Table 2.

(A) Estrogen production levels in MCF-7 and MCF-7_{CO}

Cells	Substrates	Concentrations*,†	
		E2	E1
MCF-7	AD	0.02	0.18
	TST	0.07	0.16
MCF-7_{CO}	AD	0.04	0.34
	TST	0.15	0.05

(B) Estrogen production ratios in MCF-7 and MCF-7_{CO}

Cells	Substrates	Conversion (%)*	
		AD→E1	TST→E2
MCF-7	AD	0.05	0.28
	TST	0.63	0.02
MCF-7_{CO}	AD	0.09	0.41
	TST	0.44	0.04

(C) mRNA levels of 17β-HSDs and 5α-reductases in MCF-7 and MCF-7_{CO} cells

Cells	mRNA levels (mean ± SD), %				
	HSD17B1	HSD17B2	HSD17B3	HSD17B4	HSD17B5
MCF-7	0.178 ± 0.076	0.040 ± 0.005	0.066 ± 0.114	0.016 ± 0.027	0.187 ± 0.091
MCF-7_{CO}	0.553 ± 0.047	0.009 ± 0.016	0.170 ± 0.061	0.014 ± 0.024	0.323 ± 0.172
P	0.047‡	0.030*	0.233	0.927	0.293

Abbreviations: AD, androstenedione; E2, estradiol; E1, estrone; TST, testosterone; MCF-7_{CO}, after coculture with 32N for 24 h.
*Mean of $n = 2$.
† In picograms per milliliter per 10^6 cells.
‡ Significant difference; $n = 3$; in percent of RPL13A.

human breast carcinoma tissues. Therefore, the possible effects of ASFs secreted from stromal cells on aromatase expression in parenchymal or carcinoma cells remained largely unknown possibly due to the reported low or no detectable aromatase enzyme levels of MCF-7 cells (26–29). Exogenous human epidermal growth factor (26), transforming growth factor (26), and keratinocyte growth factor (36) have all been reported to stimulate aromatase activity in MCF-7 cells. In our study, coculture of MCF-7 cells with stromal cells derived from human breast carcinoma tissues markedly induced the aromatase expression and activity in MCF-7 cells. Although we have not investigated which of the specific ASFs influence the expression of endogenous aromatase in MCF-7 cells, the ASFs described above could all be secreted from fibroblastic stromal cells adjacent to carcinoma, especially at the sites of stromal invasion, and have an influence. The precise ASFs involved would require further investigations to clarify their actual role.

The signals mediated through various nuclear receptors, including orphan nuclear receptors, have been postulated to influence aromatase activity and expression in breast carcinoma or parenchymal cells (10, 11, 37, 38). In our study, we showed that aromatase expression was closely associated with ERRα, VDR, GCNF, and TRβ expression in breast carcinoma cells. ERRα has been previously reported to be a positive regulator for aromatase gene expression in SK-BR-3 breast carcinoma cells (11), but not in the 3T3-L1 preadipocyte cells (39). ERRα is also known to bind to silencer elements located between promoter I.3 and II of the aromatase gene, which results in increased aromatase transcript levels in SK-BR-3 cells (11). The results of our study, including microarray expression profiling analyses, as well as those of a previously reported study (23), all showed that ERRα expression is positively correlated with aromatase expression in human breast carcinoma or parenchymal cells, but not with stromal cells or whole breast tissue containing both carcinoma and stromal cells (23). Therefore, ERRα is considered a key regulator of intratumoral estrogen production in human breast carcinoma or parenchymal cells, but not necessarily in stromal cells. However, there have been no studies reported on the possible correlations between aromatase gene expression and VDR, TRβ, or GCNF genes. VDR was well known as one of the estrogen target genes with an estrogen-responsive element in its promoter lesion (40). Therefore, the expression of VDR may be induced by estrogens synthesized by aromatase in human breast carcinoma cells. A statistically significant negative correlation was detected between aromatase and GCNF. GCNF was reported to be able to inhibit ERRα-mediated transactivation in human placental

choriocarcinoma cell lines (41). Therefore, GCNF may inhibit aromatase expression through the down-regulation of ERRα-mediated transactivation. The significance of TRβ expression in breast carcinoma cells has remained largely unknown. The mRNA levels of ERRα, TRβ, and GCNF in MCF-7 cells were, however, not increased following the coculture with stromal cells. Therefore, ASFs released from stromal cells may increase aromatase mRNA transcript levels through an interaction with these nuclear receptors above, but it awaits further investigations for clarification.

In the estrogen production assays, a relatively high rate of conversion into estradiol was detected in MCF-7_{CO}, but not MCF-7 cells alone following the treatment with testosterone as the aromatase substrate. Aromatase catalyzes testosterone into estradiol but not into estrone, whereas estrone is converted from androstenedione by aromatase (42). In our study, the level of estrone was decreased in MCF-7_{CO} compared with MCF-7 alone following the treatment with testosterone. Estrone is therefore considered to be converted from testosterone via androstenedione by 17β-HSD2. We also showed that the low level of 17β-HSD2 mRNA and the high level of 17β-HSD1 were detected in MCF-7_{CO}, but not MCF-7 alone. These findings all suggest that the rate of conversion into estrone in MCF-7_{CO} was lower than that in MCF-7 cells alone following the treatment with testosterone but not with androstenedione as the aromatase substrate. Interleukins were also shown to regulate 17β-HSDs mRNA and activity in human breast carcinoma cells (39, 43). Therefore, 17β-HSD1 expression may also be regulated by the factors released from stromal or carcinoma cells in addition to aromatase.

The coculture system used in our study could provided important information with regard to the evaluation of the intratumoral microenvironment such as cell-cell interactions and these soluble factors (44). It is also considered a useful model for examining the effect of medications on breast carcinoma patients. The adhesive microenvironment, including cell-matrix interactions and cell-cell interactions, plays an important role in the development of both the normal mammary gland and breast carcinoma (44). The results of our study also showed that aromatase inhibitors were more effective on aromatization increased by coculture of MCF-7 cells than in stromal 32N cells alone. These results suggest that the aromatase in parenchymal or carcinoma cells is the more important target for aromatase inhibitors in breast cancer patients because of the more effective decreases in aromatase activity in carcinoma cells. Therefore, it is clinically important to evaluate the localization of aromatase in breast carcinoma tissues to evaluate the possible efficacy of aromatase inhibitor treatment. However, it awaits further examinations to clarify all the interactions among the different cell types in human breast carcinomas.

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5α-Reductase type 1 and aromatase in breast carcinoma as regulators of *in situ* androgen production

Takashi Suzuki[1]*, Yasuhiro Miki[1], Takuya Moriya[1], Jun-ichi Akahira[1], Takanori Ishida[2], Hisashi Hirakawa[3], Yuri Yamaguchi[4], Shin-ichi Hayashi[5] and Hironobu Sasano[1]

[1]*Department of Pathology, Tohoku University School of Medicine, Sendai, Japan*
[2]*Department of Surgery, Tohoku University School of Medicine, Sendai, Japan*
[3]*Department of Surgery, Tohoku Kosai Hospital, Sendai, Japan*
[4]*Research Institute for Clinical Oncology, Saitama Cancer Center, Saitama, Japan*
[5]*Department of Molecular Medical Technology, Tohoku University School of Medicine, Sendai, Japan*

