible for the metabolism of 5-fluorouracil (5-FU) and its derivatives, including capecitabine. To examine the alteration in TP expression before and after neoadjuvant chemotherapy, 92 patients with primary breast cancer (T2-4N0-1M0) were enrolled in this study; 14 were treated with adriamycin and cyclophosphamide (AC) or epirubicin and cyclophosphamide (EC); 58 with 5-FU, adriamycin, and cyclophosphamide (FAC) or 5-FU, epirubicin, and cyclophosphamide (FEC); and 20 with FEC followed by docetaxel/taxotere (TXT-containing regimen). Thymidine phosphorylase upregulation was seen in 54.4% and 32.6% of patients in tumour cells and stromal cells, respectively. Increases in TP expression were found only in the AC/EC and TXT-containing regimen groups. In conclusion, it was strongly suggested that unlike 5-FU-containing regimens, the taxane and AC combination therapies upregulate TP expression in primary breast cancer. Thymidine phosphorylase upregulation by several anticancer drugs implies the importance of individualised strategies for sensitisation of tumour tissues to 5-FU and its derivatives.
British Journal of Cancer (2004) **90,** 2338–2343. doi:10.1038/sj.bjc.6601845 www.bjcancer.com
Published online 4 May 2004
© 2004 Cancer Research UK

Keywords: TP; preoperative chemotherapy; fluorouracil; immunohistochemistry; upregulation

Thymidine phosphorylase (TP) is an enzyme that is responsible for nucleoside metabolism, antiapoptosis activity, and promotion of angiogenesis. Thymidine phosphorylase acts mainly in the salvage cascade of DNA metabolism in response to various types of stresses. Thymidine phosphorylase functions in the prevention of hypoxia-induced apoptosis according to recent experimental analyses (Ikeda *et al*, 2003). In addition, it has been documented that a metabolite of thymidine generated by TP, 2-deoxy-D-ribose (2-DDR), acts as a potent chemotactic factor on the endothelium, which results in the promotion of neovascularisation (Haraguchi *et al*, 1994). In fact, in a variety of tumour tissues, overexpression of TP was found to correlate significantly with an increase in neovascularisation (Toi *et al*, 1995; Tanigawa *et al*, 1996; Matsuura *et al*, 1999) and poor prognosis (Maeda *et al*, 1996; Takebayashi *et al*, 1996; Koukourakis *et al*, 1998; Toi *et al*, 1999).

The regulation of TP has been also studied from various points of view. Generally, TP is upregulated by stress such as hypoxia (Griffiths *et al*, 1997), radiation (Sawada *et al*, 1999), and chemotherapeutic damage (Sawada *et al*, 1998; Endo *et al*, 1999). Several types of cytokines such as interleukin (IL)-1, tumour necrosis factor (TNF)-α, and interferon (IFN)-γ also upregulate the expression of TP in both nonmalignant and malignant cells (Eda *et al*, 1993). Therefore, it is likely that these factors have important functions in stress-induced TP upregulation.

Thymidine phosphorylase has also been studied as a key enzyme involved in nucleoside metabolism. In particular, TP is known to be essential for the activation of capecitabine from the intermediate form 5′-deoxy-5-fluorouridine (5′-DFUR) to the active form 5-fluorouracil (5-FU). Experimental studies showed that 5′-DFUR is much more active in TP-transfected cells than in mock-transfected cells (Patterson *et al*, 1995; Evrard *et al*, 1999). It is also true that 5′-DFUR is more effective for TP-overexpressing tumour xenografts than for tumour xenografts expressing normal or low levels of TP (Morita *et al*, 2001; Ishikawa *et al*, 1998). Furthermore, several preliminary studies also confirmed that TP expression in tumour cells was a predictive factor for favourable prognosis in cancer patients treated with 5′-DFUR (Yamamoto *et al*, 1996; Ishii *et al*, 1996; Koizumi *et al*, 1999; Nishimura *et al*, 2002). In primary breast cancer, an analysis of the relationship between TP expression and the therapeutic effect of 5′-DFUR as a retrospective study in a prospective clinical randomised study has recently been reported, where patients who received no systemic adjuvant treatment were compared with those who received treatment with 5′-DFUR alone. It concluded that TP is a promising marker for predicting the survival benefit from 5′-DFUR treatment in early breast cancer patients (Tominaga *et al*, 2002).

*Correspondence: Dr Ma Toi, E-mail: maktoi77@wa2.so-net.ne.jp.
Received 29 December 2003; accepted 15 March 2004; published online 4 May 2004

On the other hand, a hypothesis that TP modulation could enhance the therapeutic activity of 5′-DFUR/capecitabine has been tested at the experimental level. In various types of tumour xenograft models, the combination of capecitabine and various TP modulating chemotherapeutic agents achieved synergistic effects (Sawada *et al*, 1998; Fujimoto-Ouchi *et al*, 2002). Differences in the duration between the induction chemotherapy, with respect to TP modulation, and capecitabine treatment elicited different tumour responses, indicating that TP modulation is time dependent (Fujimoto-Ouchi *et al*, 2001) and that the timing of capecitabine treatment after the initial chemotherapy is important. In a clinical situation, it was demonstrated that therapy with capecitabine plus TXT achieved a significantly higher response and longer time to progression (TTP) than TXT therapy alone in the first-line treatment of metastatic breast cancer patients (O'Shaughnessy *et al*, 2002). This clinical finding would reflect on the basic hypothesis that TXT sensitises tumours to the effect of capecitabine. This suggests the importance of considering TP modulation from the point of sensitising breast cancer tumours to 5-FU derivatives such as capecitabine and 5′-DFUR, because the likelihood of their efficacy might be increased for TP upregulated tumours.

Issues related to TP modulation in human tumour tissues, however, are still largely unknown. Very few papers have touched on this crucial question. Thus, in the present study, we examined TP expression prior to and after the administration of chemotherapy in a neoadjuvant setting of primary breast cancer treatment. We will demonstrate that TP expression is modulated significantly by certain chemotherapies in a defined patient population.

MATERIALS AND METHODS

Patient characteristics

Between January 1, 1998 and December 30, 2002, women at the Tokyo Metropolitan Komagome Hospital and the National Kyushu Cancer Hospital who had primary, palpable, operable breast cancer (T2-4N0-1M0, according to the tumour, node, metastasis staging system) were included in this study. All patients were diagnosed by core needle biopsy or excisional biopsy prior to starting chemotherapy, and all patients were informed about the investigational nature of the study, which had been approved by the institutional review board. Written informed consent was obtained from each woman before entering her into the trial. All patients received either partial mastectomy or modified radical mastectomy with full dissection of axillary nodes after the treatment by neoadjuvant chemotherapy. Both biopsied and surgically resected samples were sufficient for accurate histological diagnosis and measurement of biomarkers.

Treatment regimens

Patients were treated with anthracycline-containing regimens or a taxane-containing regimen. The anthracycline-containing regimens consisted of adriamycin (ADR) and cyclophosphamide (CPA), (AC); epirubicin (EPI) and CPA (EC) or 5-FU, ADR, and CPA (FAC); and 5-FU, EPI, and CPA (FEC). Patients were given chemotherapy every 21 days with either the AC (ADR 50 mg m^{-2} and CPA 500 mg m^{-2}), EC (EPI 75 mg m^{-2} and CPA 600 mg m^{-2}), FAC (5-FU 500 mg m^{-2}, ADR 50 mg m^{-2}, and CPA 500 mg m^{-2}), and FEC (5-FU 500 mg m^{-2}, EPI 100 mg m^{-2}, and CPA 500 mg m^{-2}) or the TXT-containing regimen (FEC followed by TXT 75 mg m^{-2} or TXT 60 mg m^{-2}).

