^3H_2O released/min/mg protein. Protein concentration of the supernatant of the cell homogenate was determined by BCA Protein Assay Kit (Takara Bio Inc., Otsu, Japan).

Human breast cancer xenograft models. The premenopausal breast cancer xenograft model was established as follows. A suspension of MCF-7 cells (5x10^6 cells/mouse) was subcutaneously inoculated into the right flank of the BALB/c-nu/nu mice which had been subcutaneously implanted with slow-release estrogen pellets (0.25 mg/pellet 17β-estradiol; Innovative Research of America, Sarasota, FL) the day before tumor cell inoculation. Several weeks after tumor inoculation, mice bearing a tumor of ~200-400 mm^3 in volume were selected and were randomly allocated to control and treatment groups. Each group consisted of 8 mice. The mice received 6-week oral therapy with capecitabine (days 1-14, every 3 weeks) at maximum tolerated dose (MTD; 539 mg/kg/day) or two thirds MTD (359 mg/kg/day) and/or tamoxifen daily for 6 weeks at doses of 30 or 100 mg/kg/day.

The postmenopausal breast cancer xenograft model was established as follows. BALB/c-nu/nu mice were ovariectomized and subcutaneously implanted with slow-release androstenedione pellets (1.5 mg/pellet; Innovative Research of America) the day before tumor cell inoculation. MCF-7A25F3 cells (5x10^6 cells/mouse) were subcutaneously inoculated into the right flank of the mice. Mice bearing a tumor of ~200-400 mm^3 in volume were selected and were randomly allocated to control and treatment groups of 5 mice each. The mice received 6-week oral administration of capecitabine (days 1-14, every 3 weeks) at two thirds MTD and/or letrozole, at 0.1 mg/kg/day. In each model, control mice received vehicle alone.

Tumor volumes (V) were estimated from the equation $V = ab^2/2$, where a and b are the length and width of the tumor, respectively. Tumor volumes and body weights were monitored two or three times a week starting from the first day of the treatment.

Measurement of thymidine phosphorylase (TP). The tumor tissues were homogenized with a glass homogenizer in 10 mmol/l Tris buffer (pH 7.4) containing 15 mmol/l NaCl, 1.5 mmol/l $MgCl_2$ and 50 µmol/l potassium phosphate. The homogenates were then centrifuged at 10,000 x g for 20 min at 4˚C, and the supernatants were stored at -80˚C until use. The TP level was measured by ELISA using monoclonal antibodies specific to human TP as described previously by Nishida *et al* (18). One unit corresponds to the TP level of the standard enzyme solution (extracts of human colon cancer HCT116 xenograft) which converts 5'-DFUR to 5-FU at a rate of 1 µg 5-FU/h (18). The protein concentration was determined using DC protein Assay Kit (Bio-Rad Laboratories, Hercules, CA).

Preparation of estrogen-responsive element-green fluorescence protein transfected cell line, MCF-7E10. The procedure for preparation of estrogen-responsive element-green fluorescence protein (ERE-GFP)-transfected cell line was described previously (16). Briefly, MCF-7 cells were transfected with the d2E-green fluorescent protein (GFP) vector alone (Clontech Lab., Inc., Palo Alto, CA) or carrying the ptk-estrogen-responsive element (ERE) insert using Trans IT LT-1 reagent (Takara Shuzo Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. After 24 h, the cells were subjected to selection in growth medium containing geneticin (1 mg/ml). The ERE-GFP-MCF-7 clone that expressed high levels of GFP in the presence of estrogen, but not in the absence of estrogen, was selected (MCF-7E10).

Isolation of primary stromal cells from tumors and preparation of breast cancer extract. To obtain breast cancer extract, tissue specimens obtained from three premenopausal patients were minced in phenol red-free RPMI-1640 medium (PRF-RPMI; Sigma-Aldrich Co.) and suspended. The suspension was centrifuged (600 x g, 10 min, 4˚C) and the supernatant was further subjected to centrifugation (12,000 x g, 10 min, 4˚C) to obtain breast cancer extract. The isolation procedure of cancer stromal cells is similar to that described by Ackerman *et al* (19). The breast cancer tissue specimen obtained from a postmenopausal patient was minced in PRF-RPMI and suspended. The suspension was centrifuged (600 x g, 10 min, 4˚C) and the pellet was washed with Hanks' balanced salt solution (HBSS: Sigma-Aldrich Co.) and treated with the mixture of collagenase (1 mg/ml: Nitta Gelatin Co. Ltd., Osaka Japan), bovine serum albumin (40 mg/ml: Sigma-Aldrich Co.), glucose (2 mg/ml: Wako Pure Chemical Industries, Osaka, Japan), antibiotic-antimycotic (Invitrogen, Corp.) and gentamicin (50 µg/ml: Schering-Plough, NJ) for 2-3 h at 37˚C followed by filtration through nylon mesh. The cells, including stromal cells, were recovered by centrifugation (600 x g, 10 min, 4˚C) and washed several times with HBSS. The cells were suspended in MEM-α medium (Invitrogen, Corp.) containing 10% FBS (Tissue Culture Biologicals, Turale, CA) and cultured at 37˚C in a humidified atmosphere of 5% CO_2 in air.

The study was approved by the Saitama Cancer Center (Saitama, Japan) Ethics Committee. Human breast cancer tissues were obtained from surgical specimens at the Saitama Cancer Center Hospital after obtaining informed consent from the patients.

Breast cancer extract ERE reporter gene assay and coculture ERE reporter gene assay. The breast cancer extract ERE reporter gene assays were carried out according to the method of Yamaguchi *et al* (20). The MCF-7E10 cells were cultured with 5% (v/v) breast cancer tissue extract for 4 days in the presence of 5'-DFUR and/or 4-OHT. The cells were collected by mild trypsinization, and the number of cells expressing GFP was counted under fluorescence microscopy (16).

Coculture ERE reporter gene assays were performed as previously described (16). Briefly, MCF-7E10 cells and the stromal cells were precultured in PRF-RPMI containing 10% dextran-coated, charcoal-treated FBS (DCC-FBS; Hyclone Lab., UT) for 72 h. Harvested stromal cells were seeded at 5x10^4/ml in a 24-well multidish, and the next day, 5x10^4 MCF-7E10 cells were seeded on top of the adipose stromal cells with testosterone at 10^{-7} mol/l as a substrate for aromatase. After being cocultured for 4 days in the presence of 5'-DFUR, an intermediate of capecitabine, and/or letrozole, the cells were collected by mild trypsinization, and the number of cells expressing GFP was counted under fluorescence microscopy. Data are expressed as a percentage of cells expressing GFP. The isobole method was performed in each experiment to analyze the mode of interaction between capecitabine and

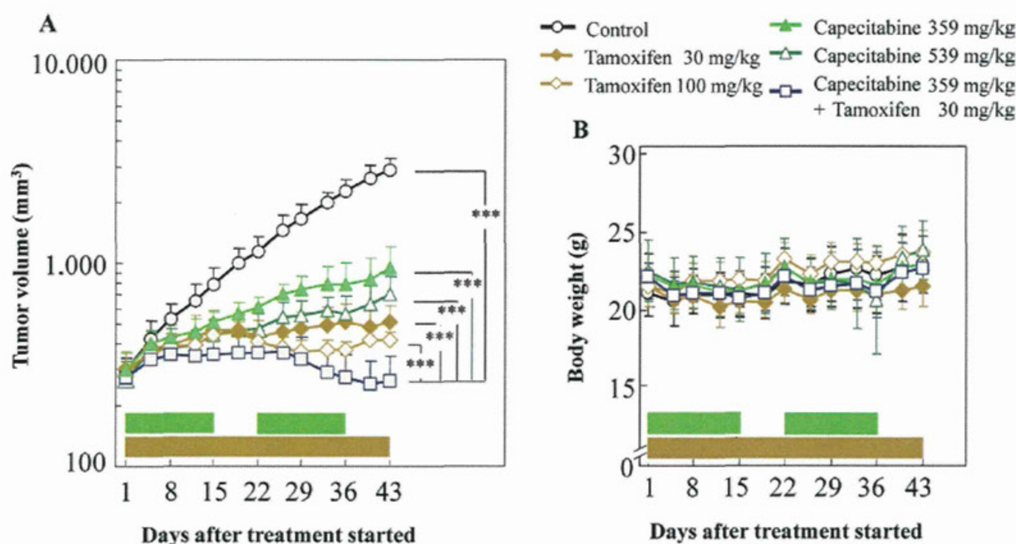

Figure 1. Antitumor activity of combination therapy of capecitabine with tamoxifen in a premenopausal breast cancer xenograft model. Mice were randomized into 6 groups of 8 mice each. Capecitabine (359 or 539 mg/kg) was orally administered once a day from day 1 to day 14 and day 22 to day 35 (green horizontal bars). Tamoxifen (30 or 100 mg/kg) was orally administered once a day for 6 weeks (brown horizontal bar). Data points indicate mean ± SD of tumor volume (A) and mean ± SD of body weight (B). Statistically significant differences versus capecitabine 359 mg/kg + tamoxifen 30 mg/kg group are shown with asterisks: ***p<0.001 by Wilcoxon test.

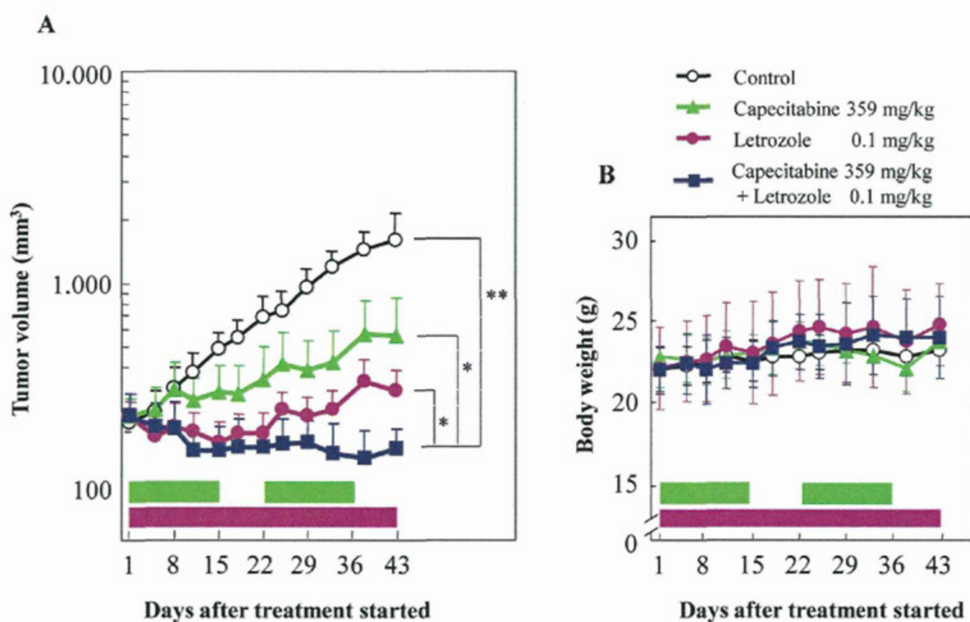

Figure 2. Antitumor activity of combination therapy of capecitabine with letrozole in a postmenopausal breast cancer xenograft model. Mice were randomized into 4 groups of 5 mice each. Capecitabine (359 mg/kg) was orally administered once a day from day 1 to day 14 and day 22 to day 35 (green horizontal bars). Letrozole (0.1 mg/kg) was orally administered once a day for 6 weeks (purple horizontal bar). Data points indicate mean ± SD of tumor volume (A) and mean ± SD of body weight (B). Statistically significant differences versus capecitabine 359 mg/kg + letrozole 0.1 mg/kg group are shown with asterisks: *p<0.05, **p<0.01 by Wilcoxon test.