Previous *in vitro* studies demonstrated that bioactive androgen 5α-dihydrotestosterone (DHT) exerted antiproliferative effects through an interaction with androgen receptor (AR) in breast carcinoma cells. However, AR status has not been examined in association with DHT concentration in breast carcinoma tissues, and significance of androgenic actions remains unclear in breast carcinomas. Therefore, in our study, we first examined intratumoral DHT concentrations in 38 breast carcinoma tissues using liquid chromatography/electrospray tandem mass spectrometry. Intratumoral DHT concentration was positively associated with 5α-reductase type 1 (5αRed1), and negatively correlated with aromatase. We then examined clinical significance of AR and 5αRed1 status in 115 breast carcinoma tissues by immunohistochemistry. Breast carcinomas positive for both AR and 5αRed1 were inversely associated with tumor size or Ki-67. These patients showed significant associations with a decreased risk of recurrence and improved prognosis for overall survival, and the AR / 5αRed1 status was demonstrated an independent prognostic factor. Moreover, we examined possible regulation of DHT production by aromatase in *in vitro* studies. DHT synthesis from androstenedione in MCF-7 cells was significantly inhibited by coculture with aromatase-positive stromal cells, which was significantly reversed by addition of aromatase inhibitor exemestane. These results suggest that intratumoral DHT concentration is mainly determined by 5αRed1 and aromatase in breast carcinoma tissues, and antiproliferative effect of DHT may primarily occur in the cases positive for both AR and 5αRed1. Aromatase inhibitors may be more effective in these patients, possibly due to increasing local DHT concentration with estrogen deprivation.

Key words: breast cancer; androgen; androgen receptor; 5α-reductase; aromatase

It is well-known that sex-steroids play important roles in the development of hormone-dependent human breast carcinomas. Among these sex-steroids, estrogens immensely contribute to growth of breast carcinoma through binding with estrogen receptor (ER).[1] In contrast, androgens are considered to predominantly exert antiproliferative effects *via* androgen receptor (AR) in breast carcinoma cells,[2–5] although some divergent findings have been reported.[6] AR is expressed in a majority of human breast carcinoma tissues,[7–11] suggesting important roles of androgens in breast carcinomas.

5α-dihydrotestosterone (DHT) binds with the highest affinity to AR, and together with testosterone promotes AR transcriptional activity.[12] Androgen concentrations have been previously examined in breast cancers by 2 groups,[13,14] and the potent androgen DHT was demonstrated to be significantly higher in breast carcinoma tissues than in plasma in these studies. Androgen-producing enzymes, such as17β-hydroxysteroid dehydrogenase type 5 (17βHSD5, conversion from circulating androstenedione to testosterone) and 5α-reductase (5αRed, reduction of testosterone to DHT), were expressed in breast carcinoma tissues,[15] and *in situ* production of DHT has been proposed in breast cancer tissues.[16]

Androgen actions are mediated through an interaction with androgens and AR. Therefore, it becomes very important to evaluate both AR expression and intratumoral DHT concentration in the breast carcinoma tissues in order to obtain a better understanding of the androgenic actions. However, such an examination has not been reported. In our study, we first examined DHT concentrations in 38 breast carcinoma tissues, and correlated these findings with various clinicopathological parameters of the patients. These results demonstrated a strong association between intratumoral DHT concentration and 5αRed1, and we subsequently examined AR and 5αRed1 status in 115 breast carcinoma tissues by immunohistochemistry. Moreover, because the intratumoral DHT concentration was also inversely associated with aromatase expression, we further examined a possible modulation by aromatase on DHT production by cell culture studies.

Material and methods

Patients and tissues

Specimens (38) of invasive ductal carcinoma were obtained from female patients who underwent mastectomy from 2002 to 2004 in the Departments of Surgery at Tohoku University Hospital and Tohoku Kosai Hospital, Sendai, Japan (a mean age, 57; and range of age, 32–86). Specimens for DHT extraction or RNA isolation were snap-frozen and stored at −80°C, and those for immunohistochemistry were fixed with 10% formalin and embedded in paraffin-wax. The histological grade of each specimen was evaluated based on the method of Elston and Ellis.[17] Informed consent was obtained from all patients prior to their surgery and examination of the specimens used in our study.

Specimens (115) of invasive ductal carcinoma were obtained from female patients who underwent mastectomy from 1984 to 1989 in Department of Surgery, Tohoku University Hospital, Sendai, Japan. A mean age of the patients was 53 years (range, 27–82). None of the patients examined in our study used oral contraceptives, and the patients did not receive irradiation or chemotherapy prior to surgery. Review of the charts of patients revealed that 104 patients received adjuvant chemotherapy, while none of the patient received tamoxifen therapy after the surgery. The mean follow-up time was 106 months (range, 5–154 months). Disease-free survival data were available for all patients. All specimens were fixed with 10% formalin and embedded in paraffin-wax. Snap-frozen tissues were not available for examination in these cases.

Research protocols for our study were approved by the Ethics Committee at both Tohoku University School of Medicine and Tohoku Kosai Hospital.

***Correspondence to:** Department of Pathology, Tohoku University School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan. Fax: +81-22-717-8051. E-mail: t-suzuki@patholo2.med.tohoku.ac.jp
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Liquid chromatography/electrospray tandem mass spectrometry

Concentrations of DHT and estradiol were measured by liquid chromatography/electrospray tandem mass spectrometry (LC-MS/MS) analysis, in Teizo Medical (Kawasaki, Japan). Briefly, breast carcinoma specimens (~40 mg for each sample) were homogenized in 1 ml of distilled water. After addition of 100 pg of DHT-$^2H_3$ (CDN Isotope, Quebec, Canada) (Hayashi Junyaku, Tokyo, Japan) as internal standard, steroids were extracted with diethyl ether from the homogenate. The separated organic layer was evaporated, and then dissolved in picolinoic anhydride in tetrahydrofran solution (100 μl) with triethylamine (20 μl). After application to a Bond Elut C_{18} column, steroid derivatives were eluted with 80% acetonitrile solution. The derivative-DHT fraction was dissolved in the elution solvent of LC.

In our study, we used an LC (Agilent 1100, Agilent Technologies, Waldbronn, Germany) coupled with an API 4000 triple-stage quadrupole mass spectrometer (Applied Biosystems, Ontario, Canada) operated with electron spray ionization in the positive-ion mode, and the chromatographic separation was performed on Cadenza CD-C_{18} column (3 × 150 mm^2, 3.5 μm, Imtakt, Kyoto, Japan). The injection volume was 20 μl. The mobile phase consisted of solvents A (0.1% formic acid in water (v/v)) and B (acetonitrile), and delivered at flow rate of 0.4 ml/min. Total run time was 10 min. We used mixture of solvents A and B (30:70 (v/v)) as an initial condition. After injection, it was followed by a linear gradient to 100% solvent B for 4 min, and this condition was maintained for 3 min. The system was returned to the initial proportion within 0.05 min, and maintained for the final 2.95 min of each run. The retention time for the derived DHT was 5.8 min. Ion spray voltage was 4.5 kV, and turbo gas temperature was 450°C in ionization conditions. For multiple reaction monitoring mode, the instrument monitored the *m/z* 262 (I.S.: 268) as ion produced from 383.3 (I.S.: 487.2) for DHT-derivatives. The minimum amount of DHT that can be detected was 0.2 pg in our study.