Efficacy assessment

Responses of the primary tumours to each chemotherapy regimen were evaluated according to the criteria established by the Japanese Breast Cancer Society (The Japanese Breast Cancer Society, 2000), which are essentially the same as those of the World Health Organization. A complete response (CR) is defined as the disappearance of tumour; partial response (PR) refers to a decrease in tumour size of 50% or more; no change (NC) indicates a decrease in tumour size of 50% or less or an increase of tumour size by less than 25%; and progressive disease (PD) indicates an increase in tumour size of 25% or more.

The grading of the pathological efficiency of chemotherapy, which was evaluated microscopically by a skilled pathologist, was also categorised according to the criteria established by the Japanese Breast Cancer Society (The Japanese Breast Cancer Society, 2000). The three grades are defined as follows: Grade 3 is the complete disappearance of variable cancer cells on the examined specimens; Grade 2, the apparent degeneration of two out of three or more of the population of observed cancer cells; Grade 1, the presence of degenerated cells in less than two out of three of examined tumour cells; and Grade 0, the presence of no degenerative cancer cells on specimens.

Immunohistochemical assessment

All samples were retrospectively processed with haematoxylin–eosin staining, negative control staining, and immunostaining for TP in our laboratory. Thymidine phosphorylase antibody was obtained from Roche Diagnostics (Basel, Switzerland), and the method for immunohistochemistry followed the protocol given in the immunohistochemistry kit 'Anti-TP Antibody, Formalin-Grade' (Roche Diagnostics Corporation, USA). The TP-stained slides were assessed for tumour cells and stromal cells according to the criteria defined in the kit. Staining intensities were scored as one of the four grades 0, 1+, 2+, and 3+, and staining patterns were scored as one of the five grades 0, 1+, 2+, 3+, and 4+.

Oestrogen receptor (ER) status progesterone receptor (PR) was also determined by an immunohistochemical method as described previously (Saji *et al*, 2002). Tumours containing 10% or more receptor-positive cells were scored as being receptor-positive.

Statistical methods

All patients with tissue staining data were included in the analysis. The statistical analyses for the TP-immunostained preparations were conducted as follows. The four grades of staining intensities were scored as 0, 1, 2, and 3. Similarly, the five grades of staining patterns were scored as 0, 1, 2, 3, and 4. Thymidine phosphorylase up- or down-regulation was evaluated as the difference between the sample score after chemotherapy minus the sample score prior to chemotherapy for each patient. Samples with score differences greater than 1 were evaluated as 'upregulated', and less than -1 as 'downregulated.' Score differences in the range between -1 and 1 were evaluated as 'no change.' Scores of staining intensities and staining patterns were analysed, and the summation of staining intensity and pattern scores were also analysed. After checking the distribution of the score differences, the *t*-test was used to compare the means.

For the contingency tables, Fisher's exact test was used to assess the potential different distribution. To relate the score differences with the treatment groups, we used the Mantel–Haenszel test for contingency tables and the *t*-test to compare the means. Since the known prognostic factors such as tumour size were distributed differently in each treatment group, tumour size was used as a stratified factor for both the Mantel–Haenszel and *t*-test. Bonferroni's correction was applied to adjust the *P*-values of the pairwise comparisons between each treatment group.

All analyses were carried out by using SAS 8.2, and alpha was set at 0.05.

© 2004 Cancer Research UK

RESULTS

Patient characteristics

A total of 92 patients were enrolled in this study. All the 92 patients were eligible and provided tissue staining results. The patient characteristics are shown in Table 1. Imbalances were observed for tumour size and number of patients, *n*, between the treatment groups, which would not affect the results of the present study, because no correlation was observed with TP regulation as reported below. At initial diagnosis, the average age of the women in this study was 51 years (range, 28–74 years). With respect to tumour size, those of 11 patients were less than 3.0 cm and those of 81 patients were greater than 3.1 cm. In all, 79% of patients had positive nodal status and 64.1% of patients had oestrogen-receptor-positive tumours.

Among the patients, 14 were treated with AC or EC, 58 were treated with FAC or FEC, and 20 were treated with the TXT-containing regimen.

Table 1 Patients' characteristics and overall response rate

Characteristics	*n*	Regimen (%) AC/EC	FAC/FEC	TXT	*P*-value*
Menopausal status					
Pre	46	5 (10.9)	27 (58.7)	14 (30.4)	
Post	46	9 (19.6)	31 (67.4)	6 (13.0)	0.107
Tumour size					
−3.0 cm	11	0 (0.0)	2 (18.2)	9 (81.8)	
3.1 cm-	81	14 (17.3)	56 (69.1)	11 (13.6)	<0.001
Number of nodes involved					
0	19	4 (21.1)	5 (26.3)	10 (52.6)	
1–3	18	2 (11.1)	9 (50.0)	7 (38.9)	
4-	55	8 (14.6)	44 (80.0)	3 (5.4)	<0.001
Oestrogen receptor					
+	59	9 (15.3)	35 (59.3)	15 (25.4)	
−	33	5 (15.2)	23 (69.7)	5 (15.1)	0.571
Progesterone receptor					
+	39	6 (15.4)	26 (66.7)	7 (17.9)	
−	53	8 (15.1)	32 (60.4)	13 (24.5)	0.789
Cycle (median)	92	2–4 (4.0)	2–6 (3.0)	7–8 (8.0)	—
Response rate	92	50.0%	41.4%	70.0%	—
(95% CI)		(23.0–77.0)	(28.6–55.1)	(45.7–88.1)	—

AC = adriamycin (ADR) and cyclophosphamide (CPA); EC = epirubicin (EPI) and CPA; FAC = 5-fluorouracil (5-FU), ADR, and CPA; FEC = 5-FU, EPI, and CPA, TXT = docetaxel-containing regimen, CI = confidence interval, *Fisher's exact test.

Thymidine phosphorylase immunohistochemistry

We used the difference in each patient's tissue staining scores before and after chemotherapy to assess TP up- or down-regulation (Figure 1). Thymidine phosphorylase scores, staining intensities, and staining patterns from both tumour cells and stromal cells were available. No correlations were observed between the tumour and stromal scores. TP changes were seen in response to chemotherapy; TP levels in tumour and stromal cells were upregulated in 50 patients (54.4%) and 30 patients (32.6%), and downregulated in 15 patients (16.3%) and 29 patients (31.5%), respectively.

Table 2 shows the correlation between TP changes and patients' characteristics (Table 2A: tumour, 2B: stroma, respectively). An association between them was seen only in tumour size for stromal TP ($P = 0.020$). On the other hand, there were no significant differences for relationships for the number of nodes involved, ER status, or menopausal status.

Table 3 shows the relation between TP changes and treatment groups. TP changes were lowest in the FAC/FEC group and highest in the AC/EC group. Adjusted *P*-values of pairwise comparisons by Bonferroni's correction suggest that the TP score changes in the FAC/FEC group are significantly different from those in the AC/EC group (tumour: $P = 0.0001$, stromal: $P = 0.0001$). Nevertheless, no association was observed between scores of tumour and stroma, and the association with treatment regimen was similar for both tumour and stroma.