4-OHT/letrozole according to the method of Berenbaum MC (21).

Statistical analysis. The Wilcoxon test was used to detect the statistical differences in tumor volume, body weight and TP level. Statistical analyses were carried out using the SAS preclinical package (version 5.0; SAS Institute Inc., Tokyo, Japan). Differences were considered to be significant at p<0.05.

Results

Establishment of MCF-7 aromatase-transfectant clone. Three aromatase-transfectant clones were selected by *in vitro* screening. The 3 clones were inoculated into ovariectomized BALB/c-nu/nu mice which had been implanted with slow-release estradiol or androstenedione pellets to determine the tumor growth in response to each hormone *in vivo*. The clone, MCF-7A25F3,

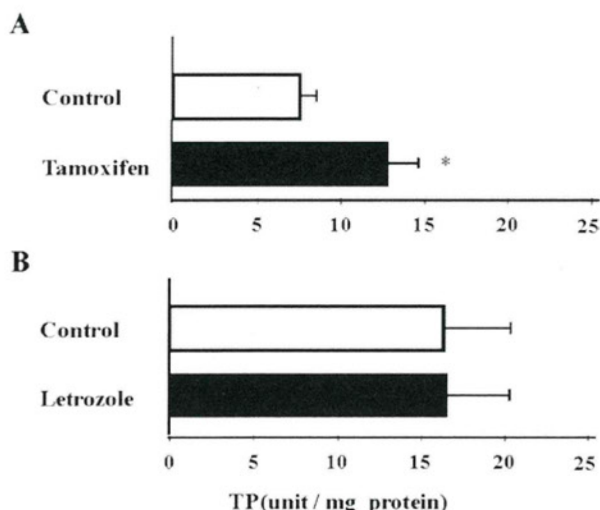

Figure 3. TP activity in tumor tissue after tamoxifen or letrozole treatment. Mice inoculated with MCF-7 or MCF-7A25F3 cells were randomly assorted to groups of 4 or 5 mice each. Tamoxifen (A) or letrozole (B) was orally administered once a day for 3 weeks and tumor tissues were excised on day 22. TP levels in tumor tissues were measured by ELISA. Data bars indicate mean ± SD. Statistically significant differences versus control group are shown with asterisks: *p<0.05 by Wilcoxon test.

which showed the highest tumor growth was selected (data not shown). The MCF-7A25F3 cells were maintained in the same medium as MCF-7 cells supplemented with 10% DCC-FBS and 350 μg/ml geneticin. The tumor of parent MCF-7 cells grew in BALB/c-nu/nu mice with estrogen pellet but not in BALB/c nu/nu mice with androstenedione pellet (data not shown). The aromatase activity in MCF-7A25F3 cell homogenate was 420-fold higher (50.4 fmol/min/mg protein) than that of parent MCF-7 (0.12 fmol/minute/mg protein) measured by the ^3H_2O release from 1β-^{3}H-androstenedione. To confirm the androstenedione-dependent growth of MCF-7A25F3 cells, tumor growth inhibition by letrozole was examined *in vivo*. MCF-7A25F3 cells were inoculated into BALB/c nu/nu mice which had been ovariectomized and subcutaneously implanted with androstenedione pellets. BALB/c nu/nu mice received daily oral administration of 0.03-3 mg/kg of letrozole for 6 weeks. Letrozole significantly inhibited the tumor growth in a dose-dependent manner with the maximum effect at 1-3 mg/kg (data not shown).

Antitumor activity of capecitabine in combination with tamoxifen in a premenopausal xenograft model. The antitumor activity of capecitabine in combination with tamoxifen was evaluated using MCF-7 premenopausal breast cancer xenograft model. The dosages of these agents were determined by a preliminary dose-finding experiment (data not shown). To detect an interaction between the two agents distinctly, suboptimal doses of each drug were used in combination therapy. The MTD for capecitabine administered daily for 2 weeks in 3-week cycles in this model was determined as 539 mg/kg/day in a previous experiment (22,23). The daily oral administration of tamoxifen for 6 weeks showed maximum antitumor activity at a dose of 100 mg/kg. Therefore, in the present combination therapy experiments, 359 mg/kg (2/3 MTD) for capecitabine, and the dose of 30 mg/kg (1/3 of maximum effective dose) for tamoxifen were selected.

As shown in Fig. 1A, both capecitabine and tamoxifen showed only moderate antitumor activities at selected doses for each agent. In contrast, the antitumor activity of combination therapy was significantly superior to the antitumor activities of monotherapy of each agent (p<0.001). In addition, the combination therapy produced statistically significant inhibition of tumor growth compared with tamoxifen at 100 mg/kg (p<0.001) or capecitabine at 539 mg/kg in monotherapy. None of the treatment groups in this experiment showed significant weight loss during the treatments (Fig. 1B).

Antitumor activity of capecitabine in combination with letrozole in a postmenopausal xenograft model. The anti-tumor activity of capecitabine in combination with letrozole was evaluated in the postmenopausal breast cancer xenograft model using MCF-7A25F3 cells. To detect the interaction between the two agents distinctly, the dose of 0.1 mg/kg for letrozole, which was 1/30 of the maximum effective dose (data not shown) and the dose of 359 mg/kg (2/3 MTD) for capecitabine, were used for combination therapy as a suboptimal dose in each monotherapy. The combination therapy of capecitabine with letrozole showed significantly higher antitumor activity than each monotherapy (Fig. 2A). None of the treatment groups in this experiment showed significant weight loss during the treatments (Fig. 2B).

TP levels in the tumor tissues. Mice inoculated with MCF-7 or MCF-7A25F3 cells were randomly assorted into groups of 4 and 5 mice each, respectively. The TP levels in tumor tissues were measured 22 days after treatment started. The TP level of MCF-7 tumor was significantly increased (12.8±2.0 U/mg protein) by tamoxifen (30 mg/kg/day) treatment for 3 weeks compared with vehicle-treated group (7.6±1.1 U/mg protein) (Fig. 3A). On the other hand, the TP level of MCF-7A25F3 tumor was higher (16.4±3.8 U/mg protein) than that of MCF-7 parent tumor and no further upregulation of TP was observed by 0.1 mg/kg of letrozole treatment (16.6±3.8 U/mg protein) (Fig. 3B).

Breast cancer extract ERE reporter gene assay and coculture ERE reporter gene assay. The inhibitory effect of 5'-DFUR in combination with 4-OHT, an active form of tamoxifen, on estrogen-responding cells was evaluated using breast cancer extract ERE reporter gene assay (Fig. 4A). The percentage of GFP-positive cells was decreased by the addition of 5'-DFUR or 4-OHT in a concentration-dependent manner. The analysis by isobole method showed a synergistic effect of 5'-DFUR and 4-OHT (Fig. 4B).

The inhibitory effect of 5'-DFUR in combination with letrozole on estrogen-responding cells was evaluated using coculture ERE reporter gene assay (Fig. 4C). The percentage of GFP-positive cells was decreased by the addition of 5'-DFUR or letrozole in a concentration-dependent manner. The isobole analysis showed an additive effect of 5'-DFUR and letrozole (Fig. 4D).

Discussion

The combination effects of antiestrogens and chemotherapeutic agents have been controversial for many years. Specifically,

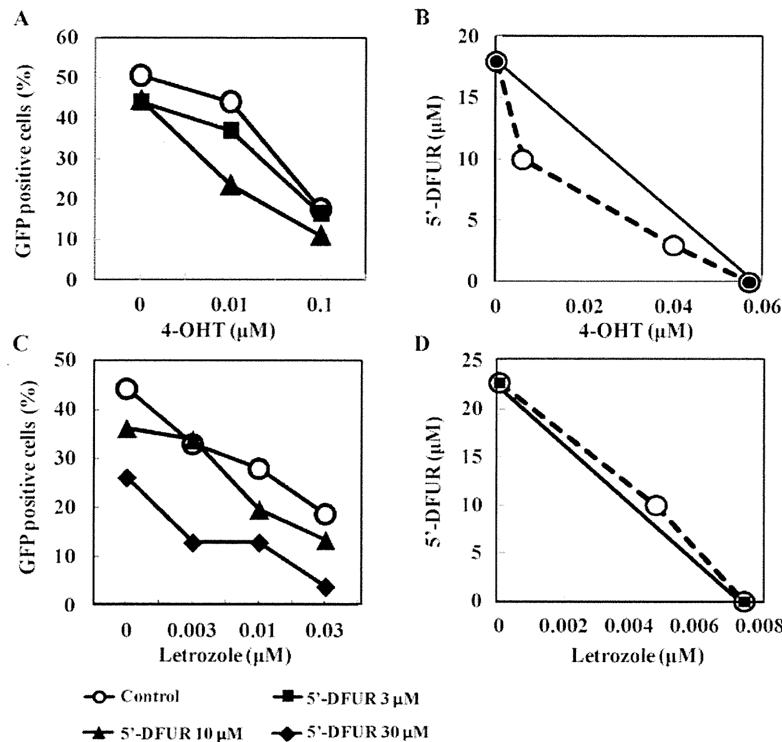

Figure 4. Combination effect of 5'-DFUR with 4-OHT, or 5'-DFUR with letrozole in ERE reporter gene assay. The MCF-7E10 cells were cultured with various concentrations of 5'-DFUR and 4-OHT in the presence of breast cancer extract for 4 days, and the number of cells expressing GFP was counted. Data were expressed as a percentage of GFP-expressing cells against the observed number of MCF-7E10 cells (A). Isobolograms of the interactions between 5'-DFUR and 4-OHT were taken at the point of 30% GFP-positive cells (B). The MCF-7E10 cells were cultured with various concentrations of 5'-DFUR and letrozole in the presence of breast cancer stromal cells for 4 days, and the number of cells expressing GFP was counted. Data were expressed as a percentage of GFP-expressing cells against the observed number of MCF-7E10 cells (C). Isobolograms of the interactions between 5'-DFUR and letrozole were taken at the point of 30% GFP-positive cells (D).

chemoendocrine combinations of tamoxifen with chemotherapy containing 5-FU revealed both positive and negative results. Fisher *et al* reported the prolongation of the disease-free interval in the therapy of tamoxifen with L-phenylalanine mustard and 5-FU in patients with primary operable breast cancer (4). The IBCSG Trial VII reported that early CMF added to tamoxifen significantly improved 5-year disease-free survival in post-menopausal breast cancer patients (5). On the other hand, Rivkin *et al* reported that combination treatment of CMFVP (cyclophosphamide, methotrexate, fluorouracil, vincristine, predonisone) chemotherapy with tamoxifen has not been shown to be superior to tamoxifen alone in the treatment of postmenopausal women with node-positive, ER-positive, operable breast cancer (2). Pritchard *et al* reported that there were no significant differences in overall survival, recurrence-free survival, locoregional recurrence-free survival and distant recurrence-free survival between the tamoxifen treatment group and tamoxifen plus CMF group in postmenopausal breast cancer patients (3). Perry *et al* reported that no difference was evident among postmenopausal patients in overall response rate and duration of responses between CAF (cyclophosphamide, adriamycin, 5-fluorouracil) and CAF plus tamoxifen, and that no difference of response rates was shown among premenopausal patients between CAF and CAF plus tamoxifen (24). However, in *in vitro*, a study on mammary carcinoma cell lines showed that the cytotoxic effects of cotreatment of 5'-DFUR with tamoxifen were dependent on tamoxifen concentration and treatment duration (25). Since 5'-DFUR is known to be converted to 5-FU by TP and it was also reported that tamoxifen upregulated TP in breast cancer cells (26), data suggest that there is a mechanism for a potentially favorable outcome of chemoendocrine therapy with tamoxifen and such chemotherapeutic agents as were activated by TP.