Immunohistochemistry

Monoclonal antibodies for ERα (ER1D5), progesterone receptor (PR; MAB429), AR (AR441) and Ki-67 (MIB1) were purchased from Immunotech (Marseille, France), Chemicon (Temecula, CA) and DAKO (Carpinteria, CA), respectively. Monoclonal antibody for steroid sulfatase (STS) was kindly provided from Dr. T. Nakata (Pharmaceuticals Company, Kyowa Hakko Kogyo, Tokyo, Japan), and characteristics of the antibody and the application for immunohistochemistry were described previously.[18] Rabbit polyclonal antibodies for HER2 (A0485) was obtained from DAKO. Rabbit polyclonal antibodies for 17βHSD1 (kindly provided by Dr. M. Poutanen (University of Oulu, Oulu, Finland)),[19] 17βHSD5 (kindly provided from Dr. V. Luu-The (CHUL Research Center, Quebec, Canada)),[15] and 5αRed1 and 5αRed2 (kindly provided by Dr. D.W. Russell (University of Texas Southwestern Medical Center, Dallas, TX), respectively)[15] were also employed in our study, and the characteristics of these polyclonal antibodies have been previously reported.[15,19] We could not perform immunohistochemistry for aromatase in our study, because immunohistochemical findings of aromatase are currently inconsistent in breast carcinoma tissues, according to the different nature of aromatase antibodies employed.[16]

A Histofine Kit (Nichirei, Tokyo, Japan), which employs the streptavidin-biotin amplification method, was used for immunohistochemistry in our study. Antigen retrieval for ERα, PR, AR, HER2 and Ki-67 immunostaining was performed by heating the slides in an autoclave at 120°C for 5 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate, pH 6.0). The dilution of primary antibodies used in our study was as follows: ERα; 1/50, PR; 1/30; AR; 1/100, HER2; 1/200, Ki-67; 1/50, STS; 1/9,000, 17βHSD1; 1/500, 17βHSD5; 1/1,000, 5αRed1; 1/1,000 and 5αRed2; 1/1,000. The antigen-antibody complex was visualized with 3.3′-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris-HCl buffer (pH 7.6), and 0.006% H_2O_2), and counterstained with hematoxylin. Normal rabbit or mouse IgG was used instead of the primary antibody as a negative control of immunohistochemistry, and no specific immunoreactivity was detected in these sections (data not shown).

Immunoreactivity of ERα, PR, AR and Ki-67 was detected in nuclei of carcinoma cells, and the immunoreactivity was evaluated in more than 1,000 carcinoma cells for each case, and subsequently the percentage of immunoreactivity, *i.e.* labeling index (LI), was determined. Cases with ERα LI or AR LI of more than 10% were considered ERα- or AR-positive breast carcinomas, according to a report by Allred *et al.*[20] Immunoreactivity for steroidogenic enzymes was detected in the cytoplasm of carcinoma cells, and cases that had more than 10% of positive carcinoma cells were considered positive.

Reverse transcription polymerase chain reaction for aromatase

Total RNA was extracted using TRIzol reagent (InVitrogen Life Technologies,Gaitherburg, ND), and a reverse transcription kit (SUPERSCRIPT II Preamplification system (Gibco-BRL, Grand Island, NY) was used in the synthesis of cDNA. Reverse transcription polymerase chain reaction (RT-PCR) for aromatase was performed using the Light Cycler (Roche Diagnositics GmbH, Germany) thermal cycler, and that for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also performed as an internal standard. Settings for the PCR thermal profile were as follows: initial denaturation at 95°C for 1 min followed by 35 amplification cycles of 95°C for 1 sec, annealing at 60°C for 15 sec and elongation at 72°C for 15 sec. The primer sequences used in our study are as follows: aromatase (X13589) (FWD 5′-GTGAAAAAGGGGACAAACAT-3′ (cDNA position, 1,286–1,305) and REV 5′-TGGAATCGTCT-CAGAAGTGT-3′ (cDNA position, 1,481–1,500))[21] and GAPDH (M33197) (FWD 5′-TGAACGGGAAGCTCACTGG-3′ (cDNA position, 731–750)) and REV 5′-TCCACCACCCTGTTGCTGTA-3′ (cDNA position, 1,018–1,038)).[21] As a positive control, placental tissues were used for aromatase,[21] and negative control experiments lacked cDNA substrate to check for the possibility of exogenous contaminant DNA.

Cell culture

MCF-7 human breast carcinoma cells were cocultured with or without primary stromal (74T) cells isolated from breast carcinoma tissue.[22] These cells were separately seeded at 5 × 10^4 cells in a Transwell-Clear culture dish (12-well multidish) (Corining, Corning, NY), and cultured in RPMI-1640 (Sigma-Aldrich, St Louis, MO) with 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS) for 3 days. The cells were precultured in phenol red-free RPMI-1640 without FBS for 24 hr, and subsequently, treated with 10 or 100 nM of androstenedione (Sigma-Aldrich) in the absence or presence of indicated concentration of exemestane (kindly provided from Pfizer (New York, NY)). Serum concentration of androstenedione is 4–7 nM in women,[23] and 1–100 nM concentrations of androstenedione are frequently used in *in vitro* studies.[24] Following 24 hr of incubation, 2 ml of the medium was removed, and concentrations of DHT and estradiol in the medium were measured using the LC-MS/MS analysis described above. The minimum concentration of DHT and estradiol detected was 0.1 pg/ml, respectively.

Statistical analysis

An association between intratumoral concentrations of DHT or estradiol and clinicopathological factors was evaluated using Mann–Whitney's *U* test or the Spearman rank correlation. An association between AR/5αRed1 status and clinicopathological parameters were evaluated using a cross-table using the χ^2 test or unpaired 2 group *t*-test. Overall, disease-free survival curves were generated according to the Kaplan–Meier method and the statistical significance was calculated using the log-rank test. Univariate and multivariate analyses were evaluated by a proportional hazard model (COX) using PROC PHREG in our SAS software. Statisti-

cal analyses in coculture experiments were performed using a one-way ANOVA and Bonferroni test. *p*-values less than 0.05 were considered significant in our study.

Results

Intratumoral concentration of DHT in 38 breast carcinomas

We first evaluated intratumoral concentration of DHT in 38 breast carcinoma tissues by LC-MS/MS analysis. The median with min–max values were 152 (8–990) pg/g. Associations between intratumoral DHT concentration and expression of steroidogenic enzymes were summarized in Table I. Intratumoral DHT concentration was positively associated with 5αRed1 ($p = 0.003$) and negatively correlated with aromatase ($p = 0.03$) in the 38 cases examined, but it was not significantly associated with other steroidogenic enzymes, such as 17βHSD5, 5αRed2, STS and 17βHSD1. As shown in Table II, intratumoral DHT concentration was not significantly associated with any clinicopathological parameters examined, including AR status ($p = 0.47$), in all the 38 cases examined, but it was negatively correlated with tumor size ($p = 0.047$) or Ki-67 LI ($p = 0.045$) in the AR-positive breast carcinoma ($n = 29$).

TABLE I – ASSOCIATION BETWEEN INTRATUMORAL CONCENTRATION OF DHT AND STEROIDOGENIC ENZYME EXPRESSION IN 38 BREAST CARCINOMAS

Value	*n*	DHT concentration (pg/g)	
		Median (min–max)	*p* value
Androgen-producing enzymes			
17βHSD5			
Positive	22	181 (8–990)	
Negative	16	138 (48–463)	0.30
5αRed1			
Positive	23	258 (41–990)	
Negative	15	78 (8–288)	*0.003*
5αRed2			
Positive	6	220 (73–762)	
Negative	32	151 (8–990)	0.26
Estrogen-producing enzymes			
STS			
Positive	22	168 (8–990)	
Negative	16	138 (23–463)	0.53
Aromatase[1]			
Positive	28	138 (8–610)	
Negative	10	229 (119–990)	*0.03*
17βHSD1			
Positive	21	141 (8–762)	
Negative	17	162 (23–990)	0.84

Data were statistically analyzed utilizing Mann–Whitney's *U* test. *p* values less than 0.05 were considered significant, and described as italic.
[1]Expression of aromatase was evaluated by RT-PCR analysis. Expression of all other enzymes was evaluated by immunohistochemistry.