In the AC or EC group, TP was upregulated in the tumour and stromal cells of 92.9 and 85.7% of patients, respectively; however,

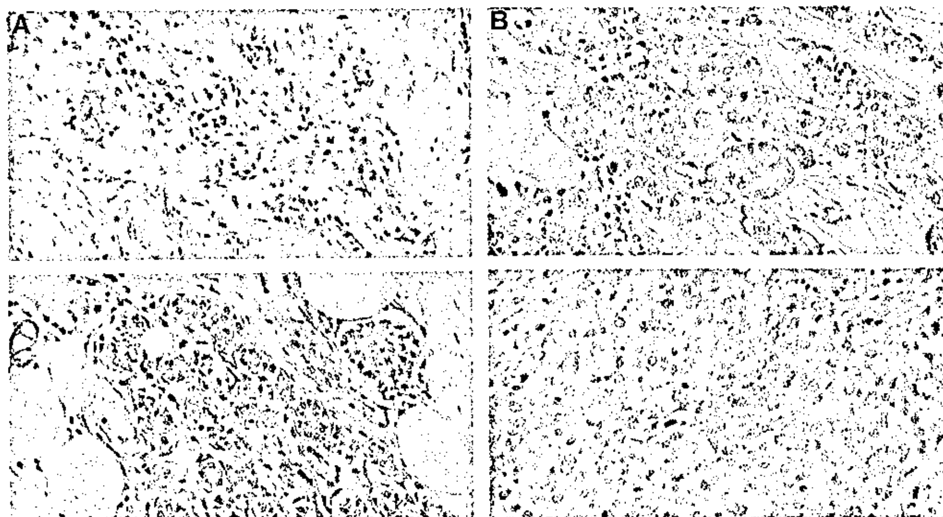

Figure 1 TP expression status of pre- and post-treatment. (**A**) An invasive ductal carcinoma: TP expression was upregulated remarkably by the treatment with FEC (5-FU, epirubicin, and cyclophosphamide) followed by docetaxel. Tumour TP score: pretreatment; 0 (upper), post-treatment; 7 (bottom), yielding a score difference of 7. The treatment achieved PR. (**B**) An invasive ductal carcinoma; TP expression was not changed remarkably by the treatment with FEC followed by docetaxel. Tumour TP score: pretreatment; 6 (upper), post-treatment; 5 (bottom), yielding a score difference of −1. The treatment achieved PR.

© 2004 Cancer Research UK

Table 2 Association of (**A**) tumour TP changes and (**B**) stromal TP changes with patients' characteristics

(A) Tumour TP changes

	Tumour TP			
	Up (%)	**NC (%)**	**Down (%)**	***P*-value***
Menopausal status				
Pre	29 (63.0)	9 (19.6)	8 (17.4)	
Post	21 (45.7)	18 (39.1)	7 (15.2)	0.122
Tumour size				
Median (range)	6.2 (1.5–18.0)			
−3.0 cm	6 (54.6)	2 (18.2)	3 (27.3)	
3.1 cm −	44 (54.3)	25 (30.9)	12 (14.8)	0.456
Number of nodes involved				
0	13 (68.4)	5 (26.3)	1 (5.3)	
1–3	9 (50.0)	5 (27.8)	4 (22.2)	
4−	28 (50.9)	17 (30.9)	10 (18.2)	0.578
Oestrogen receptor				
Positive	36 (61.0)	16 (27.1)	7 (11.9)	
Negative	14 (42.4)	11 (33.3)	8 (24.2)	0.157

(B) Stromal TP changes

	Stromal TP			
	Up (%)	**NC (%)**	**Down (%)**	***P*-value***
Menopausal status				
Pre	15 (32.6)	16 (34.8)	15 (32.6)	
Post	15 (32.6)	17 (37.0)	14 (30.4)	1.000
Tumour size				
Median (range)	6.2 (1.5–18.0)			
−3.0 cm	0 (0.0)	7 (63.6)	4 (36.4)	
3.1 cm−	30 (37.0)	26 (32.1)	25 (30.9)	0.020
Number of nodes involved				
0	10 (52.6)	3 (15.8)	6 (31.6)	
1–3	4 (22.2)	9 (50.0)	5 (27.8)	
4−	16 (29.1)	21 (38.2)	18 (32.7)	0.173
Oestrogen receptor				
Positive	21 (35.6)	20 (33.9)	18 (30.5)	
Negative	9 (27.3)	13 (39.4)	11 (33.3)	0.736

TP = thymidine phosphorylase; Up = upregulated; NC = no change; Down = down-downregulated; *Fisher's exact test.

Table 3 Tumour TP changes by each regimen

Regimen	***n***	**Gain in TP score (mean)**	**Up (%)**	**NC (%)**	**Down (%)**
AC/EC					
Tumour	14	4.3	13 (92.9)	1 (7.1)	0 (0.0)
Stroma	14	3.6	12 (85.7)	2 (14.3)	0 (0.0)
FAC/FEC					
Tumour	58	0.7	24 (41.4)	22 (37.9)	12 (20.7)
Stroma	58	−0.9	10 (17.2)	25 (43.1)	23 (39.7)
TXT-containing regimen					
Tumour	20	1.8	13 (65.0)	4 (20.0)	3 (15.0)
Stroma	20	0.0	8 (40.0)	6 (30.0)	6 (30.0)
Total					
Tumour	92	—	50 (54.4)	27 (29.3)	15 (16.3)
Stroma	92	—	30 (32.6)	33 (35.9)	29 (31.5)

	Tumour		Stroma	
Regimen compared	**t-test**	**M–H**	**t-test**	**M–H**
AC/EC vs FAC/FEC	<0.0001	0.0114	<0.0001	<0.0001
FAC/FEC vs TXT	0.2287	0.5700	0.0580	0.0021
AC/EC vs TXT	0.1527	0.5616	0.0339	0.7773

Up = upregulated; NC = no change; Down = downregulated; AC = adriamycin (ADR) and cyclophosphamide (CPA); EC = epirubicin (EPI) and CPA; FAC = 5-fluorouracil (5-FU), ADR, and CPA; FEC = 5-FU, EPI, and CPA; P-values with Bonferroni's correction, adjusted by tumour size; M–H = Mantel–Haenszel test.

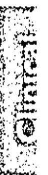

Table 4 Relationship between TP changes and response

	n	**Up (%)**	**NC (%)**	**Down (%)**	***P*-value***
Tumour					
Responder	45	24 (53.3)	16 (35.6)	5 (11.1)	
Nonresponder	47	26 (55.3)	11 (23.4)	10 (21.3)	0.383
Stroma					
Responder	45	14 (31.1)	14 (31.1)	17 (37.8)	
Nonresponder	47	16 (34.0)	19 (40.5)	12 (25.5)	0.461

Up = upregulated; NC = no change; Down = downregulated; *Mantel–Haenszel test adjusted by tumour size.

TP was not downregulated in any patient. In the FAC or FEC group, tumour TP was upregulated in 41.4% of patients and downregulated in 20.7%. In the TXT-containing regimen, tumour TP was upregulated in 65.0% of patients and downregulated in 15.0%.

Clinical response rates

Of the 92 patients available for analysis, an overall response rate (ORR) of of 50.0%. (95% confidence interval (CI): 23.0–77.0%) was achieved by patients who were treated with AC or EC, an ORR of 41.4% (95% CI: 28.6–55.1%) by the patients treated with FAC or FEC, and an ORR of 70.0% (95% CI: 45.7–88.1%) by those patients given the TXT-containing regimen, as shown in Table 1.

The relationship between ORR and TP status is shown in Table 4. There was no correlation observed between clinical response and TP status, for either tumour or stromal cells ($P=0.383$ and $P=0.461$, respectively).