In this study, we showed the antitumor efficacy of chemo-endocrine therapy using capecitabine as a chemotherapeutic agent, in premenopausal as well as postmenopausal breast cancer xenograft models. Capecitabine has been approved for breast cancer in many countries due to proven efficacy and tolerability in patients with metastatic breast cancer (27,28). Tamoxifen has been the most widely used anti-hormonal agent for many years in the first line treatment of ER-positive breast cancers, and has a profound impact on breast cancer mortality (8,9,29). Letrozole has been used for the breast cancers of postmenopausal women, and Mouridsen *et al* demonstrated the superiority of letrozole to tamoxifen in first-line endocrine therapy in postmenopausal breast cancer patients (30).

In the present study, we examined the antitumor efficacy of combination therapy of capecitabine with tamoxifen or with letrozole in premenopausal and postmenopausal status, respectively. For a premenopausal model, ER-positive MCF-7 human breast cancer cells were used. For a postmenopausal model, we prepared the MCF-7 aromatase transfectant clone, MCF-7A25F3. The feature of MCF-7A25F3 cells as aromatase

transfectants was confirmed by the estradiol- and androstenedione-dependent growth *in vivo* and the increased (420-fold) aromatase activity in MCF-7A25F3 cells compared with parent MCF-7 cells.

Using the established premenopausal and postmenopausal human breast cancer xenograft models, we evaluated the antitumor efficacy of combination therapy of capecitabine with tamoxifen or letrozole. In each model, dose and schedule of capecitabine was determined according to the study of Sawada *et al* (22,23). To make the combination effect clear, MTD or sub-MTD of capecitabine and suboptimal dose of tamoxifen or letrozole were used according to the preliminary experiments (data not shown). In both models, combination therapy clearly showed the supra-additive antitumor activity to either monotherapy. In the premenopausal model, the combination therapy of capecitabine at 359 mg/kg (2/3 MTD) with tamoxifen at 30 mg/kg showed a significantly higher antitumor activity than each monotherapy (Fig. 1A). The combination therapy showed higher antitumor activity than MTD (539 mg/kg) of capecitabine or 100 mg/kg of tamoxifen. In the postmenopausal model, the combination therapy of capecitabine at 2/3 MTD with letrozole at 0.1 mg/kg showed significantly higher antitumor activity than either monotherapy (Fig. 2A). Concerning the therapeutic experiment of breast cancer aromatase transfectant with letrozole, Long *et al* reported that suppression of tumor growth was observed for at least 44 weeks in MCF-7Ca aromatase transfectant xenograft model in which letrozole was subcutaneously injected daily (10 μg/day) (31). However, we showed the antitumor effect of letrozole by oral administration, which is consistent with the clinical administration route. No augmentation of toxicity in terms of body weight loss was observed in the combination therapy in either model (Figs. 1B and 2B). The results indicate that both chemo-endocrine therapy regimens may be applicable to breast cancer patients.

Capecitabine generates the active substance 5-FU selectively in tumors by using three enzymes located in the liver and in tumors (22,23,32). The final step is the conversion of the intermediate metabolite 5'-DFUR to 5-FU by TP in tumors and this conversion appeared to be a rate-limiting step for the efficacy of capecitabine. Ishikawa *et al* reported that the efficacy of capecitabine correlated with TP activity in tumor tissues using human cancer xenograft models (6). TP is upregulated in tumors by receiving stress such as hypoxia, radiation and chemotherapeutic damage. Several types of cytokines such as interleukin-1, tumor necrosis factor-α, interferon-γ also upregulate the expression of TP in malignant cells (33). Endo *et al* have previously reported that several types of antitumor drugs, such as taxanes, cyclophosphamide and oxaliplatin, upregulated TP in tumor tissues and that capecitabine in combination with these TP upregulators showed synergistic antitumor activity in xenograft models (32). Ustunomiya *et al* reported the upregulation of TP in T47D breast cancer cells treated with tamoxifen *in vitro* (26) and also Evans *et al* reported the induction of hypoxia by tamoxifen in MCF-7 xenografts (34). These findings prompted us to examine the TP activity in the tumor tissue of mice treated with tamoxifen and letrozole. Consequently, we found that the TP activity in MCF-7 tumor tissue of mice treated with tamoxifen was significantly increased compared to that of control mice in the premenopausal model (Fig. 3A). Thus, the increased efficacy of combination therapy of capecitabine with tamoxifen may, at least partly, be explained by the upregulation of TP by tamoxifen in tumor tissue facilitating the conversion of capecitabine to 5-FU. On the other hand, in the postmenopausal model, the increased efficacy of combination therapy of capecitabine with letrozole could not be explained by TP upregulation because no TP increase was observed by letrozole treatment, suggesting that mechanisms other than TP upregulation are involved in the efficacy of this combination therapy (Fig. 3B).

Next, we tried to analyze the effect of the combinations capecitabine with tamoxifen or capecitabine with letrozole *in vitro*. For this purpose, we established ERE reporter gene assay, to mimic premenopausal or postmenopausal breast cancer (16,20). For the combination effect of capecitabine with tamoxifen, MCF-7E10 cells were cultured with breast cancer extract in the presence of various concentrations of 5'-DFUR and 4-OHT, an active metabolite of tamoxifen (35). To make the assay system closer to the clinical conditions of premenopausal breast cancer, human breast cancer extracts were used as a source of estrogen and other unknown factors which may affect the ER signals and cell proliferation. Both 5'-DFUR and 4-OHT decreased the percentage of GFP-positive cells in a dose-dependent manner. The analysis by isobole method showed the mode of the combination effect of the two drugs to be synergistic. The mechanism of the synergy was considered to come from the TP upregulation in MCF-7E10 cells by tamoxifen facilitating the generation of 5-FU from 5'-DFUR. The combination effect of capecitabine with letrozole was examined using coculture ERE reporter gene assay which corresponds to postmenopausal breast cancer. In this assay, exogenously added testosterone was converted into estrogen by aromatase in stromal cells isolated from breast cancer tissues. Both 5'-DFUR and letrozole decreased the percentage of GFP-positive cells in a dose-dependent manner. The analysis by isobole method showed the mode of the combination effect of the two drugs to be additive. The results indicated that 5'-DFUR and letrozole work independently and do not interfere with each other. Unexpectedly, the results of the *in vitro* letrozole experiment did not coincide with the *in vivo* results. The reason for this discrepancy could not be delineated in the present study.

In conclusion, several types of therapeutic agents including chemotherapeutic agents, endocrine therapy agents and molecular targeted medicines are now available for the treatment of breast cancer. To conduct clinical studies, research demonstrating the scientific rationale is required. In the present study, we showed the efficacy of combination therapy of capecitabine with tamoxifen or letrozole for a premenopausal and postmenopausal breast cancer model, respectively. The usefulness of these combinations in patients needs to be confirmed in clinical trials.

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HORM CANC (2013) 4:1–11
DOI 10.1007/s12672-012-0126-6

ORIGINAL PAPER

Intratumoral Estrogen Concentration and Expression of Estrogen-Induced Genes in Male Breast Carcinoma: Comparison with Female Breast Carcinoma

Kiyoshi Takagi · Takuya Moriya · Masafumi Kurosumi · Kimako Oka · Yasuhiro Miki · Akiko Ebata · Takashi Toshima · Shoji Tsunekawa · Hiroyuki Takei · Hisashi Hirakawa · Takanori Ishida · Shin-ichi Hayashi · Junichi Kurebayashi · Hironobu Sasano · Takashi Suzuki

Received: 10 August 2012 / Accepted: 8 October 2012 / Published online: 18 October 2012
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Abstract It is speculated that estrogens play important roles in the male breast carcinoma (MBC) as well as the female breast carcinoma (FBC). However, estrogen concentrations or molecular features of estrogen actions have not been reported in MBC, and biological significance of estrogens remains largely unclear in MBC. Therefore, we examined intratumoral estrogen concentrations, estrogen receptor (ER) α/ERβ status, and expression profiles of estrogen-induced genes in MBC tissues, and compared these with FBC. 17β-Estradiol concentration in MBC (n=4) was significantly (14-fold) higher than that in non-neoplastic male breast (n=3) and tended to be higher than that in FBC (n=7). Results of microarray analysis clearly demonstrated that expression profiles of the two gene lists, which were previously reported as estrogen-induced genes in MCF-7 breast carcinoma cell line, were markedly different between MBC and FBC. In the immunohistochemistry, MBC tissues were frequently positive for aromatase (63 %) and 17β-hydroxysteroid dehydrogenase type 1 (67 %), but not for steroid sulfatase (6.7 %). A great majority (77 %) of MBC showed positive for both ERα and ERβ, and its frequency was significantly higher than FBC cases. These results suggest that estradiol is locally produced in MBC

K. Takagi (✉) · T. Suzuki
Department of Pathology and Histotechnology,
Tohoku University Graduate School of Medicine,
2-1 Seiryo-machi, Aoba-ku,
Sendai, Miyagi-ken 980-8575, Japan
e-mail: k-takagi@med.tohoku.ac.jp

T. Moriya
Department of Pathology 2, Kawasaki Medical School,
Kurashiki, Japan

M. Kurosumi
Department of Pathology, Saitama Cancer Center,
Saitama, Japan

K. Oka · H. Hirakawa
Department of Surgery, Tohoku Kosai Hospital,
Sendai, Japan

Y. Miki · H. Sasano
Department of Anatomic Pathology,
Tohoku University Graduate School of Medicine,
Sendai, Japan

A. Ebata · T. Ishida
Department of Surgical Oncology,
Tohoku University Graduate School of Medicine,
Sendai, Japan

T. Toshima
Department of Surgery, Tohoku Rosai Hospital,
Sendai, Japan

S. Tsunekawa
Department of Surgery, Kansai Electric Power Hospital,
Osaka, Japan

H. Takei
Division of Breast Surgery, Saitama Cancer Center,
Saitama, Japan

S.-i. Hayashi
Department of Molecular and Functional Dynamics,
Tohoku University Graduate School of Medicine,
Sendai, Japan

J. Kurebayashi
Department of Breast and Thyroid Surgery,
Kawasaki Medical School,
Kurashiki, Japan

H. Sasano
Department of Pathology, Tohoku University Hospital,
Sendai, Japan

tissue by aromatase. Different expression profiles of the estrogen-induced genes may associate with different estrogen functions in MBC from FBC, which may be partly due to their ERα/ERβ status.