Immunohistochemistry for AR and 5αRed1 in 115 breast carcinomas

In the LC-MS/MS analysis, 5αRed1 was suggested a potent regulator of DHT concentration in breast carcinoma tissues. Because androgenic actions are mainly mediated through an interaction with DHT and AR, we then performed immunohistochemistry for AR and 5αRed1 in 115 breast carcinoma tissues. AR immunoreactivity was detected in the nucleus of carcinoma cells, while 5αRed1 immunoreactivity was detected in the cytoplasm carcinoma cells (Figs. 1*a* and 1*b*), and these immunolocalizations were in good agreement with results of a previous report.[15] A number of positive cases for AR and 5αRed1 was 80 (70%) and 66 cases (57%), respectively. We subsequently classified the carcinomas into following 2 groups according to AR and 5αRed1 status: +/+, cases positive for both AR and 5αRed1 ($n = 53$), and others ($n = 62$).

Associations between AR and 5αRed1 status and clinicopathological parameters in 115 breast carcinomas were summarized in Table III. A group of +/+ was inversely associated with tumor size ($p = 0.0003$) or Ki-67 LI ($p = 0.01$), and positively correlated with ERα LI ($p = 0.03$) and PR LI ($p = 0.01$). No significant associations were detected between AR/5αRed1 status and other clinicopathological parameters examined, including patient age, menopausal status, clinical stage, lymph node status, histological grade and HER2 status in our study.

TABLE II – ASSOCIATION BETWEEN INTRATUMORAL CONCENTRATION OF DHT AND CLINICOPATHOLOGICAL PARAMETERS IN 38 BREAST CARCINOMAS

Value	DHT concentration (pg/g) in 38 breast carcinomas			DHT concentration (pg/g) in 29 AR-positive breast carcinomas		
	n	Median (min–max)	*p* value	*n*	Median (min–max)	*p* value
Age[1] (32–86 years)	38		0.60	29		0.74
Menopausal status						
Premenopausal	10	191 (105–762)		16	175 (48–762)	
Postmenopausal	28	138 (8–990)	0.23	13	138 (8–700)	0.93
Tumor size[1] (8–72 mm)	38		0.32	29		*0.047* ($r = -0.38$)
Lymph node status						
Positive	23	138 (48–990)		16	175 (48–762)	
Negative	13	192 (8–700)	0.73	13	258 (8–990)	0.93
Histological grade						
1 + 2	27	174 (8–762)		24	190 (8–762)	
3	11	138 (23–990)	0.64	5	258 (23–288)	0.60
ERα status						
Positive	28	181 (8–762)		24	138 (48–990)	
Negative	10	130 (60–990)	0.79	5	138 (101–288)	0.64
ERα LI[1] (0–96%)	38		0.27	29		0.38
PR LI[1] (0–96%)	38		0.48	29		0.55
AR status						
Positive	29	181 (8–762)				
Negative	9	130 (60–990)	0.47			
AR LI[1] (0–79%)	38		0.45	29		0.22
HER2						
Positive	12	139 (23–990)		9	138 (23–7628)	
Negative	26	168 (8–610)	0.68	20	214 (8–610)	0.99
Ki-67 LI[1] (3–46%)	38		0.26	29		*0.045* ($r = -0.36$)

p values less than 0.05 were considered significant, and described as italic.
[1]Data were statistically analyzed utilizing the Spearman rank correlation, and the median values were not calculated. All other value were evaluated by Mann–Whitney's *U* test.

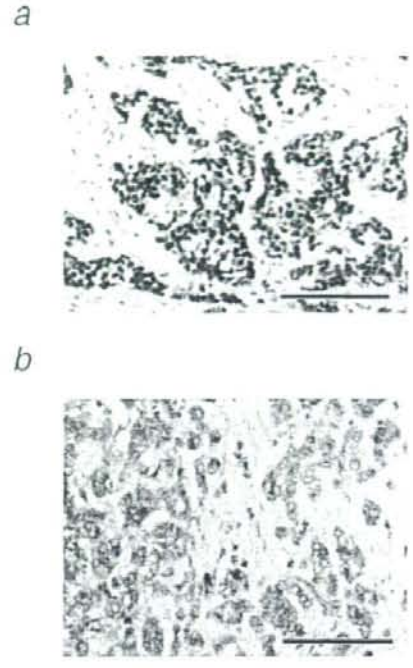

FIGURE 1 – Immunohistochemistry for AR (*a*) and 5αRed1 (*b*) in the invasive ductal carcinoma. Immunoreactivity for AR was detected in nuclei of carcinoma cells (*a*), and that of 5αRed1 was positive for the cytoplasm of these cells. Bar = 100 μm, respectively.

TABLE III – ASSOCIATION BETWEEN AR AND 5αRED1 STATUS AND CLINICOPATHOLOGICAL PARAMETERS IN 115 HUMAN BREAST CARCINOMAS

Value	AR/5αRed1 status		*p* value
	+/+ (*n* = 53)	Others (*n* = 62)	
Age[1] (years)	52.0 ± 1.7	54.0 ± 1.5	0.38
Menopausal status			
Premenopausal	29 (25%)	26 (23%)	
Postmenopausal	24 (21%)	36 (31%)	0.24
Stage			
1 + 2	48 (42%)	52 (46%)	
3	5 (4%)	10 (9%)	0.43
Tumor size[1] (mm)	20.8 ± 1.2	29.1 ± 1.8	*0.0003*
Lymph node status			
Positive	18 (16%)	33 (29%)	
Negative	35 (30%)	29 (25%)	0.07
Histological grade			
1 + 2	38 (33%)	32 (29%)	
3	15 (13%)	30 (26%)	0.052
ERα LI[1]	50.1 ± 4.3	36.5 ± 4.6	*0.03*
PR LI[1]	53.6 ± 4.7	36.5 ± 4.5	*0.01*
Ki-67 LI[1]	21.9 ± 1.5	30.6 ± 2.6	*0.01*
HER2			
Positive	16 (14%)	22 (19%)	
Negative	37 (32%)	40 (35%)	0.69

AR/5αRed1 status was evaluated by immunohistochemistry, and "+/+" represents breast carcinomas positive for both AR and 5αRed1. All other values represent the number of cases and percentage, and were evaluated using a cross-table using the χ^2 test. *p* values less than 0.05 were considered significant, and described as italic.

[1]Data are presented as mean ± 95% confidence interval (95% CI), and were evaluated by unpaired two group *t*-test.

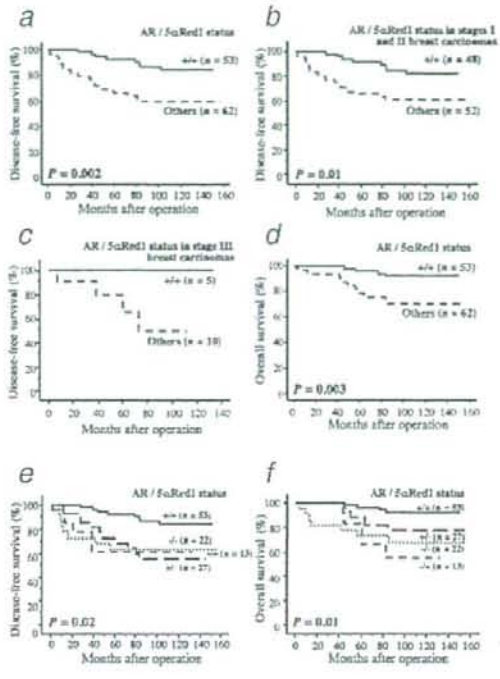

FIGURE 2 – Disease-free (*a–c*, *e*) and overall (*d*, *f*) survival curves of 115 patients with breast carcinoma according to AR and 5αRed1 status (Kaplan–Meier method). According to AR/5αRed1 status, breast carcinomas were classified into 2 groups (+/+, cases positive for both AR and 5αRed1, and others) (*a–d*) or 4 groups (+/+, +/−, AR-positive but 5αRed1-negative cases; −/+, AR-negative but 5αRed1-positive cases and −/−, cases negative for AR or 5αRed1) (*e*, *f*). *p* values were evaluated by a log-rank test. *p* value was not calculated in (*c*), because no patient had recurrent in the group of "+/+" breast cancer patients.