Pathological response rate

Of the 87 patients available for analysis, a grade 2 response was achieved by 14.3%. of patients who were treated with AC or EC (95% CI: 1.78–42.8%), 12.1% of those treated with FAC or FEC (95% CI: 4.99–23.3%), and 6.7% of those treated with the TXT-containing regimen (95% CI: 0.17–32.0%). Overall, a grade 2 response of 11.5% (95% CI: 5.65–20.1%) was seen in this study. There was no significant correlation between the pathological responses of grade 2 and TP changes in both tumour and stromal cells ($P=0.600$ and $P=0.273$, respectively).

DISCUSSION

Although the predictive value of TP expression in tumour tissues has been studied extensively for 5-FU or 5-FU-containing treatments, there is still little known about changes in TP levels in human tumours in response to chemotherapy. In this study, we showed that TP expression is often enhanced in primary breast

© 2004 Cancer Research UK

tumours in response to neoadjuvant chemotherapy. In particular, we found that TP was frequently upregulated in response to treatment by an EC/AC- or TXT-containing regimen. These results seem to be compatible with the data for human cancer xenograft experiments where taxanes, CPA, and mitomycin-C showed the potent ability to upregulate TP (Sawada *et al*, 1998; Endo *et al*, 1999). TXT also caused TP upregulation as a neoadjuvant in advanced breast cancer patients (Kurosumi *et al*, 2000), a result that also seems to be compatible with the clinical data. Thymidine phosphorylase in tumour cells tended to be co-upregulated with TP in tumour-associated stromal cells such as macrophages, indicating a possible role for microenvironmental factors in this response. In previous studies looking at correlations between TP and various immune mediators in the human breast tumour microenvironment, TP expression was associated significantly with expressions of TNF-α (Leek *et al*, 1998), IL-1α (Eda *et al*, 1993), and monocyte chemoattractant protein (MCP)-1 (Saji *et al*, 2002). From the molecular profile, it is known that multiple copies of potential Sp-1 binding sites are clustered upstream of the start site for the initiation of TP transcription (Zhu *et al*, 2002). Therefore, it is possible that TP upregulation would be triggered by increases in the intratumoural concentrations of these immune mediators in response to chemotherapy. As chemotherapy causes massive damage in tumour cells, the immune cells, especially macrophages, are activated to eliminate the damaged cells. In this process, it is estimated that large amounts of chemical immune mediators are produced by tumour-associated macrophages in the tumour microenvironment. Since hypoxia and hypoglucose are also characterised as stimuli of TP expression (Griffiths *et al*, 1997), these physical factors might help to enhance TP expression in association with local hyper-cytokinaemia.

For those patients treated with FAC or FEC, the 5-FU-containing regimens, we found no increased frequency of TP upregulation after chemotherapy. There are at least two possible explanations for this phenomenon. Firstly, the high concentration of 5-FU might downregulate TP expression. It is known that high concentrations of pyrimidine substrate change or downregulate the expression levels of nucleoside metabolism enzymes. There are few reports investigating the effect of high concentrations of 5-FU on TP; however, this mechanism is likely to be involved.

Secondarily, 5-FU might selectively kill or suppress TP-overexpressing cells. Many basic and clinical studies have indicated that 5-FU-contaning regimens are more effective for TP-overexpressing tumour cells as compared with TP-less-expressing tumour cells (Fox *et al*, 1997; Evrard *et al*, 1999; Gasparini *et al*, 1999; Morita *et al*, 2001; Yang *et al*, 2002). Therefore, these two scenarios should be further studied. Thymidine phosphorylase is stress-induced and, basically, TP is shown to be an enzyme that contributes to cell survival, because 2-DDR, a metabolite of thymidine via TP, induces neovascularisation and contributes to antiapoptosis (Haraguchi *et al*, 1994). After exposure to chemotherapy, TP might also function as mechanism for survival by the tumour cells. Based upon this hypothesis, a sequential treatment consisting of TP-upregulating chemotherapy followed by TP-dependent chemotherapy, such as by capecitabine, might be a reasonable therapeutic approach. In fact, the combination treatment with taxane and capecitabine showed a synergistic effect in animal experiments (Sawada *et al*, 1998) and induced a significant improvement in the survival of metastatic breast cancer patients (O'Shaughnessy *et al*, 2002). Therefore, the examination of TP expression in detail might provide various ideas to consider about optimal combinations in dosage and timing between capecitabine and other chemotherapeutic drugs. For example, a TC or TAC regimen might be promising to induce maximal TP expression. Furthermore, in cases where TP is not upregulated after the initial chemotherapy, the subsequent capecitabine monotherapy might not be effective.

In conclusion, TP is frequently up- or down-regulated after EC/AC- or taxane-containing chemotherapy in primary breast tumour tissues. The upregulated levels of TP are less for 5-FU-containing regimens. Thymidine phosphorylase is indeed upregulated by several anticancer drugs in human breast cancer cells, including both tumour and stromal cells; however, there are variations in the level. Thus, it is important to consider an individual strategy for sensitisation of breast tumour tissues to 5-FU by chemotherapy through TP.

REFERENCES

Eda H, Fujimoto K, Watanabe S, Ura M, Hino A, Tanaka Y, Wada K, Ishitsuka H (1993) Cytokines induce thymidine phosphorylase expression in tumor cells and make them more susceptible to 5′-deoxy-5-fluorouridine. *Cancer Chemother Pharmcol* 32: 333–338

Endo M, Shinbori N, Fukase Y, Sawada N, Ishikawa T, Ishitsuka H, Tanaka Y (1999) Induction of thymidine phosphorylase expression and enhancement of efficacy of capecitabine or 5′-deoxy-5-fluorouridine by cyclophosphamide in mammary tumor models. *Int J Cancer* 83: 127–134

Evrard A, Cuq P, Ciccolini J, Vian L, Cano JP (1999) Increased cytotoxicity and bystander effect of 5-fluorouridine in human colorectal cancer cells transfected with thymidine phosphorylase. *Br J Cancer* 80: 1726–1733

Fox SB, Engels K, Comley M, Whitehouse RM, Turley H, Gatter KC, Harris AL (1997) Relationship of elevated tumour thymidine phosphorylase in node-positive breast carcinomas to the effects of adjuvant CMF. *Ann Oncol* 8: 271–275

Fujimoto-Ouchi K, Sekiguchi F, Tanaka Y (2002) Antitumor activity of combination of anti-HER-2 antibody trastuzumab and oral fluoropyrimidines capecitabine/5′-dFUrd in human breast cancer models. *Cancer Chemother Pharmacol* 49: 211–216

Fujimoto-Ouchi K, Tanaka Y, Tominaga T (2001) Schedule dependency of antitumor activity in combination therapy with capecitabine/5′-deoxy-5-fluorouridine and docetaxel in breast cancer models. *Clin Cancer Res* 7: 1079–1086

Gasparini G, Toi M, Miceli R, Vermeulen PB, Dittadi R, Bonganzoli E, Morabito A, Fanelli M, Gatti C, Suzuki H, Tominaga T, Dirix LY, Gion M (1999) Clinical relevance of vascular endothelial growth factor and thymidine phosphorylase in patients with node-positive breast cancer treated with either adjuvant chemotherapy of hormone therapy. *Cancer J Sci Am* 5: 101–111

Griffiths L, Dachs GU, Bicknell R, Harris AL, Stratford IJ (1997) The influence of oxygen tension and pH on the expression of platelet-derived endothelial cell growth factor/thymidine phosphorylase in human breast tumor cells grown *in vitro* and *in vivo*. *Cancer Res* 15: 570–572

Haraguchi M, Miyadera K, Uemura K, Sumizawa T, Furukawa T, Yamada K, Akiyama S (1994) Angiogenic activity of enzymes. *Nature* 368: 198