Introduction

Male breast carcinoma (MBC) is an uncommon disease, and its incidence is less than 1 % of that in female breast carcinoma (FBC). However, it has been increasing in recent years [1]. Because of the low incidence, MBC has not been studied well, and limited information is available regarding the epidemiology, pathogenesis, and treatment [2]. Therefore, it is very important to examine the biological features of MBC in order to improve clinical outcome of the patients.

It is well known that estrogens contribute immensely to the development and/or progression of FBC. Concentration of biologically active estrogen estradiol is significantly high in FBC tissues, and it is locally produced from circulating inactive steroids by estrogen-producing enzymes, such as aromatase (conversion from circulating androstenedione to estrone or testosterone to estradiol), steroid sulfatase (STS; hydrolysis of circulating estrone sulfate to estrone), and 17β-hydroxysteroid dehydrogenase type 1 (17βHSD1; conversion of estrone to estradiol) [3]. Estrogen actions are initiated by binding of estrogens with estrogen receptors (i.e., ERα or ERβ), followed by transactivation of the target genes. Various estrogen-responsive genes have been identified in the breast carcinoma [4, 5], and analyses of these genes have greatly contributed to better understanding of molecular functions of estrogen actions in FBC [6]. The estrogen actions are considered to be mainly mediated through ERα in FBC [7, 8], and endocrine therapies, such as anti-estrogens (tamoxifen, etc.), aromatase inhibitors, and gonadotropin-releasing hormone (GnRH) agonists, are used in patients with ERα-positive FBC patients.

Estrogens are also speculated to play important roles in MBC, and tamoxifen is used in MBC patients as an endocrine therapy [9]. Various studies have demonstrated frequent expression of ERα in MBC tissues as well as ERβ and progesterone receptor (PR) [10–12], and immunolocalization of aromatase has been also reported in MBC [13]. However, intratumoral concentration of estrogens or expression of other estrogen-producing enzymes has not been reported in MBC. Moreover, no information is available regarding the expression profiles of estrogen-responsive genes in MBC, to the best of our knowledge. Therefore, it remains unclear whether estrogen actions and/or effectiveness of endocrine therapy in MBC could be the same as that in FBC.

Therefore, in this study, we examined intratumoral concentrations of estrogens, immunolocalization of estrogen-producing enzymes, and expression profiles of estrogen-induced genes in MBC tissues, and compared these findings with those in FBC, in order to examine the significance of estrogens in MBC.

Materials and Methods

Patients and Tissues

Two sets of tissue specimens were used in this study. The first set is composed of 14 snap-frozen specimens. Among these, four MBC tissues were obtained from patients who underwent surgical treatment from 2009 to 2010 at Tohoku University Hospital (Sendai, Japan), Tohoku Kosai Hospital (Sendai, Japan), Tohoku Rosai Hospital (Sendai, Japan), and Kansai Electric Power Hospital (Osaka, Japan). The mean age of these patients was 65 years (range, 62–67). Three non-neoplastic breast tissues were also collected from patients who underwent surgical treatment at Tohoku University Hospital, Tohoku Kosai Hospital, and Saitama Cancer center (Saitama, Japan; mean age, 65 years; range, 62–67 years), which were not matched with the carcinoma specimens. As a control group, seven specimens of FBC were obtained from postmenopausal patients who underwent surgical treatment from 2001 to 2003 at Tohoku University Hospital (mean age, 57 years; range, 50–69 years). These specimens were stored at −80 °C for subsequent hormone assays. Eight specimens of MBC and FBC were also used in microarray analysis.

The second set is composed of 102 specimens of breast carcinomas fixed in 10 % formalin and embedded in paraffin wax. Among these, 30 MBC tissues were obtained from patients who underwent surgical treatment from 1975 to 2010 at Tohoku University Hospital, Tohoku Kosai Hospital, Tohoku Rosai Hospital, Saitama Cancer Center, Sendai, and Kawasaki Medical School Hospital (Okayama, Japan). As a control group, we also used 72 FBC tissues collected from postmenopausal women who underwent surgical treatment from 1984 to 1992 at Tohoku University Hospital.

Research protocol was approved by Ethics Committee at Tohoku University School of Medicine.

Liquid Chromatography/Electrospray Tandem Mass Spectrometry (LC-MS/MS)

Concentrations of estradiol, estrone, testosterone, and androstenedione were measured by LC-MS/MS analysis in ASKA Pharma Medical Co., Ltd. (Kawasaki, Japan), as described previously [14, 15]. In the evaluation of estradiol concentration, we measured only 17β-estradiol, but not 17α-estradiol in this study. Briefly, tissue specimens were homogenized in 1 mL of distilled water, and steroid fraction was extracted with diethyl ether. In this study, we used an LC (Agilent 1100, Agilent Technologies, Waldbronn, Germany) coupled with an API 4000 triple-stage quadrupole

mass spectrometer (Applied Biosystems, Foster City, CA, USA) operated with electron spray ionization in the positive-ion mode, and the chromatographic separation was performed on Cadenza CD-C18 column (3×150 mm, 3.5 mm, Imtakt, Kyoto, Japan).

Laser-Capture Microdissection (LCM)/Microarray Analysis

Gene expression profiles of MBC and FBC cells were examined by microarray analysis. Four MBC and four FBC tissues were subjected to the study. LCM was conducted using the MMI Cellcut (Molecular Machines and Industries, Flughofstrase, Glattbrugg, Switzerland) according to previous reports [14, 16]. Briefly, breast carcinoma specimens (one specimen for each case) were embedded in Tissue-Tek optimal cutting temperature compound (Sakura Finetechnical Co., Tokyo, Japan), and serial sections were made at a thickness of 10 μm. Sections were stained with toluidine blue according to manufacturer's recommendation, and subsequently, breast carcinoma cells in each specimen (approximately 5,000 cells) were dissected under light microscopy and laser transferred from the serial sections. The total RNA (approximately 200 ng) was subsequently extracted from these cell fractions isolated by LCM using the RNeasy® Micro Kit (QIAGEN, Mannheim, Germany). Gene expression profiles were examined by microarray analyses. Whole Human Genome Oligo Microarray (G4112F, ID: 012391, Agilent Technologies), containing 41,000 unique probes, was used in this study, and sample preparation and processing were performed according to the manufacturer's protocol.

In our present study, we focused upon the expression profiles of two gene lists which were previously reported as estrogen-induced genes in FBC cell line MCF-7 [4, 5]. One was Frasor's list which consisted of 50 genes [4], and the other was Creighton's list which consisted of 63 genes [5]. If a gene was represented multiple times on the platform, the probe with strongest positive correlation with ESR1 (ERα) was selected. In order to compare the expression profiles of these genes, unsupervised hierarchical clustering analysis was performed using the Cluster and TreeView programs (the software copyright Stanford University 1998–1999, http://rana.stanford.edu) to generate tree structures based on the degree of similarity, as well as matrices comparing the levels of expression of individual genes in each specimens. Expression of genes was statistically evaluated by Student's t test, and $P<0.05$ was considered significant in this study.

Immunohistochemistry

The characteristics of primary antibody of aromatase [13], STS [17], and 17βHSD1 [15] were described previously. Monoclonal antibodies for ERα (ER1D5), ERβ (14C8), PR (MAB429), and Ki-67 (MIB1) were purchased from Immunotech (Marseille, France), Gene Tex (San Antonio, TX, USA), Chemicon (Temecula, CA, USA), and DAKO (Carpinteria, CA, USA), respectively. Rabbit polyclonal antibody for HER2 (A0485) was obtained from DAKO. Rabbit polyclonal antibody for receptor interacting protein 140 (RIP140) and retinoic acid receptor α (RARα) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

A Histofine Kit (Nichirei Biosciences, Tokyo, Japan), which employs the streptavidin-biotin amplification method, was used in this study. Immunoreactivity of estrogen-producing enzymes was detected in the cytoplasm, and the cases that had more than 10 % of positive cells were considered positive [18, 19]. Immunoreactivity of ERα, ERβ, PR, Ki-67, RIP140, and RARα was detected in the nucleus. These immunoreactivities were evaluated in more than 1,000 carcinoma cells, and subsequently, the percentage of immunoreactivity, i.e., labeling index (LI), was determined [20]. HER2 immunoreactivity was evaluated according to a grading system proposed in HercepTest (DAKO), and the cases with strongly circumscribed membrane staining of HER2 in more than 10 % carcinoma cells (i.e., score 3+) were considered positive in this study.

Results

Tissue Concentration of Estrogens and Androgens in MBC

We first examined tissue concentration of sex steroids in non-neoplastic male breast, MBC, and FBC tissues by LC-MS/MS. Median with minimum–max value of the estradiol level was 37.0 (8.0–74.0)pg/g in non-neoplastic male breast, 523 (267–633)pg/g in MBC, and 190 (15.7–540) pg/g in FBC (Fig. 1a). Tissue concentration of estradiol was significantly ($P=0.03$ and 14-fold) higher in MBC than non-neoplastic male breast tissues. Moreover, intratumoral estradiol concentration was 2.8-fold higher in MBC than in FBC tissues, although P value did not reach a significant level ($P=0.09$). On the other hand, tissue concentration of estrone was in 83.0 (56.0–359)pg/g in non-neoplastic male breast, 134 (67.0–280)pg/g in MBC, and 75.0 (13.0–555)pg/g in FBC, respectively, and the estrone level in MBC was not significantly different from that in non-neoplastic male breast or FBC ($P=0.72$ and $P=0.71$, respectively; Fig. 1b).

Tissue concentration of testosterone was high both in non-neoplastic male breast [1,519 (23.0–3,287)pg/g] and MBC [2,540 (1,454–3,483)pg/g], compared to that in FBC [133 (70.0–240)pg/g; $P=0.008$ in MBC vs. FBC], but no significant difference was detected between these two groups ($P=0.48$; Fig. 1c). Androstenedione has similar levels in these three groups [620 (53–7,525)pg/g in non-neoplastic male breast, 1,021 (291–1,805)pg/g in MBC, and 561 (160–5,785)pg/g in FBC] in this study (Fig. 1d).