Correlation between AR/5αRed1 status and clinical outcome of the patients

A +/+ group of AR/5αRed1 status was significantly associated with a decreased risk of recurrence ($p = 0.002$ in a log-rank test) in 115 breast carcinoma patients (Fig. 2*a*), and similar tendency was detected regardless of the clinical stage in our study (Figs. 2*b* and 2*c*). In a univariate analysis by COX (Table IV), lymph node status ($p < 0.0001$), AR/5αRed1 status ($p = 0.004$), tumor size ($p = 0.01$) and HER2 ($p = 0.047$) were turned out significant prognostic parameters for disease-free survival of the patients, and a subsequent multivariate analysis demonstrated that only lymph node status ($p = 0.0001$) and AR/5αRed1 status ($p = 0.03$) were independent prognostic factors with relative risks over 1.0 (Table IV). AR status alone ($p = 0.08$) or 5αRed1 alone ($p = 0.08$) did not reach a significant level in a univariate analysis for the disease-free survival in our study.

Overall survival curve was demonstrated in Figure 2*d*, and the +/+ group was significantly associated with a better clinical outcome of the patients ($p = 0.003$ in a log-rank test). Utilizing a univariate analysis (Table V), lymph node status ($p = 0.001$), AR/5αRed1 status ($p = 0.01$), tumor size ($p = 0.02$) and HER2 ($p = 0.03$) were detected significant prognostic factors for overall survival of the patients. Following multivariate analysis demonstrated lymph node status ($p = 0.002$) and AR/5αRed1 status ($p = 0.03$) as independent prognostic factors for overall survival. AR status

TABLE IV – UNIVARIATE AND MULTIVARIATE ANALYSES OF DISEASE-FREE SURVIVAL OF 115 BREAST CANCER PATIENTS EXAMINED

Variable	Univariate *p*	Multivariate *p*	Relative risk (95% CI)
Lymph node status (positive *vs.* negative)	*<0.0001*[1]	*0.0001*	6.5 (2.5–16.7)
AR/5αRed1 status (+/+ *vs.* others)	*0.004*[1]	*0.03*	2.6 (1.2–5.9)
Tumor size (≥20 mm *vs.* <20 mm)	*0.01*[1]	0.54	
HER2 (positive *vs.* negative)	*0.047*[1]	0.79	
AR status (negative *vs.* positive)	0.08		
5αRed1 (negative *vs.* positive)	0.08		
Ki-67 LI (≥10 *vs.* < 10)	0.15		
Histological grade (3 *vs.* 1, 2)	0.23		
ERα status (positive *vs.* negative)	0.33		

Univariate and multivariate analyses were evaluated by a proportional hazard model (COX) using PROC PHREG in our SAS software. Significant values were described as italic.

[1]Data were examined in a following multivariate analysis.

TABLE V – UNIVARIATE AND MULTIVARIATE ANALYSES OF OVERALL SURVIVAL OF 115 BREAST CANCER PATIENTS EXAMINED

Variable	Univariate *p*	Multivariate *p*	Relative risk (95% CI)
Lymph node status (positive *vs.* negative)	*0.001*[1]	*0.002*	21.9 (3.3–81.7)
AR/5αRed1 status (+/+ *vs.* others)	*0.01*[1]	*0.03*	3.5 (1.3–10.9)
Tumor size (≥20 mm *vs.* <20 mm)	*0.02*[1]	0.93	
AR status (negative *vs.* positive)	*0.03*		
HER2 (positive *vs.* negative)	*0.03*[1]	0.89	
Histological grade (3 *vs.* 1, 2)	0.06		
5αRed1 (negative *vs.* positive)	0.09		
Ki-67 LI (≥10 *vs.* < 10)	0.36		
ERα status (positive *vs.* negative)	0.49		

Univariate and multivariate analyses were evaluated by a proportional hazard model (COX) using PROC PHREG in our SAS software. Significant values were described as italic.

[1]Data were examined in a following multivariate analysis.

alone was also turned out a significant prognostic factor for overall survival ($p = 0.03$), but not an independent factor when this variable was included in a multivariate analysis instead of AR/5αRed1 status ($p = 0.21$). 5αRed1 alone was not a significant prognostic factor ($p = 0.09$) for overall survival.

Significant associations between AR/5αRed1 status and clinical outcome of the patients were also detected when AR/5αRed1 status was further categorized into 4 groups (+/+, +/−, AR-positive but 5αRed1-negative cases; −/+, AR-negative but 5αRed1-positive cases and −/−, cases negative for AR or 5αRed1) (Figs. 2*e* and 2*f*).

Inhibition of DHT production by aromatase in cell culture experiments

In LC-MS/MS analysis in breast carcinoma tissues, intratumoral DHT concentration was inversely associated with aromatase expression (Table I). These results suggest possible regulation of DHT production by aromatase in the breast carcinoma, but such findings have not been reported yet to our best knowledge. To examine this hypothesis, we used MCF-7 breast carcinoma cells, which expressed 17βHSD5 and 5αRed1 but with very low level of aromatase, and 74T stromal cells which highly expressed aromatase but with low levels of 17βHSD5 and 5αRed1 by RT-PCR (data not shown).

When cells were treated with 10 nM androstenedione for 24 hr, estradiol production was negligible in MCF-7 cells alone (1.1 ± 0.3 pg/ml), but was increased at 49-fold (43.0 ± 11.8 pg/ml; $p < 0.001$ *vs.* MCF-7 alone) in the medium cocultured with MCF-7 and 74T cells (Fig. 3*a*). The production of estradiol was significantly inhibited by addition of an aromatase inhibitor, exemestane, in a dose-dependent manner ($p < 0.01$ in addition of 10 nM exemestane, and $p < 0.001$ in addition of 100 nM exemestane). On the other hand, DHT was markedly synthesized in MCF-7 cells alone in treatment with 10 nM androstenedione for 24 hr (60.0 ± 11.9 pg/ml), but was decreased into 13% (7.5 ± 5.7 pg/ml, $p < 0.001$ *vs.* MCF-7 alone) when MCF-7 cells were cocultured with 74T cells (Fig. 3*b*). DHT production was dose-dependently increased by addition of exemestane ($p < 0.01$ in addition of 10 nM exemestane, and $p < 0.001$ in addition of 100 nM exemestane). DHT concentration in the medium was not significantly changed between MCF-7 and T74 cells with 100 nM exemestane and MCF-7 alone ($p = 0.31$).

A similar tendency was also detected, when cells were treated with 100 nM androstenedione for 24 hr (Figs. 3*c* and 3*d*).