Ikeda R, Furukawa T, Mitsuo R, Noguchi T, Kitazono M, Okumura H, Sumizawa T, Haraguchi M, Che XF, Uchimiya H, Nakajima Y, Ren XQ, Oiso S, Inoui I, Yamada K, Akiyama S (2003) Thymidine phosphorylase inhibits apoptosis induced by cisplatin. *Biochem Biophys Res Commun* 301: 358–363

Ishii R, Takiguchi N, Oda K, Koda K, Miyazaki M (1996) Thymidine phosphorylase expression is useful in selecting adjuvant chemotherapy for stage III gastric cancer. *Int J Cancer* 19: 717–722

Ishikawa T, Sekiguchi F, Fukase Y, Sawada N, Ishitsuka H (1998) Positive correlation between the efficacy of capecitabine and doxiflouridine and the ratio of thymidine phosphorylase to dihydropyrimidine dehydrogenase activities in tumors in human cancer xenografts. *Cancer Res* 58: 685–690

Koizumi W, Saigenji K, Nakamaru N, Okayasu I, Kurihara M (1999) Prediction of response to 5′-deoxy-5-fluorouridine (5′-DFUR) in patients with inoperable advanced gastric cancer by immunostaining of thymidine phosphorylase/platelet-derived endothelial cell growth factor. *Oncology* 56: 21–222

© 2004 Cancer Research UK

Koukourakis MI, Giatromanolaki A, Kakolyris S, O'Bvrne kj, Apostolikas N, Skarlatos J, Gatter KC (1998) Different patterns of stromal and cancer cell thymidine phosphorylase reactivity in non-small-cell lung cancer: impact on tumour neoangiogenesis and survival. *Br J Cancer* 77: 1696–1703

Kurosumi M, Tabei T, Suemasu K, Inoue K, Kusawake T, Sugamata N, Higashi Y (2000) Enhancement of immunohistochemical reactivity for thymidine phosphorylase in breast carcinoma cells after administration of docetaxel as a neoadjuvant chemotherapy in advanced breast caner. *Oncol Rep* 7: 945–948

Leek RD, Lannders R, Fox SB, Ng F, Harris AL, Lewis CE (1998) Association of tumour necrosis factor alpha and its receptors with thymidine phosphorylase expression in invasive breast carcinoma. *Br J Cancer* 77: 2246–2251

Maeda K, Chung YS, Ogawa Y, Takatsuka S, Kang SM, Ogawa M, Sawada T, Onoda N, Kato Y, Sowa M (1996) Thymidine phosphorylase/platelet derived endothelial cell growth factor expression associated with hepatic metastasis in gastric cancer. *Br J Cancer* 73: 884–888

Matsuura T, Kuratate I, Teramachi K, Osaki M, Fukuda Y, Ito H (1999) Thymidine phosphorylase expression is associated with both increase of intratumoral microvessels and decrease of apoptosis in human colorectal carcinomas. *Cancer Res* 59: 5037–5040

Morita T, Masuzaki A, Tokue A (2001) Enhancement of sensitivity to capecitabine in human renal carcinoma cells transfected with thymidine phosphorylase cDNA. *Int J Cancer* 92: 451–456

Nishimura G, Terada I, Kobayashi T, Ninomiya I, Kitagawa H, Fushida S, Fujimura T, Kayahara M, Shimizu K, Ohta T, Miwa K (2002) Thymidine phosphorylase and dihydropyrimidine dehydrogenase levels in primary colorectal cancer show a relationship to clinical effects of 5′-deoxy-5-fluorouridine as adjuvant chemotherapy. *Oncol Rep* 9: 479–482

O'Shaughnessy BJ, Miles D, Vukelja S, Moiseyenko V, Ayoub JP, Cervantes G, Fumoleau P, Jones S, Lui WY, Mauriac L, Twelves C, Hazel GV, Verma S, Leonard R (2002) Superior survival with capecitabine plus docetaxel combination therapy in anthracycline-pretreated patients with advanced breast cancer: phase III trial results. *J Clin Oncol* 20: 2812–2823

Patterson AV, Zhang H, Moghaddam A, Bicknell R, Talbot DC, Stratford IJ, Harris AL (1995) Increased sensitivity to the prodrug 5′-deoxy-5-fluorouridine and modulation of 5-fluoro-2′-deoxyuridine sensitivity in MCF-7 cells transfected with thymidine phosphorylase. *Br J Cancer* 72: 669–675

Saji H, Koike M, Yamori T, Saji S, Seki M, Matsushima K, Toi M (2002) Significant correlation of monocyte chemoattractant protein-1 expression with neovascularization and progression of breast carcinoma. *Cancer* 92: 1085–1091

Saji S, Omoto Y, Shimizu C, Warner M, Hayashi Y, Horiguchi S, Watanabe T, Hayashi S, Gustafsson JA, Toi M (2002) Expression of estrogen receptor (ER) (beta) cx protein in ER (alpha)-positive breast cancer: specific correlation with progesterone receptor. *Cancer Res* 62: 4849–4853

Sawada N, Ishikawa T, Sekiguchi F, Tanaka Y, Ishitsuka H (1999) X-ray irradiation induces thymidine phosphorylase and enhances the efficacy of capecitabine (Xeloda) in human cancer xenografts. *Clin Cancer Res* 5: 2948–2953

Sawada N, Ishikawa T, Fukase Y, Nishida M, Yoshikubo T, Ishitsuka H (1998) Induction of thymidine phosphorylase activity and enhancement of capecitabine efficacy by Taxol/Taxotere in human cancer xenografts. *Clin Cancer Res* 4: 1013–1019

Takebayashi Y, Akiyama S, Akiba S, Yamada K, Miyadera K, Sumizawa T, Yamada Y, Murata F, Aikou T (1996) Clinicopathologic and prognostic significance of an angiogenic factor, thymidine phosphorylase, in human colorectal carcinoma. *J Natl Cancer Inst* 88: 1110–1117

Tanigawa N, Amaya H, Matsumura M, Katoh Y, Kitaoka A, Aotake T, Shimomatusya T, Rosenwasser OA, Iki M (1996) Tumor angiogenesis and expression of thymidine phosphorylase/platelet derived endothelial cell growth factor in human gastric carcinoma. *Cancer Lett* 108: 281–290

The Japanese Breast Cancer Society (2000) *General Rules for Clinical and Pathological Recording of Breast Cancer*, 14th edn. Tokyo: Kanehara Shuppan

Toi M, Inada K, Hoshina S, Suzuki H, Kondo S, Tominaga T (1995) Vascular endothelial growth factor and platelet-derived endothelial cell growth factor are frequently coexpressed in highly vascularized human breast cancer. *Clin Cancer Res.* 1: 961–964

Toi M, Ueno T, Masumoto H, Saji H, Funata N, Koike M, Tominaga T (1999) Significance of thymidine phosphorylase as a marker of protumor monocytes in breast cancer. *Clin Cancer Res.* 5: 1131–1137

Tominaga T, Toi M, Ohashi Y, Abe O (2002) Prognostic and predictive value of thymidine phosphorylase activity in early-stage breast cancer patients. *Clin Breast Cancer* 3: 55–64

Yamamoto Y, Toi M, Tominaga T (1996) Prediction of the effect of 5′-deoxy-5-fluorouridine by the status of angiogenic enzyme thymidine phosphorylase expression in recurrent breast cancer patients. *Oncol Rep* 3: 863–865

Yang Q, Barbaresch M, Mori I, Mauri F, Muscara M, Nakamura M, Nakamura Y, Yoshimura G, Sakurai T, Caffo O, Galligioni E, Palma PD (2002) Prognostic value of thymidine phosphorylase expression in breast carcinoma. *Int J Cancer* 97: 512–517

Zhu GH, Lenzi M, Schwartz EL (2002) The Sp1 transcription factor contributes to the tumor necrosis factor-induced expression of the angiogenic factor thymidine phosphorylase in human colon carcinoma cells. *Oncogene* 21: 8477–8485

© 2004 Cancer Research UK

Presidential Symposium I

Current Status of Antibody Therapy for Breast Cancer

Masakazu Toi*[1], Masahiro Takada*[1], Hiroko Bando*[1], Kazumi Toyama*[1], Hiroyasu Yamashiro*[1], Shinichiro Horiguchi*[2], and Shigehira Saji*[1]

*[1]Department of Surgery, and *[2]Department of Pathology, Tokyo Metropolitan Cancer and Infectious Disease Center, Komagome Hospital, Japan.