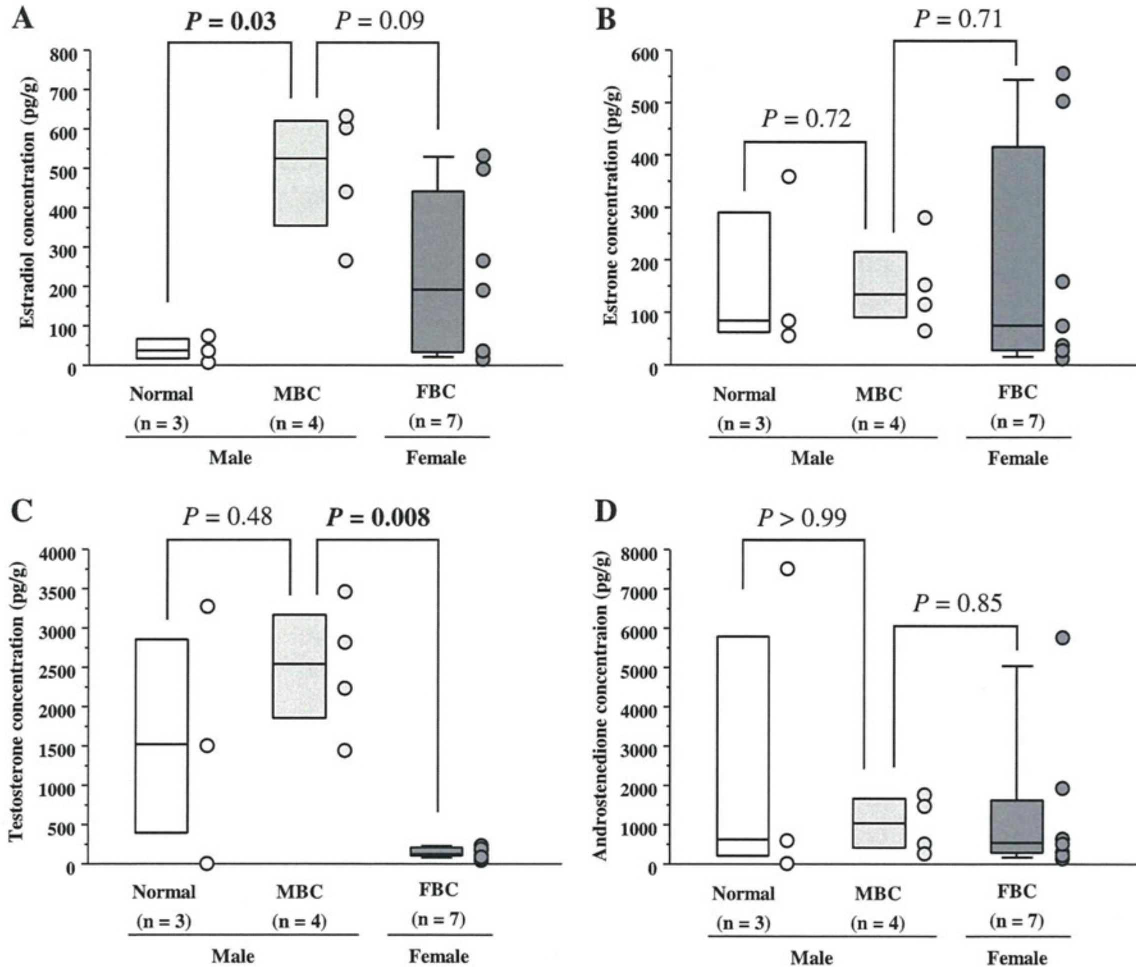

Fig. 1 Tissue concentration of estradiol (**a**), estrone (**b**), testosterone (**c**), and androstenedione (**d**) in non-neoplastic male breast, MBC, and FBC tissues. Each value was represented as a *circle*, and the grouped data were shown as *box-and-whisker plots*. The median value is demonstrated by a *horizontal line* in the box plot, and the *gray box* denotes the 75th (upper margin) and 25th percentiles of the values (lower margin). The *upper* and *lower bars* indicated the 90th and tenth percentiles, respectively. Statistical analysis was done by Mann–Whitney's *U* test; *P* values <0.05 were considered significant and indicated in *bold*

Expression Profiles of Estrogen-Induced Genes in MBC Compared with Those of FBC

We then performed microarray analysis in order to examine gene expression profiles of MBC cells isolated by LCM. Statistical analysis using Student's *t* test demonstrated that 12,295 probes showed significantly different expression between MBC and FBC cases. We then focused upon the expression profiles of two gene lists which were previously reported as estrogen-induced genes in FBC cell line MCF-7 (i.e., Frasor's list [4] and Creighton's list [5]) in order to examine molecular characteristics of estrogen actions in MBC. In the Frasor's list, 28 out of 50 (56 %) genes showed significantly different expression levels in MBC compared to FBC, and among these genes, 14 genes were highly expressed in MBC while 14 genes were lowly expressed (Table 1). In the Creighton's list, expression levels of 32 genes out of 63 (51 %) genes were significantly different between in MBC and FBC, and 18 genes were highly expressed in MBC while the other 14 genes were lowly expressed (Table 2). Five genes (RASGRP1, RARA, ADCY9, CXCL12, and NRIP1) were also included in these two gene lists, and expression levels of NRIP (P=0.0045) and ADCY9 (P=0.046) were significantly higher in MBC than FBC, and those of RARA (P=0.0012), RASGRP1 (P=0.011), and CXCL12 (P=0.012) were significantly lower in MBC.

As demonstrated in Fig. 2, results of unsupervised hierarchical cluster analysis revealed that MBC (n=4) and FBC cases (n=4) formed independent clusters regardless of the gene lists examined.

Immunolocalization of Estrogen-Producing Enzymes in MBC

We next immunolocalized estrogen-producing enzymes in 30 MBC tissues. Immunoreactivity of aromatase (Fig. 3a),

Table 1 List of genes identified as estrogen-induced genes by Frasor et al. (Frasor's list) [4]

Symbol	*P*	MBC vs. FBC	Symbol	*P*	MBC vs. FBC
CCND1	**0.041**	**L**	TGIF2	0.076	–
MYBL2	**0.027**	**L**	EGR3	0.36	–
RASGRP1[a]	**0.011**	**L**	**CXCL12**[a]	**0.012**	L
PKMYT1	0.13	–	GLRB	0.23	–
CBFA2T3	0.36	–	CHEK2	0.051	–
CDC20	**0.046**	**L**	FOS	0.056	–
IGFBP5	0.18	–	SLK	0.056	–
CCBP2	**0.0064**	**L**	**ELL2**	**<0.0001**	**H**
MYC	**0.015**	**L**	**RFC4**	**0.0084**	**H**
CCNA2	**0.0097**	**L**	**ADCY9**[a]	**0.046**	**H**
POLE2	**0.019**	**L**	**MYB**	**0.011**	**H**
BRCA2	**0.022**	**L**	**BIRC5**	**0.047**	**H**
RARA[a]	**0.0012**	**L**	**NRIP1**[a]	**0.0045**	**H**
HOXC5	**0.0043**	**L**	**MCM3**	**0.0021**	**H**
CALCR	**0.0023**	**L**	**RBBP7**	**0.0031**	**H**
POLA2	**0.011**	**L**	**RAB31**	**0.0022**	**H**
AREG	**0.0021**	**H**	WISP2	0.52	–
PCNA	**0.0093**	**H**	MCM2	0.52	–
OSTF1	**0.0039**	**H**	MCM5	0.31	–
GADD45B	**0.048**	**H**	CDC2	0.051	–
VEGF	0.27	–	AURKA	0.33	–
PPP2R1B	0.30	–	BUB1	0.76	–
STC2	**0.020**	**H**	TMF1	0.66	–
TSPAN5	0.088	–	CDC6	0.81	–
IGFBP4	0.12	–	JAK1	0.96	–

Comparison of gene expression between MBC and FBC was performed by Student's *t* test. $P<0.05$ was considered positive and described as *boldface*

"H" means that the gene is highly expressed in MBC compared to FBC, and "L" means that the gene is lowly expressed in MBC compared to FBC

[a]Genes contained by both Frasor's and Creighton's lists

STS (Fig. 3b), and 17βHSD1 (Fig. 3c) was detected in the cytoplasm of carcinoma cells in MBC tissues, but STS immunoreactivity was weaker and focal. The number of positive cases was as follows: aromatase, 19/30 (63 %); STS, 2/30 (6.7 %); and 17βHSD1, 20/30 (67 %). Non-neoplastic mammary glands and intratumoral stroma were negative for aromatase (Fig. 3d), STS, and 17βHSD1 in this study.

Immunolocalization of ERs and Estrogen-Induced Genes in MBC Compared with FBC

We also evaluated an association of several immunohistochemical parameters between MBC ($n=30$) and FBC tissues ($n=72$). As shown in Table 3, ERα and ERβ LIs were significantly ($P<0.0001$ and $P=0.001$) higher in MBC than FBC. When cases with ER LI of 10 % were considered ER-positive breast carcinoma [17, 18], all MBC cases examined were positive for ERα, while 67 % (48/72) of FBC were positive for ERα. In addition, a great majority (77 %) of MBC cases showed double positive for ERα and ERβ, and its frequency was significantly ($P=0.0009$) higher than that in FBC (39 %). PR LI was also significantly ($P=0.011$) higher in MBC than FBC, and it was positively associated with ERα LI [$P=0.03$ and $r^2=0.16$ (data not shown)]. On the contrary, Ki67 LI was significantly ($P=0.019$) lower in MBC than FBC. HER2 status was not significantly different between these in this study.

Since our microarray analyses demonstrated different expression profiles of estrogen-induced genes in MBC from those in FBC (Fig. 2), we also performed immunohistochemistry for two representative genes included in both Frasor's and Creighton's lists [RARA (RARα) and NRIP1 (RIP140)] to confirm the results. RARα immunoreactivity was sporadically detected in the nuclei of MBC cells (Fig. 4a), and its LI was significantly ($P=0.0034$ and 0.62-fold) lower in MBC than FBC (Fig. 4b). On the other hand, RIP140 immunoreactivity was frequently detected in the nuclei of MBC cells (Fig. 4c), and RIP140 LI in MBC was significantly ($P=0.002$ and 1.91-fold) higher than FBC (Fig. 4d).