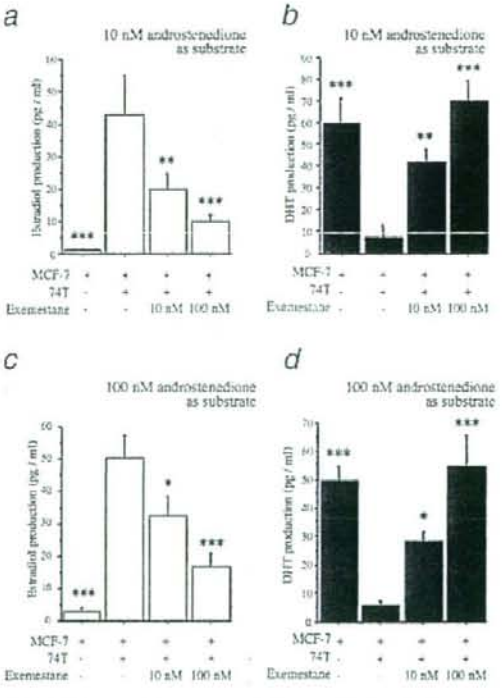

FIGURE 3 – Regulation of estradiol (*a*, *c*) and DHT (*b*, *d*) synthesis by aromatase in MCF-7 cells cocultured with aromatase-expressed 74T stromal cells. These cells were treated with androstenedione (10 nM (*a*, *b*) or 100 nM (*c*, *d*)) in absence or presence of aromatase inhibitor exemestane (10 or 100 nM) for 24 hr, and subsequently, concentrations of estradiol and DHT in the medium was evaluated by LC-MS/MS. Data are presented as mean ± SD ($n = 3$). *, ** and *** $p < 0.05$, $p < 0.01$ and $p < 0.001$ *vs.* coculture of MCF-7 and 74T cells without exemestane (second column), respectively.

Discussion

In our present study, the median intratumoral DHT concentration determined in 38 breast carcinomas was 152 pg/g with a range from 8 to 990 pg/g. This was similar to the results from 2 other groups.[13,14] The intratumoral DHT concentration was not significantly altered according to the menopausal status in our study, which was also consistent with a previous report by Mistry *et al.*[13] Results of our study demonstrated a significant positive association between intratumoral DHT concentration and 5αRed1. DHT is synthesized from testosterone by 5αRed and 2 isoforms of 5αRed, *i.e.* 5αRed1 and 5αRed2, have been characterized in mammals.[25] 5αRed activity was 4–8 times higher in breast cancer tissues than in nontumorous breast tissues,[26] and immunoreactivity of 5αRed1 was detected in 58% of breast carcinomas, while that of 5αRed2 was found only in 15% cases.[15] Therefore, it is suggested that intratumoral DHT concentration was mainly determined by 5αRed1 in breast carcinoma tissues regardless of the menopausal status.

On the other hand, the intratumoral DHT concentration was not significantly associated with AR status in our study. In previous studies, AR expression was suppressed by DHT with a reduction in AR mRNA stability in AR-positive MDA453 breast carcinoma cells,[27] and AR levels were also regulated by *cis*-acting element, CpG methylation, and histone acetlyation.[12,28,29] On the other hand, regulatory mechanisms of 5αRed1 remain largely unclear; however, the rat 5αRed1 gene was reported under positive regulation by growth hormone and DHT.[30] These data indicate that intratumoral DHT concentration and AR expression are differently regulated in the breast carcinomas. DHT actions are mediated through an interaction with DHT and AR. Therefore, it is suggested that effects of DHT primarily exist in breast carcinoma tissues positive for both AR and 5αRed1, and AR status alone does not necessarily reflect the androgenic actions.

In our study, breast carcinomas double-positive for AR and 5αRed1 were inversely associated with tumor size and Ki-67. Previous *in vitro* studies have demonstrated that DHT inhibited cell proliferation of various breast carcinoma cells.[2–5] These antiproliferative effects were independent on the presence of estradiol, and were associated with an increase in a proportion of cells in G0/G1 phase in MCF-7 cells.[4] DHT also caused accumulation of cyclin-dependent kinase inhibitor p27 in CAMA-1 cells,[3] and DHT treatment resulted in a rapid fall in tumor volume of ZR75-1 cells injected into athymic mice.[31] Our present results are in good agreement with results of these previous studies, and suggest that DHT is mainly involved in the growth inhibition in breast carcinomas.

Correlation between AR status and clinical outcome of breast carcinoma patients has been examined by several groups,[7,9,32] but the results were not necessarily consistent. Bryan *et al.*[32] found a significant association between AR status evaluated by AR assays and overall survival of the patients. Soreide *et al.*,[7] however, did not detect any significant correlation between AR status and relapse-free survival. In more recent study, Kuenen-Boumeester *et al.*[9] performed immunohistochemistry for AR in 153 breast carcinomas, and reported that AR status was a significant prognostic factor for disease-free survival, but was not an independent factor following a multiple analysis. In our present study, AR/5αRed1 status was an independent prognostic factor for both disease-free and overall survival as well as a lymph node status, a well-established diagnostic modality.[33] If DHT is involved in growth inhibition through AR, residual cancer cells following surgical treatment in AR and 5αRed1 double-positive breast carcinomas possibly grow slowly in the presence of locally produced DHT, which may subsequently result in a better clinical outcome of these patients. Inconsistent results regarding the correlation between AR status and prognosis in previous studies may partly be due to different ratios of breast carcinomas positive for both AR and 5αRed1 examined.

Previous studies demonstrated a significant association of intratumoral concentrations between DHT and testosterone in breast carcinomas,[13,14] suggesting that the intratumoral DHT concentrations were influenced by amounts of the precursor. In our present study, aromatase expression was inversely associated with intratumoral DHT concentrations in breast carcinoma tissues. In coculture studies, aromatase regulated both DHT and estradiol synthesis regardless of the concentration of androstenedione as a substrate (Fig. 3). Aromatase catalyzes the conversion of androstenedione and testosterone, which are precursors of DHT, to estrone and estradiol, respectively. However, DHT itself is nonaromatizable. Previously, Spinola *et al.*[34] reported that treatment with an aromatase inhibitor (4-hydroxyandrostenedione) markedly elevated intratumoral testosterone concentrations in dimethylbenz(a)anthracene-induced rat mammary tumors, and Sonne-Hansen and Lykkesfeldt[35] recently showed that aromatase preferred testosterone as a substrate in MCF-7 cells. In addition, an aromatase inhibitor letrozole was demonstrated to block conversion of androgens into estrogens with a subsequent increment of intraovarian androgens.[36,37] Therefore, aromatase is suggested a negative regulator for *in situ* production of DHT in breast carcinoma tissues by possibly reducing concentrations or availability of the precursor testosterone. Administration of androgens combined with antiestrogen has been more effective than that of antiestrogen alone in breast cancer patients, and the additive inhibitory effects were exerted in part by different mechanisms.[38] In addition, results of recent large multicenter trials all demonstrated superior efficacy of aromatase inhibitors, such as anastrolozole letrozole and exemestane, compared to antiestrogen tamoxifen,[39–41] although it might be due to agonistic effects of tamoxifen in estrogen-deprived environment.[42] Therefore, results of our present study suggest that treatment of aromatase inhibitors may be more effective in breast carcinoma patients positive for both AR and 5αRed1, because of additional antiproliferative effects through increasing local DHT concentration with estrogen deprivation. It awaits further examinations to clarify the clinical importance of AR/5αRed1 status as a marker of response to aromatase inhibitors in breast cancer patients.