Antibody therapy with trastuzumab has greatly impacted breast cancer treatment. Combination treatment with trastuzumab is regarded currently as a first-line therapy for metastatic breast cancers that overexpress Her-2. It has become routine practice to examine the status of Her-2 expression in primary tumors. The impact of this therapy might be as great as that of endocrine therapy from a historical point of view. A number of new approaches using trastuzumab for seeking individualized treatment are being tested in current clinical trials. We reviewed recent advances in trastuzumab treatment and discuss the future of antibody therapy for breast cancer.

Breast Cancer 11:10-14, 2004.

Key words: Her-2, Antibody, Trastuzumab, Breast cancer

Rationale of Clinical Development of Trastuzumab

It is widely accepted that primary breast cancer over-expressing Her-2 have a poor prognosis. Her-2 positive tumors are unlikely to respond to hormone therapy[1-4]. Since the expression level of Her-2 differs enormously between Her-2 positive tumors and Her-2 negative tumors, Her-2 is thought to be an ideal molecular target to treat. The Her-2 positive tumors often show more than 10 thousand times higher Her-2 protein expression compared with normal breast duct epithelium, which results in the therapeutic efficacy of anti-Her-2 therapy. This biological approach enables us to realize an individualized therapy for breast cancer.

From the beginning of clinical development, trastuzumab was used only for the patients with Her-2 overexpressing tumors, which was remarkably different from other previous anti-cancer agents[5-9]. The strategy used for trastuzumab was more like that used for hormone therapy, not for cytotoxic chemotherapy, although trastuzumab was used in combination with chemotherapy. The agents for combination with trastuzumab were chosen based upon *in vitro* and *in vivo* experimental data. In particular, the UCLA group made a significant contribution in producing the data and constructing the strategy[10, 11]. It was clarified that taxane, vinorelbine and platinums showed synergistic combination effects with trastuzumab against various types of Her-2 overexpressing cultured human breast cancer cells, whereas anthracyclines showed additive effects and FUs showed a rather antagonistic effect. In addition, a recent study confirmed that trastuzumab sensitizes the effect of paclitaxel *in vitro* and *in vivo* for Her-2 over-expressing human breast cancer cells[12].

Attention has been paid to standardizing the methods and assessment of Her-2 status determination. In the process of standardization, fluorescence *in situ* hybridization (FISH) assay was established as the most reliable methodology to identify overexpression of Her-2[13-15]. Various immunohistochemical (IHC) assays were tested and compared with FISH. At present, the consensus is that screening for Her-2 overexpression should be carried out by IHC assays like the Hercep test and FISH should be used to complement IHC for borderline cases, for example IHC 2+. In the clinical trials, the centralized evaluation system has become popular globally to obtain standardized high-quality data. It might be true that

Reprint requests to Masakazu Toi, Department of Surgery, Tokyo Metropolitan Komagome Hospital, 3-18-22, Honkomagome, Bunkyo-ku, Tokyo 113-8677, Japan.
E-mail: maktoi77@wa2.so-net.ne.jp

Abbreviations:
ER, Estrogen receptor; PgR, Progesterone receptor; ADR, Adriamycin; Epi, Epirubicin; CPA, Cyclophosphamide

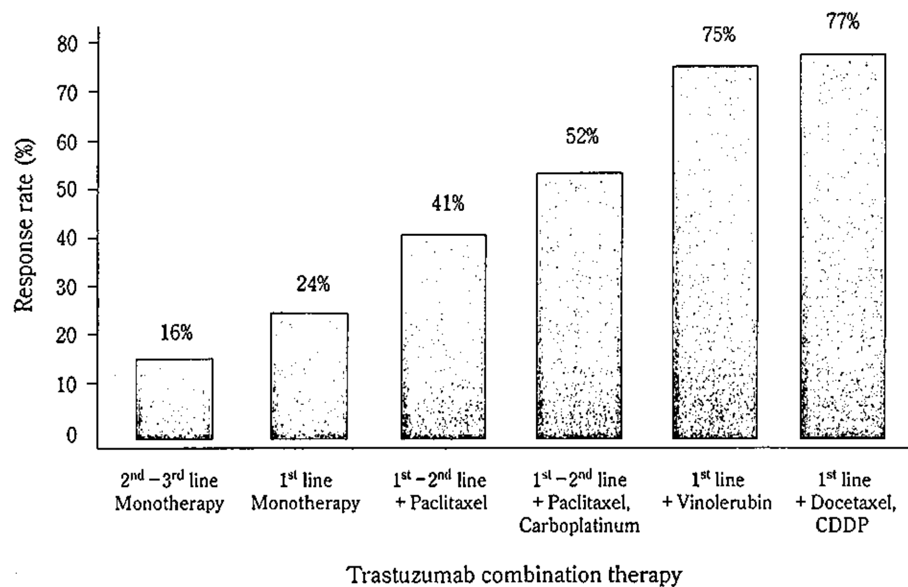

Fig 1. Increase in tumor response to trastuzumab combination therapy.

Her-2 testing has advanced more remarkably than any other diagnostic tool for cancer.

Several key questions are being tested in the on-going large-scale international collaboration studies. In particular, three important issues, the sensitizing effect of trastuzumab on taxanes, possible synergy between trastuzumab and platinums, and the time-dependent effect of trastuzumab, are under investigation in the adjuvant studies. A meta-analysis of these adjuvant trials promises to provide answers regarding the optimal use of trastuzumab for primary breast cancer within several years. A variety of translational research data are also incorporated into the clinical trials, which will enable more precise prediction and monitoring of the response and toxicity.

Recent Advances in Metastatic Disease

As shown in Fig 1, the response rates achieved with trastuzumab in combination with various types of chemotherapy for metastatic disease have increased. A pivotal study showed that trastuzumab in combination with paclitaxel achieved a 41% response rate, which was notably higher than that of monotherapy[7]. Recent regimens such as combination with vinorelbine and docetaxel and cisplatinum, have achieved clinical response rates of 70%. It is noteworthy that this increase in clinical response is compatible with the tendency observed in the preclinical experimental data[16]. More surprisingly, it was also confirmed that the survival advantage as assessed by time-to-progression (TTP) improved along with the improvement in response rate (Fig 2). According to a randomized clinical trial comparing paclitaxel plus trastuzumab and paclitaxel plus trastuzumab plus carboplatinum as first-line therapy for metastatic diseases, the median TTP of the three agent combination was 11.2 months whereas that of the two agent combination was 8.9 months, a statistically significant difference. Since similar results were obtained from two other clinical trials of taxanes plus platinum and trastuzumab in combination, the combination of trastuzumab with taxane and carboplatinum is thought to be one of the most potent regimens for Her-2 overexpressing tumors. This affect on metastatic diseases is being tested currently in the adjuvant setting.