Discussion

To the best of our knowledge, this is the first study to have demonstrated intratumoral estrogen concentrations in MBC tissues. In the present study, tissue concentration of estradiol

Table 2 List of genes identified as estrogen-induced genes by Creighton et al. (Creighton's list) [5]

Symbol	*P*	MBC vs. FBC	Symbol	*P*	MBC vs. FBC
ATAD2	**0.0074**	**L**	PAK1IP1	0.61	–
CISH	0.056	–	CA12	0.80	–
GREB1	0.051	–	MYBL1	0.23	–
RASGRP1[a]	**0.011**	**L**	IRS1	0.37	–
ADSL	**0.0048**	**L**	KLF10	0.94	–
FLJ22624	**0.026**	**L**	**ADCY9**[a]	**0.046**	**H**
IGF1R	**0.015**	**L**	**FLJ11184**	**0.0064**	**H**
BRIP1	**0.0079**	**L**	**TIPARP**	**0.0045**	**H**
IL17RB	**0.0082**	**L**	TPBG	0.076	–
TEX14	**0.0004**	**L**	ZWILCH	0.25	–
PLK4	**0.012**	**L**	**MCM4**	**0.046**	**L**
RARA[a]	**0.0012**	**L**	**CXCL12**[a]	**0.012**	**L**
PTGES	0.066	–	**DSU**	**0.024**	**L**
SNX24	**0.016**	**L**	OLFM1	0.11	–
HSPB8	0.38	–	EEF1E1	0.43	–
TFF1	0.45	–	LOC56902	0.079	–
SIAH2	0.25	–	**NOL7**	**0.041**	**H**
OGFOD1	0.83	–	**SDCCAG3**	**0.030**	**H**
WDHD1	0.32	–	**PPIF**	**0.0046**	**H**
ZNF259	0.50	–	**MRPS2**	**0.024**	**H**
SLC39A8	0.83	–	**ALG8**	**0.0066**	**H**
WHSC1	0.63	–	**SLC9A3R1**	**0.014**	**H**
CTNNAL1	0.17	–	**XBP1**	**0.021**	**H**
DLEU1	0.18	–	CSPP1	0.76	–
FER1L3	**0.019**	**H**	THBS1	0.66	–
LRRC54	**0.024**	**H**	ENST00000379534	0.90	–
SGK3	**0.0068**	**H**	ENST00000278505	0.35	–
CTPS	**0.0059**	**H**	PPAT	0.61	–
LRP8	0.054	–	**MYB**	**0.029**	**H**
FHL2	**0.0005**	**H**	THRAP2	0.20	–
NRIP1[a]	**0.0045**	**H**	TPD52L1	0.57	–
DNAJC10	**0.042**	**H**			

Comparison of gene expression between MBC and FBC was performed by Student's *t* test. $P<0.05$ was considered positive and described as *boldface*

"H" means that the gene is highly expressed in MBC compared to FBC, and "L" means that the gene is lowly expressed in MBC compared to FBC

[a]Genes contained by both Frasor's and Creighton's lists

was significantly higher (14-fold) in MBC [523 (267–633) pg/g] than the non-neoplastic male breast tissues (Fig. 1a), whereas estrone, testosterone, and androstenedione levels did not significantly change between in these two groups (1.6-fold, 0.83-fold, and 1.6-fold, respectively). Serum estradiol concentration in men is known to be similar to that in postmenopausal women [21]. Chetrite et al. [22] previously showed that estradiol level was significantly higher in breast carcinomas in postmenopausal women [388±106 pg/g (mean±SEM)] than in the areas considered as morphologically normal in the same patients, which is currently explained by intratumoral production of estradiol [3]. Although serum estradiol level in MBC patients has been reported twofold higher than that in healthy subjects [23], our present results suggest possible local production of estradiol in MBC tissues as well as FBC.

In the breast carcinoma of postmenopausal women, intratumoral estradiol is produced by aromatase and/or STS pathways [24]. In our present study, aromatase immunoreactivity was detected in 63 % of MBC cases. Its frequency was in good consistent with a previous report [13], and similar to that in FBC reported previously (55–77 %) [25, 26]. The positivity of 17βHSD1 immunoreactivity in MBC in our present study (67 %) was also similar to previous reports in FBC (47–61 %) [27, 28]. On the other hand, STS immunoreactivity was detected only in 7 % of MBC cases in this study, which was much lower (approximately 0.1-fold) than that in FBC reported (60–90 %) [29, 30]. Therefore, it is suggested that estradiol is mainly synthesized by aromatase pathway in MBC rather than STS.

Results of our present study also showed that estradiol concentration was 2.8-fold higher in MBC than postmenopausal

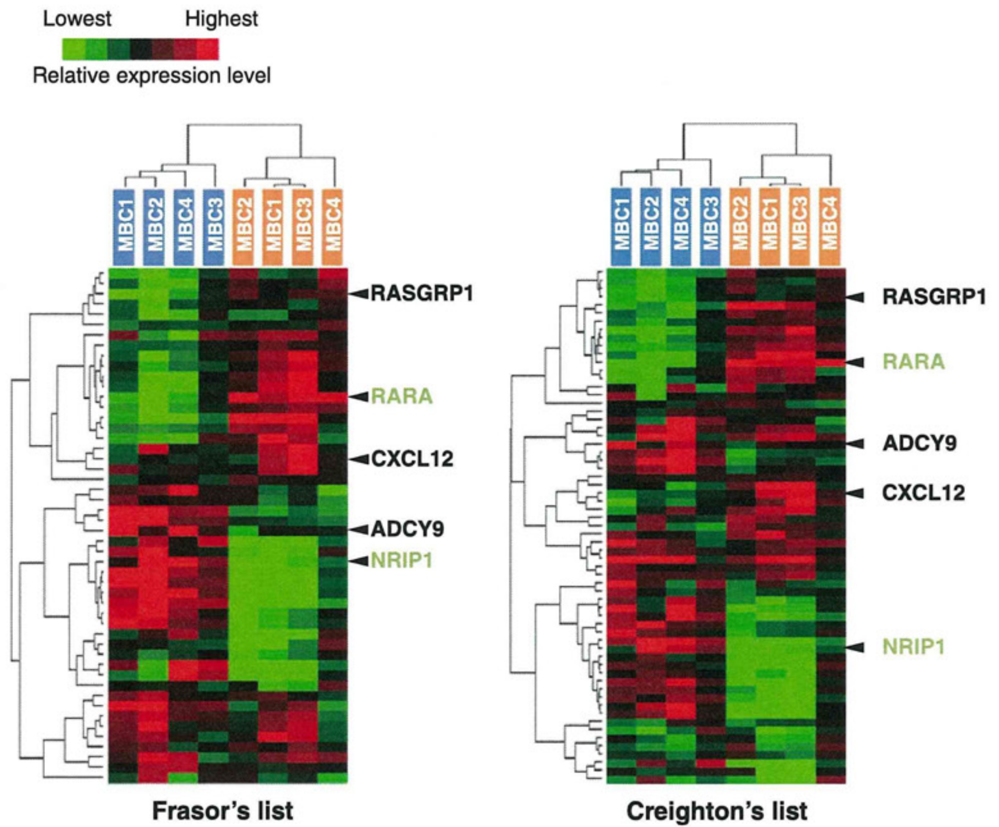

Fig. 2 Unsupervised hierarchical clustering analysis of mRNA expression levels focused on the genes which were previously reported as estrogen-induced genes [Frasor's list (*left*; 50 genes) and Creighton's list (*right*; 63 genes)]. Eight breast carcinoma samples [four MBCs (MBC1-4) and four FBCs (FBC1-4)] were used in this study, and genes and/or cases were grouped according to the similarity of gene expression, and the *shorter length of the branch* represents the higher similarity of cluster pairs. *Color of blocks* represents relative mRNA expression level of each gene, compared to the average in eight breast carcinoma samples. Five genes included in both lists (i.e., RASGRP1, RARA, ADCY9, CXCL12, and NRIP1) were indicated by *wedge*. Among these, two genes (RARA and NRIP1), which were subsequently evaluated by immunohistochemistry, were *highlighted in green*

FBC. Previously, Sonne-Hansen and Lykkesfeldt [31] reported that aromatase preferred testosterone as a substrate in MCF-7 breast carcinoma cells. In addition, plasma concentration of testosterone is approximately tenfold higher in men than postmenopausal women, while that of androstenedione is approximately1.5-fold

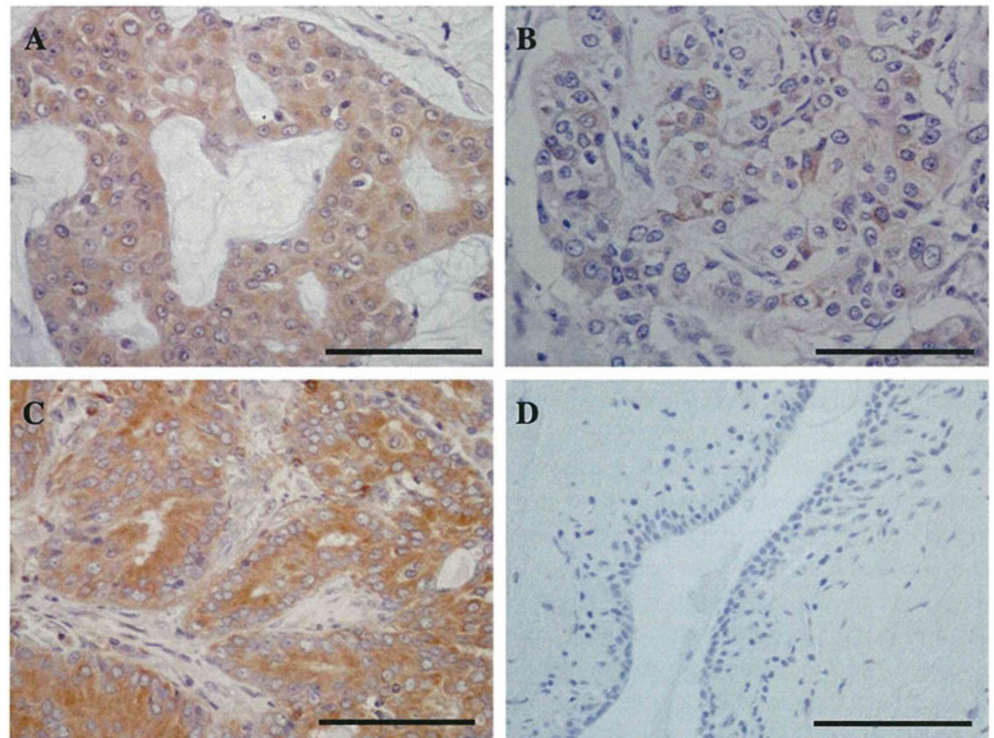

Fig. 3 Immunohistochemistry of estrogen-producing enzymes in MBC tissues. Immunoreactivity for aromatase (**a**), STS (**b**), and 17βHSD1 was visualized with 3,3′-diaminobenzidine (DAB; *brown*) and detected in the cytoplasm of carcinoma cells. Aromatase immunoreactivity was not detected in non-neoplastic mammary gland or stroma (**d**). *Bar*=100 μm, respectively

Table 3 Immunohistochemical features of MBC compared with FBC

	MBC n=30	FBC n=72	P value
ERα LI (%)[a]	90.5 (43–98.0)	40.0 (0.0–92)	**<0.0001**
ERα status			
Positive	30 (100 %)	48 (67 %)	
Negative	0 (0 %)	24 (33 %)	**<0.0001**
ERβ LI (%)[a]	27.5 (0–95)	8.5 (0–72)	**0.001**
ERβ status			
Positive	23 (77 %)	35 (49 %)	
Negative	7 (23 %)	37 (51 %)	**0.017**
ERα/ERβ status			
Positive/positive	23 (77 %)	28 (39 %)	
Others	7 (23 %)	44 (61 %)	**0.0009**
PR LI (%)[a]	43.5 (6–95)	17.5 (0–93)	**0.011**
HER2			
Positive	5 (17 %)	24 (33 %)	
Negative	25 (83 %)	48 (67 %)	0.099
Ki67 LI (%)[a]	15.5 (1.0–30)	20.0 (2.0–67)	**0.019**

[a] Data was presented as median with minimum–max or the number of cases with percentage. P value <0.05 was considered significant and described as *boldface*

higher in men [21]. Therefore, estradiol may be mainly produced from circulating testosterone by aromatase in MBC tissues. These findings also suggest that aromatase inhibitors are possibly effective in a selective group of MBC patients. A phase 2 trial used aromatase inhibitor, and GnRH analogue (SWOG-S 0511 trial) is currently ongoing in MBC patients [32].