In summary, intratumoral DHT concentrations were positively associated with 5αRed1 and inversely correlated with aromatase in 38 breast carcinomas. In the immunohistochemistry in 115 breast carcinomas, cases positive for both AR and 5αRed1 were inversely associated with tumor size and Ki-67. AR/5αRed1 status was demonstrated an independent prognostic factor for these patients. In the coculture experiments, DHT production was negatively regulated by aromatase. The results of our present study suggest that antiproliferative effects of DHT may primarily occur in breast carcinomas positive for both AR and 5αRed1, and aromatase inhibitors may be more effective in these patients by accumulation of the local DHT concentration.

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Peroxisome proliferator-activated receptor γ in human breast carcinoma: a modulator of estrogenic actions

T Suzuki, S Hayashi[1], Y Miki, Y Nakamura, T Moriya, A Sugawara[2], T Ishida[3], N Ohuchi[3] and H Sasano

Department of Pathology, Tohoku University School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai, 980-8575, Japan
[1]Department of Molecular Medical Technology, Tohoku University School of Medicine, Sendai, Japan
[2]Department of Medicine, Tohoku University School of Medicine, Sendai, Japan
[3]Department of Surgery, Tohoku University School of Medicine, Sendai, Japan

(Requests for offprints should be addressed to T Suzuki; Email: t-suzuki@patholo2.med.tohoku.ac.jp)

Abstract

It has been reported that agonists of peroxisome proliferator-activated receptor γ (PPARγ) inhibit proliferation of breast carcinoma cells, but the biological significance of PPARγ remains undetermined in human breast carcinomas. Therefore, we immunolocalized PPARγ in 238 human breast carcinoma tissues. PPARγ immunoreactivity was detected in 42% of carcinomas, and was significantly associated with the status of estrogen receptor (ER) α, ERβ, progesterone receptor, retinoic X receptors, p21 or p27, and negatively correlated with histological grade or cyclooxygenase-2 status. PPARγ immunoreactivity was significantly associated with an improved clinical outcome of breast carcinoma patients by univariate analysis, and multivariate analysis demonstrated that PPARγ immunoreactivity was an independent prognostic factor for overall survival in ERα-positive patients. We then examined possible mechanisms of modulation by PPARγ on estrogenic actions in MCF-7 breast carcinoma cells. A PPARγ activator, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), significantly inhibited estrogen-responsive element-dependent transactivation by estradiol in MCF-7 cells, which was blocked by addition of a PPARγ antagonist GW9662. Subsequent study, employing a custom-made microarray focused on estrogen-responsive genes, revealed that mRNA expression was significantly regulated by estradiol in 49 genes, but this significance vanished on addition of 15d-PGJ_2 in 16 out of 49 (33%) genes. These findings were confirmed by real-time PCR in 11 genes. 15d-PGJ_2 significantly inhibited estrogen-mediated proliferation of MCF-7 cells, and caused accumulation of p21 and p27 protein. These results suggest that PPARγ is mainly expressed in well-differentiated and ER-positive breast carcinomas, and modulates estrogenic actions.

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Introduction

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear hormone receptor superfamily, and has also been designated NR1C3 (Lemberger *et al.* 1996, Schoonjans *et al.* 1996). PPARγ functions as a transactivation factor following heterodimerization with retinoic X receptors (RXRs), and binds to its specific response elements termed peroxisome proliferating responsive elements (PPREs) of various target genes (Mangelsdorf & Evans 1995). PPARγ is one of the ligand-activated transcription factors, and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) is currently considered a naturally occurring PPARγ ligand, which activates PPARγ at μM concentrations in the human (Koeffler 2003).

It is well known that PPARγ plays essential roles in adipogenesis (Tontonoz *et al.* 1994), insulin resistance (Celi & Shuldiner 2002), and development of various organs (Barak *et al.* 1999). In addition, various *in vitro* studies have demonstrated that PPARγ ligands have a potent antiproliferative activity against a wide variety

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of neoplastic cells (Koeffler 2003). For instance, PPARγ agonist inhibits the proliferation of human breast cancers (Elstner *et al.* 1998, Mueller *et al.* 1998, Yee *et al.* 1999), and a phase II clinical trial using PPARγ ligands has been recently performed as a novel therapy for advanced breast cancer patients (Burstein *et al.* 2003).

It then becomes very important to obtain a better understanding of the clinical and/or biological roles of PPARγ in breast cancer tissues in order to improve the potential clinical efficiency of PPARγ ligand therapy for breast cancer patients. Mueller *et al.* (1998) previously demonstrated the expression of PPARγ in human primary and metastatic cancers, and Wang *et al.* (2004) reported higher amounts of PPARγ expression in breast carcinoma cells than in normal human mammary epithelial cells. However, it has been also demonstrated that PPARγ expression is significantly lower in breast cancer tissues at both mRNA (Jiang *et al.* 2003) and protein (Watkins *et al.* 2004) levels than that in normal tissues. Expression of PPARγ has been examined in human breast carcinomas by several groups, but little information is available on the clinicopathological features of PPARγ-positive breast cancers. Therefore, the biological significance of PPARγ remains largely undetermined in human breast carcinoma. In this study, we examined immunolocalization of PPARγ in 238 cases of human breast carcinoma patients, and correlated these findings with various clinicopathological parameters. As the results of immunohistochemistry demonstrated a strong association between PPARγ and estrogen receptor (ER) α in breast carcinomas, we also examined a possible modulation by PPARγ on estrogenic actions in breast cancer cells for further characterization of PPARγ in human breast carcinoma.

Materials and methods

Patients and tissues

Two hundred and thirty-eight surgical pathology specimens of invasive ductal carcinoma of the breast were retrieved from pathology archives of the Department of Surgery, Tohoku University Hospital, Sendai, Japan. Breast tissue specimens were obtained from female patients who underwent mastectomy from 1982 to 1992 with a mean age of 54.1 years (range 22–82). The patients did not receive chemotherapy or irradiation prior to surgery. Review of the charts of patients revealed that 194 patients received adjuvant chemotherapy, and 43 patients received tamoxifen therapy after surgery. The mean follow-up time was 102 months (range 2–157 months). The histological grade of each specimen was evaluated based on the method of Elston & Ellis (1991). All specimens were fixed with 10% formalin and embedded in paraffin wax. Research protocols for this study were approved by the Ethics Committee at Tohoku University School of Medicine (approved number: 2000-142).

Antibodies

Rabbit polyclonal antibody for PPARγ was raised against a synthetic peptide corresponding to amino acids 60–79 of mouse PPARγ1 (accession number; AAA62110), which also corresponds to amino acids 62–81 of human PPARγ1 (CAA62152) or 90–109 of human PPARγ2 (AAB04028). This antibody therefore recognizes both human PPARγ1 and γ2. The characterization of this antibody has been previously confirmed by both immunoblotting and immunohistochemistry (Sato *et al.* 2004). The characteristics of polyclonal antibodies for RXRα, RXRβ and RXRγ have been previously reported by the authors (Sugawara *et al.* 1995, Suzuki *et al.* 2001). Monoclonal antibodies for ERα (ER1D5), progesterone receptor (PR; MAB429), Ki-67 (MIB1), p21 (6B6), p27 (1B4), c-Myc (1-6E10), pS2 (M7184), and cyclin D1 (P2D11F11) were purchased from Immunotech (Marseille, France), Chemicon (Temecula, CA, USA), DAKO (Carpinteria, CA, USA), Pharmingen (San Diego, CA, USA), Novocastra Laboratories (Newcastle, UK), Cambridge Research Biochemical (Cambridge, UK), DAKO and Novocastra Laboratories respectively. Rabbit polyclonal antibodies for ERβ (06-629), HER2 (A0485) and cathepsin D (A0561) were obtained from Upstate Biotechnology (Lake Placid, NY, USA), DAKO and DAKO respectively. Goat polyclonal antibody for cyclooxygenase-2 (COX2) (C-20) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Immunohistochemistry