Adjuvant Trials

Four large-scale postoperative adjuvant trials are on-going (Table 1). In NSABP B-31, the usefulness of concurrent trastuzumab and paclitaxel will be clarified. The intergroup study focuses upon the issue of whether concurrent or sequential use of trastuzumab is superior in combination with weekly paclitaxel treatment. BCIRG is investigating the synergy among docetaxel, carboplatin and trastuzumab. The HERA study examines trastuzumab treatment in comparison with no treatment. Presumably three years from now, the usefulness of trastuzumab for primary breast can-

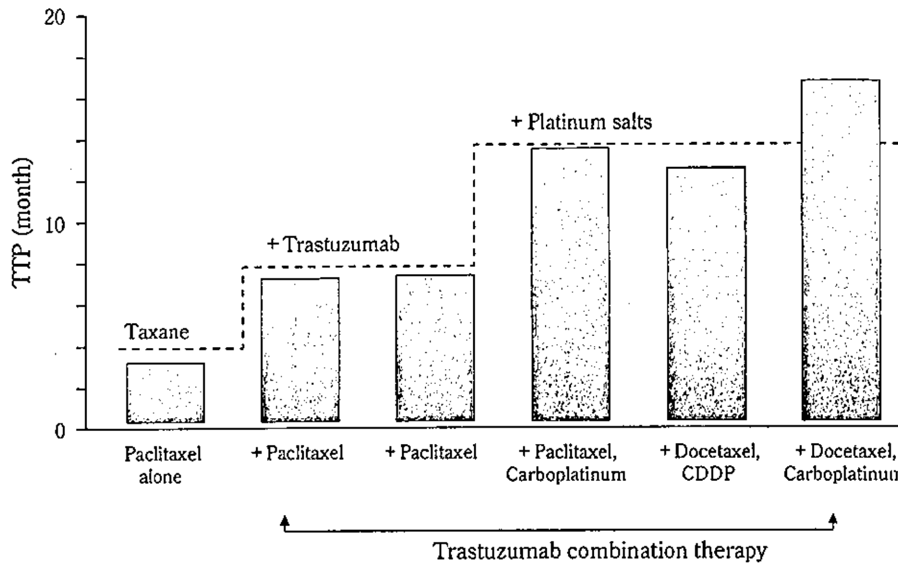

Fig 2. Survival improvement by trastuzumab combination therapy.

Table 1. Postoperative Adjuvant Studies with Trastuzumab

Study	Protocol	Implication
NSABP B-31	AC × 4 → Paclitaxel q3w × 4 AC × 4 → Paclitaxel q3w × 4 + H	Concurrent combination effect
Intergroup N9831	AC × 4 → Paclitaxel qw × 12 AC × 4 → Paclitaxel qw × 12 → H AC × 4 → Paclitaxel qw × 12 + H	Sequential combination effect
BCIRG 006	AC × 4 → Docetaxel q3w × 4 AC × 4 → Docetaxel q3w × 4 + H Carboplatin + Docetaxel q3w × 6 + H	Synergistic effect with platinum
HERA Trial	Any CT and/or RT → H q3w × 12 months Any CT and/or RT → H q3w × 24 months Any CT and/or RT → Observation	Time-depende effect

cer patients will become more obvious[9].

Issues in Clinical Practice

In clinical practice, we often encounter some issues regarding use of trastuzumab. First, an important issue is whether trastuzumab should be started as monotherapy or combination therapy. At present, as far as we know, there is no evidence in clinical trials to answer this question directly. As described already, theoretically, it would be more beneficial to use combination therapy to achieve a higher response rate and longer survival, especially for patients with life-threatening diseases such as multiple organ metastases. However, it is also true that even small population can obtain a reasonable survival outcome from monotherapy without any significant toxicity[6]. The patients with non-life-threatening diseases such as few metastases in the lungs or minor soft tissue recurrences might be candidates for monotherapy. Taken together, trastuzumab monotherapy would not be the standard of care but rather an option for non-life-threatening disease, especially as first-line therapy. In cases for which it is difficult to assess whether the disease is life-threatening or not, combination therapy should be used for avoiding loss of the survival benefit. In patients who are both hormone recep-

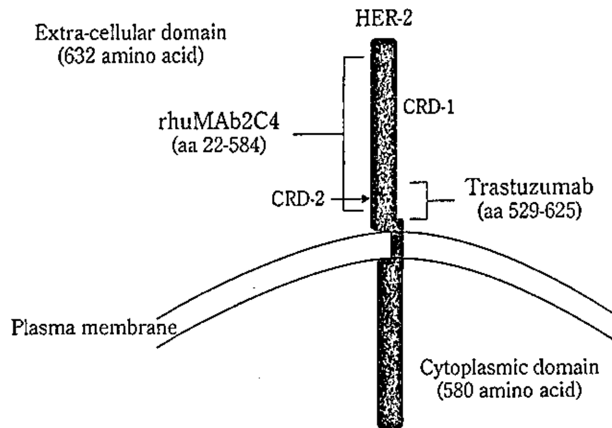

Fig 3. The difference between trastuzumab and 2C4 in the binding site of the extracellular domain of Her-2.

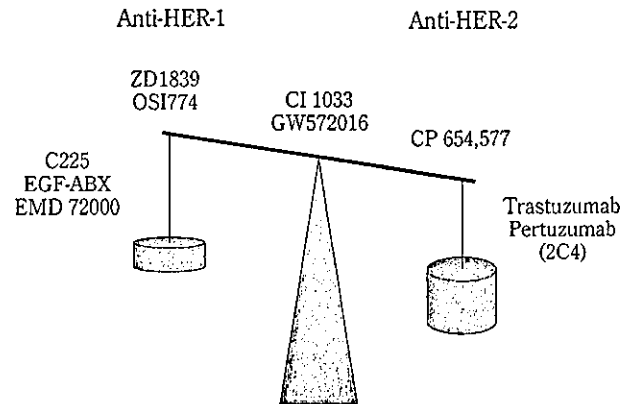

Fig 4. Her-1 inhibitors and Her-2 inhibitors.

tor and Her-2 positive, an alternative is initial hormone therapy or trastuzumab-containing treatment. At the 2002 ASCO symposium, the expert panel recommended starting with hormone therapy if the disease is not life-threatening, because there is no evidence to suggest the superiority of trastuzumab treatment over hormone therapy in hormone-dependent tumors. At the presidential symposium of the 2003 Japan Breast Cancer meeting, the panelists agreed with the same strategy. It is an interesting idea to try combination therapy with hormone therapy and trastuzumab for hormone receptor positive and Her-2 positive patients, however we haven't yet obtained enough data from clinical trials.

Future Perspectives

Although trastuzumab is a powerful tool to prolong the survival of Her-2 positive patients, about half of the patients lose disease control within a year and a half, indicating that we need novel approaches to overcome resistance to trastuzumab treatment. An approach was recently documented with a different anti-Her-2 antibody. The antibody 2C4, which detects a different site of external domain of Her-2 protein as shown in Fig 3, achieved a response in tumors resistant to trastuzumab in animal experiments[17]. This antibody is being tested in clinical trials currently. If the clinical efficacy of 2C4 is proven, the combined use of two Her-2 antibodies will be studied, which will likely block Her-2 function more completely. Similarly, the strategy of combination therapy with other Her inhibitors such as Her-1 may help to suppress the appearance of resistance (Fig 4). Experimentally, several studies proved this hypothesis successfully[18,19]. Since ligands of Her-1 such as epidermal growth factor and transforming growth factor α may possibly bind to Her-1 in the presence of trastuzumab, dual suppression of Her-2 and Her-1 might act synergistically to inhibit the Her-dependent growth of tumor cells.