The biological effects of estrogens are mediated through an initial interaction with ERα and/or ERβ, and ERs functions as hetero- or homodimers. In this study, both ERα and ERβ were more frequently immunolocalized in MBC than in FBC, which was in good agreement with previous reports [10–12]. Moreover, we also found that a great majority (77 %) of MBC cases showed double positive for ERα and ERβ, and its frequency was significantly (2.0-fold) higher than FBC cases (Table 1). Therefore, it may be possible to speculate that ERs are frequently heterodimerized in MBC tissues. Heterodimerization of ERα and ERβ modulates biological functions of each ER [33, 34], and FBC patients double positive for ERα and ERβ had longer disease-free and overall survival than those showed positive for ERα only [35, 36]. On the other hand, Weber-Chappuis et al. [37] suggested that functions of ER in MBC were different from that in FBC, and Johansson et al. [38] recently demonstrated that MBC was classified into two groups (i.e., luminal M1 and M2), those differed from the intrinsic subtypes of ER-positive FBC, by microarray analyses. Therefore, estrogen actions in MBC may not be necessarily the same as those in FBC, which is partly due to the different ERα/ERβ status from FBC.

Results of our microarray analysis did demonstrate that a majority of estrogen-induced genes (56 % in Frasor's list and 51 % in Creighton's list) showed significantly different expression between in MBC and FBC, and MBC cases formed a different cluster from FBC cases. We also confirmed these results by employing immunohistochemistry for representative genes (i.e., RARα and RIP140). Therefore, it is reasonably postulated that molecular functions of estrogens in MBC may be different from those in FBC based on the results above. However, it is also true that estrogen-induced genes examined in this study were identified in female breast cancer cell line MCF-7, and it is still not clarified whether these genes were similarly regulated by estrogen in MBC tissues or not, which also suggests that all the genes detected at markedly different levels in MBC compared to FBC were therefore not necessarily regulated by estrogens. In addition, only two genes on Creighton's list (CA12 and SIAH2) were included in the gene list, which was recently identified as MBC-specific genes by Johansson et al. [38]. Estrogen-induced genes are not determined yet in MBC because of unavailability of appropriate cell line and/or its relevant in vivo model. Therefore, further examinations are required to clarify the molecular features of estrogen actions in MBC.

Among the genes overexpressed in FBC (summarized in Tables 1 and 2), MYC (C-MYC) was well known to be associated with poor prognosis or adverse clinical outcome of ER-positive breast cancer patients [39], and RARA (RARα) upregulated 17βHSD1 and contributed to in situ production of estradiol in FBC [40]. IGF1R (insulin-like growth factor receptor) has been considered to promote breast carcinoma cell growth by interacting with estrogen signaling [41]. In addition, Ma et al. and Wang et al. independently reported that IL17RB (interleukin-17 receptor B) expression was significantly associated with increased risks of recurrence in ERα-positive breast cancer patients [42, 43]. However, among the genes highly expressed in MBC, MYB (c-myb) was associated with a good prognosis in the patients [44]. NRIP1 (RIP140) is a negative transcriptional regulator of hormone receptor [45, 46] and inhibited ERα activity in the breast carcinoma cells [43]. RBBP7 (RBAP46) also modulated estrogen responsiveness in breast carcinoma cells through an interaction with ERα [47] and inhibited an estrogen-stimulated progression of transformed breast epithelial

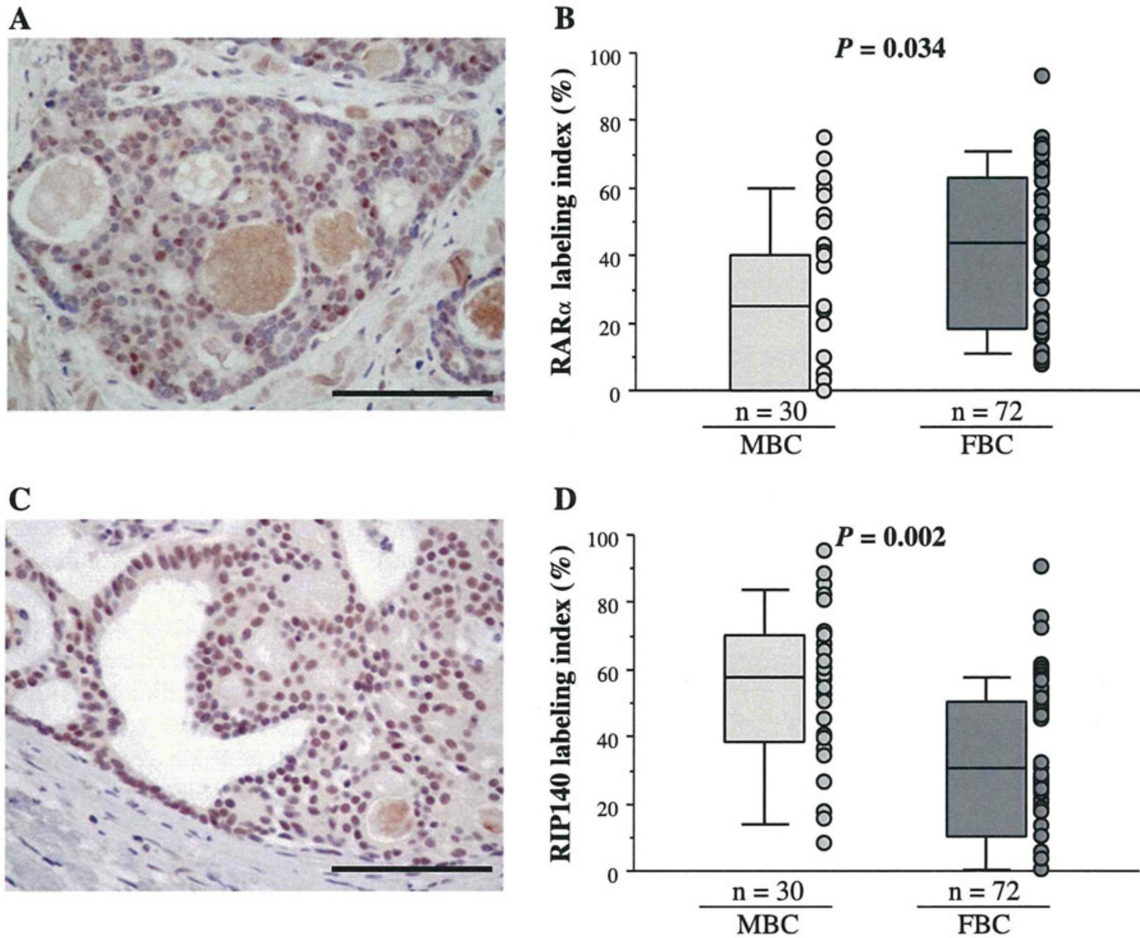

Fig. 4 Immunohistochemistry of RARα (**a**, **b**) and RIP140 (**c**, **d**) in MBC tissues. RARα (**a**) and RIP140 (**c**) immunoreactivity was visualized with DAB (*brown*) and detected in the nuclei of carcinoma cells. *Bar*=100 μm, respectively. Relative immunoreactivity of RARα and RIP140 was summarized in **b** and **d**, respectively. Each value was represented as a *circle*, and the grouped data were shown as *box-and-whisker plots*. The median value is demonstrated by a *horizontal line* in the box plot, and the *gray box* denotes the 75th (upper margin) and 25th percentiles of the values (lower margin). The *upper* and *lower bars* indicate the 90th and tenth percentiles, respectively. Statistical analysis was performed by Mann–Whitney's *U* test; *P* values <0.05 were considered significant and indicated in *bold*

cells [48]. In addition, FHL2 (four and a half LIM domains 2) was reported to inhibit proliferation and invasion of breast carcinoma cells by suppressing the function of ID3 (inhibitor of DNA binding 3), which was also known as one of the adverse prognostic factor of patients with breast cancer [49, 50]. Considering the functions of these gene above, estrogens may more efficiently promote aggressive clinical behavior in FBC than MBC, although some genes highly expressed in MBC were indeed associated with aggressive phenotypes of the breast carcinoma, such as AREG (amphiregulin) and XBP1 (X-box binding protein 1) [51, 52]. To date, tamoxifen is used as an endocrine therapy for MBC patients. However, it has been reported that expression profile of estrogen responsive gene was closely related to the response to tamoxifen in FBC patients [53]. Further examinations are required to clarify molecular functions of estrogen actions in MBC to improve the effectiveness of endocrine therapy for MBC patients.

In summary, intratumoral concentration of estradiol was significantly higher in MBC than non-neoplastic male breast tissues in this study, and aromatase and 17βHSD1 were frequently immunolocalized in MBC tissues. In addition, a great majority (77 %) of MBC cases showed positive for both ERα and ERβ, and its frequency was significantly higher than FBC cases. Results of microarray analysis revealed that expression profiles of genes known to be regulated by estrogen were markedly different between MBC and FBC. These results suggest that estradiol is mainly produced by aromatase from circulating testosterone in MBC tissues, and expression profiles of estrogen-induced genes in MBC are different from FBC, which may be partly due to their different ERα/ERβ status.

Acknowledgments We appreciate the skillful technical assistance of Mr. Katsuhiko Ono (Department of Anatomic Pathology, Tohoku University Graduate School of Medicine).

Disclosures The authors declare that there is no conflict of interest to be disclosed.

Funding This work was partly supported by Grant-in-Aid for Scientific Research (24790343) from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

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Cancer Medicine

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ORIGINAL RESEARCH

Individual transcriptional activity of estrogen receptors in primary breast cancer and its clinical significance

Tatsuyuki Gohno[1], Yuko Seino[1,2], Toru Hanamura[1], Toshifumi Niwa[1], Mitsuyo Matsumoto[1,3], Nobuo Yaegashi[3], Hanako Oba[4], Masafumi Kurosumi[4], Hiroyuki Takei[5], Yuri Yamaguchi[2] & Shin-ichi Hayashi[1,2,6]

[1]Department of Molecular and Functional Dynamics, Graduate School of Medicine, Tohoku University, Aoba-ku, Sendai, 980-8575, Japan
[2]Research Institute for Clinical Oncology, Saitama Cancer Center, Ina-machi, Saitama, 362-0806, Japan
[3]Department of Gynecology, Graduate School of Medicine, Tohoku University, Aoba-ku, Sendai, 980-8575, Japan
[4]Department of Pathology, Saitama Cancer Center, Ina-machi, Saitama, 362-0806, Japan
[5]Division of Breast Surgery, Saitama Cancer Center, Ina-machi, Saitama, 362-0806, Japan
[6]Center for Regulatory Epigenome and Disease, Graduate School of Medicine, Tohoku University, Aoba-ku, Sendai, 980-8575, Japan

Keywords
Breast cancer, ERE transcriptional activity, estrogen receptor α, Ki67, Luminal A

Correspondence
Shin-ichi Hayashi, Department of Molecular and Functional Dynamics, Graduate School of Medicine, Tohoku University, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan.
Tel: +81-22-717-7913; Fax: +81-22-717-7913; E-mail: shin@med.tohoku.ac.jp

Funding Information
This study was supported, in part, by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan; a Grant-in-Aid for Cancer Research from the Ministry of Health, Labour, and Welfare, Japan; the Advanced research for medical products Mining Programme of the National Institute of Biomedical Innovation (NIBIO); and a Grant from the Smoking Research Foundation.

Received: 1 August 2012; Revised: 1 October 2012; Accepted: 1 October 2012

***Cancer Medicine* 2012; 1(3): 328–337**

doi: 10.1002/cam4.41

Abstract

To predict the efficacy of hormonal therapy at the individual-level, immunohistochemical methods are used to analyze expression of classical molecular biomarkers such as estrogen receptor (ER), progesterone receptor (PgR), and HER2. However, the current diagnostic standard is not perfect for the individualization of diverse cases. Therefore, establishment of more accurate diagnostics is required. Previously, we established a novel method that enables analysis of ER transcriptional activation potential in clinical specimens using an adenovirus estrogen response element–green fluorescence protein (ERE-GFP) assay system. Using this assay, we assessed the ERE transcriptional activity of 62 primary breast cancer samples. In 40% of samples, we observed that ER protein expression was not consistent with ERE activity. Comparison of ERE activity with clinicopathological information revealed that ERE activity was significantly correlated with the ER target gene, PgR, rather than ER in terms of both protein and mRNA expression. Moreover, subgrouping of Luminal A-type breast cancer samples according to ERE activity revealed that ERα mRNA expression correlated with ER target gene mRNA expression in the high-, but not the low-, ERE-activity group. On the other hand, the low-ERE-activity group showed significantly higher mRNA expression of the malignancy biomarker Ki67 in association with disease recurrence in 5% of patients. Thus, these data suggest that ER expression does not always correlate with ER transcriptional activity. Therefore, in addition to ER protein expression, determination of ERE activity as an ER functional marker will be helpful for analysis of a variety of diverse breast cancer cases and the subsequent course of treatment.

Introduction

To predict the efficacy of hormonal therapy for breast cancer at the level of the individual, immunohistochemical methods are used to analyze classical molecular biomarkers such as estrogen receptor (ER), progesterone receptor (PgR), and HER2 [1–3]. Novel markers such as Ki67, FOXA1, and GATA3 are also examined and used to predict long-term outcome after neoadjuvant endocrine treatment [4–7]. However, the current diagnostic standard is not always suitable for the classification of cases. In ER-positive patients, endocrine therapy to antagonize ER signaling is ineffective in approximately 30% of cases [8]. This discrepancy could be the result of the activation of other ER-independent estrogen-related signaling pathways in these breast cancer cells, such as

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insulin-like growth factor 1 (IGF-1)- or vascular endothelial growth factor (VEGF)-mediated signaling cascades [9, 10]. Therefore, reliable diagnostic techniques or tools are required for the sensitive evaluation of likely endocrine therapy efficacy for individual patients.

ER is activated by estrogen [11, 12] or protein phosphorylation by kinases such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt [13, 14]. Activated ER induces transcription of genes containing the estrogen response element (ERE). The molecular mechanisms regulating transcriptional activity by ER have been well investigated in breast cancer cells. However, although ER protein expression has been evaluated by immunohistochemistry (IHC) [1, 2], its relationship with ERE transcriptional activity has not been reported. We have previously observed several cases in which ER protein expression and ER target gene mRNA expression do not correlate [15–18]. These results suggest that ER protein expression may not necessarily reflect the function of ER.

To explore the possibility of recategorizing breast cancers, we analyzed human breast cancer cases according to three features: ER protein expression, ERE transcriptional activity, and ER target gene mRNA expression. We have previously produced a construct in which the common ERE is ligated upstream of green fluorescence protein (GFP) cDNA, and packaged into an adenovirus vector [12, 19, 20]. Primary breast cancer cells, prepared from patients, were infected with this adenovirus vector, and the ERE transcriptional activity was measured by analyzing the GFP fluorescence, as previously described for endometrial cancer [20]. We also determined the protein and mRNA expression levels of ER and the ER target genes identified in our microarray [15–18], using formalin-fixed paraffin-embedded (FFPE) sections from the same patients. This is the first report describing the relationship between ER and its transcriptional activity using clinical samples. Our result indicates that Luminal A-type breast cancer may be classified into two or more types. These findings could be used for a novel predictive model of hormonal therapeutic effectiveness. Indeed, further subtyping of Luminal A-type breast cancer based on the functional evaluation of ER could contribute to more accurate diagnosis and the selection of more effective treatment strategies.

Materials and Methods

Tumor samples

Primary human breast cancer tissues were surgically obtained from 62 informed and consenting patients at the Saitama Cancer Center Hospital (Saitama, Japan) between 2005 and 2007 (Table 1) with approval from the Saitama Cancer Center and Tohoku University Ethics Committee (Saitama Cancer Center No. 216, Tohoku University No. 2008-442). These living cells were used for the assessment of ERE activity. FFPE sections were also prepared from these samples and used for hematoxylin and eosin staining, immunohistochemical staining, and real-time reverse transcription polymerase chain reaction (PCR). Preparation of FFPE and staining were carried out as previously [21] described.

Table 1. Patient clinicopathological information.

Characteristic	*n*
Age	
<50	27
≥50	35
Menopausal	
Pre	28
Post	33
No (men)	1
Tumor size (mm)	
<20	27
≥2	30
Unknown	5
Stage	
0	3
I	13
II	33
III	5
Unknown	8
Grade	
1	7
2	9
3	33
Unknown	13
ER	
Positive	46
Negative	13
Unknown	3
PgR	
Positive	46
Negative	13
Unknown	3
HER2	
Positive	10
Negative	47
Unknown	5

Reagents

ICI 182,780 (Fulvestrant, pure antiestrogen) and 4-hydroxytamoxifen (Tamoxifen) were purchased from Sigma-Aldrich (St. Louis, MO).

IHC of the ER, PgR, and HER2

We analyzed the expression of ER and PgR by IHC. ER was detected using monoclonal anti-ERα antibody 1D5

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(M7047; Dako, Glostrup, Denmark), and PgR using monoclonal antibody PgR 636 (M3569; Dako). Immunointensity was graded on the basis of Allred scoring [22] (ER: Fig. 1A and B; PgR: Fig. 1C and D). We also assessed HER2 positivity using the HercepTest^{TM} (Dako) and scored the results as 0, 1, 2, and 3, according to the ASCO/CAP guidelines [1, 2] (Fig. 1E and F). A HER2-positive status was defined as HER2 protein 3 or 2 and FISH ratio of more than 2.2. Histologic grading was evaluated according to the Elston and Ellis grading scheme [23] with slight modification.

ERE transcriptional activity assay in primary tumor cells: Ad-ERE-GFP assay

To assess ERE transcriptional activity in primary tumor cells, we used the Ad-ERE-GFP assay [12, 19, 20]. The isolation of tumor cells was performed as previously described by Ackerman [24] with slight modifications. Briefly, cancer tissue specimens were minced to ~1 mm^3 in size after being rinsed with phosphate-buffered saline (PBS), and digested with collagenase solution (1 mg/mL collagenase, 40 mg/mL bovine serum albumin, 2 mg/mL glucose, 1× antibiotic-antimycotic, and 50 μg/mL gentamicin in HBSS [Hank's balanced salt solution]) for 20–30 min at 37°C. The cells, including tumor cells, were washed several times with PBS, and incubated in 24-well plates with 400 μL of PRF-RPMI (phenol red-free RPMI) 1640 medium (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal calf serum (Tissue Culture Biologicals, Tulare, CA). The cells were then infected with 2×10^9 PFU (plaque forming unit) (in 293A cells) Ad-ERE-GFP, and incubated for a further 3 days at 37°C in 5% CO_2–95% air. To examine the infectivity of the adenovirus in primary tumor cells, the cells were infected with 2×10^9 PFU Ad-ERE-GFP or Ad-CMV-DsRed. Approximately 80% of cells were confirmed to be infected. To evaluate drug sensitivity, the cells were simultaneously treated with or without ICI 182,780 or 4-hydroxytamoxifen at a final concentration of 1 μmol/L at

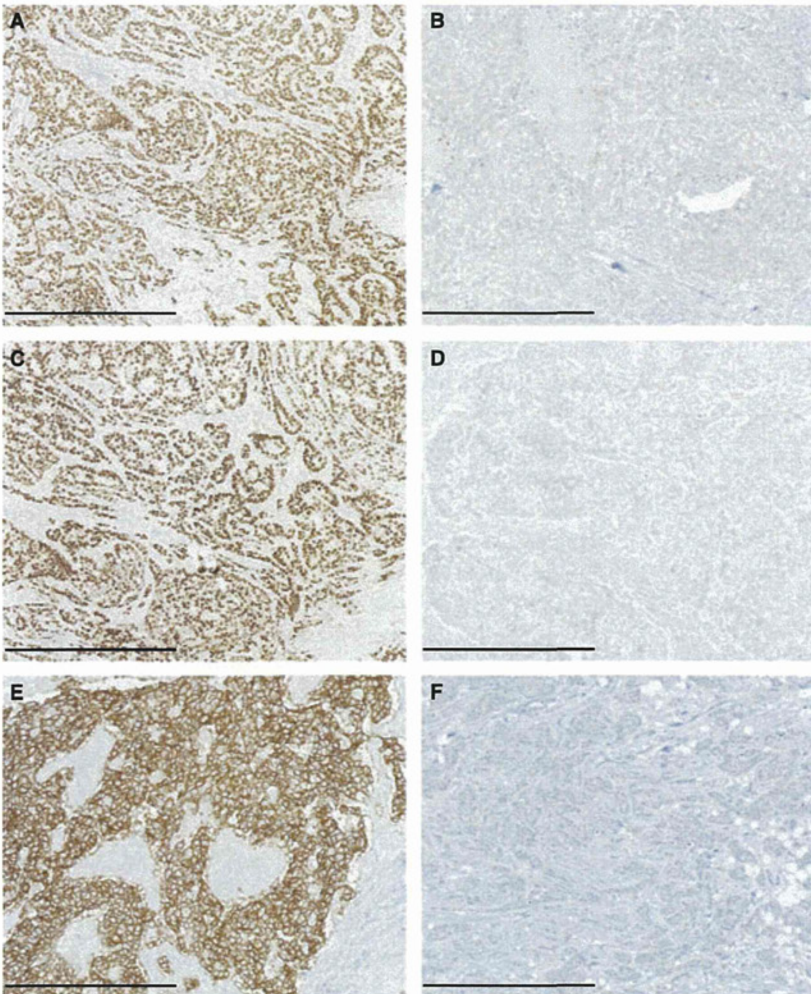

Figure 1. Representative images of IHC labeling of ER (A: positive; B: negative), PgR (C: positive; D: negative), and HER2 (E: positive; F: negative). Scale bars, 500 μm.

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