The neoadjuvant use of trastuzumab is also intriguing, and achieving the maximal effect of the agent and selecting the responders more strictly is important. It is generally accepted that early tumors are more sensitive to the treatment than advanced tumors. A recent neoadjuvant study documented a 25% pathological complete response (pCR) rate from treatment with 4-cycles of q3 paclitaxel and trastuzumab[20]. This response rate is surprisingly high because the pCR rate of q3 paclitaxel alone was much lower in previous studies[21]. It is remarkable that the pCR rate of 4 cycles of q3 paclitaxel and trastuzumab was comparable to the pCR rate of 4-cycle adriamycin plus cyclophosphamide followed by 4-cycle docetaxel in the NSABP B-27 study. The selection of the patients by Her-2 testing seems to have achieved such a high response. Next, whether the short-term response in the neoadjuvant setting promises a long-term survival benefit in the postoperative adjuvant setting should be studied.

At last, the clinical development of antibody treatment is in the dynamic phase in breast cancer. We have seen already how a novel molecular targeting therapy has changed the paradigm of breast cancer treatment significantly. Other antibodies like 2C4 and anti-vascular endothelial growth factor antibody will come to the clinical

stage in the near future. Because of the less toxic profile and the high target specificity, the antibody therapies will play increasingly important roles in the treatment of breast cancer.

References

1) Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL: Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 235:177-182, 1987.
2) Gusterson B, Gelber R, Goldhirsch A, Price KN, Save-Soderborgh J, Anbazhagan R, Styles J, Rudenstam CM, Golouh R, Reed R: Prognostic importance of c-erbB-2 expression in breast cancer. International (Ludwig) Breast Cancer Study Group. *J Clin Oncol* 10:1049-1056, 1992.
3) Muss HB, Thor AD, Berry DA, Kute T, Liu ET, Koerner F, Cirrincione CT, Budman DR, Wood WC, Barcos M, Henderson IC: c-erbB-2 expression and response to adjuvant therapy in women with node-negative early breast cancer. *N Engl J Med* 330:1260-1266, 1994.
4) Thor AD, Berry DA, Budman DR, Muss HB, Kute T, Henderson IC, Barcos M, Cirrincione C, Edgerton S, Allred C, Norton L, Liu ET: erbB-2, P53, and efficacy of adjuvant therapy in lymph node-positive breast cancer. *J Natl Cancer Inst* 90:1346-1360, 1998.
5) Cobleigh MA, Vogel CL, Tripathy D, Robert NJ, Scholl S, Fehrenbacher L, Wolterr JM, Paton V, Shak S, Lieberman G, Slamon DJ: Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpression metastatic breast cancer that has progressed after chemotherapy for metastatic disease. *J Clin Oncol* 17:2639-2648, 1999.
6) Vogel CL, Cobleigh MA, Tripathy D, Gutheil JC, Harris LN, Fehrenbach L, Slamon DJ, Murphy M, Novotny WF, Burchmore M, Shak S, Stewart SJ, Press M: Efficacy and safety of Trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. *J Clin Oncol* 20:719-726, 2002.
7) Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, Baselga J, Norton L: Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* 344:783-792, 2001.
8) Seidman A, Hudis C, Pierri MK, Shak S, Paton V, Ashby M, Murphy M, Stewart SJ, Keefe D: Cardiac dysfunction in the Trastuzumab clinical trials experience. *J Clin Oncol* 20:1215-1221, 2002.
9) Piccart-Gebhart MJ: Herceptin: the future in adjuvant breast cancer therapy. *Anti-Cancer Drug* 12:S27-S33, 2001.
10) Pegram M, Hsu S, Lewis G, Pietras R, Beryt M, Sliwkowski M, Coombs BD, Kabbinavar F, Slamon D: Inhibitory effects of combinations of HER-2/neu antibody and chemotherapeutic agents used for treatment of human breast cancers. *Oncogene* 18:2241-2251, 1999.
11) Pegram MD: Docetaxel and herceptin: foundation for future strategies. *Oncologist* 6 Suppl 3:22-25, 2001.
12) Lee S, Yang W, Lan KH, Sellappan S, Klos K, Hortobagyi G, Hung MC, Yu D: Enhanced sensitization to taxol-induced apoptosis by herceptin pretreatment in ErbB2-overexpressing breast cancer cells. *Cancer Res* 62:5703-5710, 2002.
13) Dowsett M, Bartlett J, Ellis IO, Salter J, Hills M, Mallon E, Watters AD, Cooke T, Paish C, Wencyk PM, Pinder SE: Correlation between immunohistochemistry (HercepTest) and fluorescence in situ hybridization (FISH) for HER-2 in 426 breast carcinomas from 37 centres. *J Pathol* 199:411-417, 2003.
14) O'Grady A, Flahavan CM, Kay EW, Barrett HL, Leader MB: HER-2 Analysis in Tissue Microarrays of Archival Human Breast Cancer: Comparison of Immunohistochemistry and Fluorescence In Situ Hybridization. *Appl Immunohistochem Mol Morphol* 11:177-182, 2003.
15) Umemura S, Sakamoto G, Sasano H, Tsuda H, Akiyama F, Kurosumi M, Tokuda Y, Watanabe T, Toi M, Hasegawa T, Osamura RY: Evaluation of HER2 status: for the treatment of metastatic breast cancers by humanized anti-HER2 Monoclonal antibody (trastuzumab) (Pathological committee for optimal use of trastuzumab). *Breast Cancer* 8:316-320, 2001.
16) Burstein HJ, Kuter I, Campos SM, Gelman RS, Tribou L, Parker LM, Manola J, Younger J, Matulonis U, Bunnell CA, Partridge AH, Richardson PG, Clarke K, Shulman LN, Winer EP: Clinical activity of Trastuzumab and vinorelbine in women with HER2-overexpressing metastatic breast cancer. *J Clin Oncol* 19:2722-2730, 2001.
17) Agus DB, Akita RW, Fox WD, Lewis GD, Higgins B, Pisacane PI, Lofgren JA, Tindell C, Evans DP, Maiese K, Scher HI, Sliwkowski MX: Targeting ligand-activated ErbB2 signaling inhibits breast and prostate tumor growth. *Cancer Cell* 2:127-137, 2002.
18) de Bono JS, Rowinsky EK: The ErbB receptor family: a therapeutic target for cancer. *Trends Mol Med* 8:S19-26, 2002.
19) Normanno N, Campiglio M, De LA, Somenzi G, Maiello M, Ciardiello F, Gianni L, Salomon DS, Menard S: Cooperative inhibitory effect of ZD1839 (Iressa) in combination with trastuzumab (Herceptin) on human breast cancer cell growth. *Ann Oncol* 13:65-72, 2002.
20) Burstein HJ, Harris LN, Gelman R, Lester SC, Nunes RA, Kaelin CM, Parker LM, Ellisen LW, Kuter I, Gadd MA, Christian RL, Kennedy PR, Borges VF, Bunnell CA, Younger J, Smith BL, Winer EP: Preoperative therapy with trastuzumab and paclitaxel followed by sequential adjuvant doxorubicin/cyclophosphamide for HER2 overexpressing stage II or III breast cancer: a pilot study. *J Clin Oncol* 21:46-53, 2003.
21) Smith I, Lipton L: Preoperative/neoadjuvant medical therapy for early breast cancer. *Lancet Oncol* 2:561-570, 2001.