A Histofine Kit (Nichirei, Tokyo, Japan), which employs the streptavidin-biotin amplification method was used in this study. Antigen retrieval for PPARγ, ERα,β, PR, RXRα,β,γ, HER2, Ki-67, p21, p27 and cyclin D1 immunostaining was performed by heating the slides in an autoclave at 120 °C for 5 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate, pH 6.0), ,and antigen retrieval for COX2 and pS2 immunostaining was done by heating the slides in a microwave oven for 15 min in the citric acid buffer. Dilutions of primary antibodies used in this study were as follows: PPARγ; 1/1500, ERα; 1/50,

ERβ; 1/50, PR; 1/30; RXRα; 1/4000, RXRβ; 1/4000, RXRγ; 1/2000, COX2; 1/500, HER2; 1/200, Ki-67; 1/50, p21; 1/250, p27; 1/150, c-Myc 1/600, pS2; 1/30, cyclin D1; 1/40 and cathepsin D; 1/300. The antigen–antibody complex was visualized with 3,3′-diaminobenzidine solution (1 mM, in 50 mM Tris–HCl buffer (pH 7.6) and 0.006% H_2O_2), and counterstained with hematoxylin. As a negative control, normal rabbit, mouse or goat IgG was used instead of the primary antibodies. For PPARγ immunohistochemistry, a preabsorption test was also performed as a negative control.

Scoring of immunoreactivity and statistical analysis

PPARγ, ERα,β, PR, RXRα,β,γ, Ki-67, p21, p27, c-Myc and cyclin D1 immunoreactivity was detected in the nucleus, and the immunoreactivity was evaluated in more than 1000 carcinoma cells for each case, and subsequently the percentage of immunoreactivity, i.e. labeling index (LI), was determined. Inter-observer differences were less than 5%, and the mean of the three values was obtained. Cases with PPARγ, ERα or c-Myc LIs of more than 10% were considered PPARγ-, ERα- or c-Myc-positive breast carcinomas in this study, according to a report for ERα by Allred *et al.* (1998). For p21 and p27 immunohistochemistry, the cut-off values used were 5 and 50% respectively, according to previous reports (Barbareschi *et al.* 2000, Pellikainen *et al.* 2003). Immunoreactivity for COX2 and cathepsin D was detected in the cytoplasm, and cases that had more than 10% of positive carcinoma cells were considered positive.

An association between immunoreactivity for PPARγ and clinicopathological factors was evaluated using a one-way ANOVA and a Bonferroni test or a cross-table using the chi-square test. Overall and disease-free survival curves were generated according to the Kaplan–Meier method and statistical significance was calculated using the log-rank test. An association between ERα LI and PR or cyclin D1 LI was performed utilizing a correlation coefficient (r) and regression equation. Univariate and multivariate analyses were evaluated by a proportional hazard model (Cox) using PROC PHREG in our SAS software. P values less than 0.05 were considered significant in this study.

Cell line, plasmids and chemicals

MCF-7 human breast cancer cell line was cultured in RPMI-1640 (Sigma-Aldrich, St Louis, MO, USA) with 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA). MCF-7 cells were cultured with phenol red-free RPMI 1640 medium containing 10% dextran-coated charcoal (DCC)-FBS for 3 days before treatment in the experiment. In this study, we used estrogen-responsive reporter plasmids pERE-Luc, containing *Xenopus* vitellogenin A2 estrogen-responsive element (ERE) (Saji *et al.* 2001). The pRL-TK vectors were purchased from Promega (Madison, WI, USA). 15d-PGJ_2, ciglitazone and $PGF_{2\alpha}$ were purchased from Biomol Research Laboratories (Butler Pike, PA, USA), and GW1929 and GW9662 were purchased from Sigma-Aldrich.

Luciferase assay

The luciferase assay was performed according to a previous report (Sakamoto *et al.* 2002) with some modifications. Briefly, 1 μg ptk-ERE-Luc plasmids and 200 ng pRL-TK control plasmids were used to measure the transcriptional activity of endogenous ER. Transient transfections were carried out using *Trans*IT-LT Transfection Reagents (TaKaRa, Tokyo, Japan) in MCF-7 cells, and the luciferase activity of lysates was measured using a Dual-Luciferase Reporter Assay system (Promega) and Luminescencer-PSN (AB-2200) (Atto Co., Tokyo, Japan) after incubation with growth medium with the indicated concentrations of estradiol and/or 15d-PGJ_2 for 24 h. The cells were also treated with the same volume of ethanol (final dilution – 0.1%) for 24 h as controls. The transfection efficiency was normalized against *Renilla* luciferase activity using pRL-TK control plasmids, and the luciferase activity for each sample was evaluated as a ratio (%) compared with that of controls. The statistical analyses were performed using a one-way ANOVA and Bonferroni test.

Immunoblotting

The cell protein was extracted in triple detergent lysis buffer (LK-18) at 4 °C. Twenty micrograms of the protein (whole cell extracts) were subjected to SDS-PAGE (10% acrylamide gel). Following SDS-PAGE, proteins were transferred onto Hybond P polyvinylidene difluoride membrane (Amersham Biosciences, Piscataway, NJ, USA). The blots were blocked in 5% non-fat dry skim milk for 1 h at room temperature, and were then incubated with a primary antibody for ERα, p21, p27 or β-actin (Sigma-Aldrich) for 18 h at 4 °C. After incubation with anti-mouse IgG horseradish peroxidase (Amersham Biosciences) for 1 h at room temperature, antibody–protein complexes on the blots were detected using ECL-plus western blotting detection reagents (Amersham Biosciences). Immunointensity of

Table 1 Primer sequences used in real-time PCR in this study

cDNA (Accession no.)	Sequence (position in cDNA)	Size (bp)	Reference
pS2 (NM003225)	FWD: 53–72, REV: 240–260	208	Colombel *et al.* (1999)
PDZK1 (AF012281)	FWD: 725–746, REW: 824–847	123	Yoshida *et al.* (2004)
Cyclin D1 (NM053056)	FWD: 456–475, REV: 640–659	204	Paruthiyil *et al.* (2004)
IGFBP-4 (M62403)	FWD: 697–715, REV: 756–773	77	Yoshida *et al.* (2004)
SLC7A5* (M80244)	FWD: 816–835, REV: 1025–1044	206	—
TPD52L1 (U44427)	FWD: 450–469, REV: 561–580	130	Yoshida *et al.* (2004)
PR*† (XM006190)	FWD: 1987–2006, REV: 2163–2182	195	—
Cathepsin D (BT006910)	FWD: 697–715, REV: 804–822	126	Yoshida *et al.* (2004)
TAL6 (M90657)	FWD: 246–265, REV: 299–319	74	Kao *et al.* (2003)
IGFBP-5 (NM000599)	FWD: 1090–1108, REV: 1213–1232	143	Yoshida *et al.* (2004)
SELENBP1 (U29091)	FWD: 167–186, REV: 315–333	167	Yoshida *et al.* (2004)
p21* (NM000389)	FWD: 415–434, REV: 573–592	78	—
p27 (NM004064)	FWD: 829–848, REW: 929–938	120	Schonherr *et al.* (2001)
RPL13A (NM012423)	FWD: 487–509, REV: 588–612	125	Vandesompele *et al.* (2002)

PDZK1; PDZ domain-containing-protein, IGFBP-4; insulin-like growth factor-binding protein-4, SLC7A5; solute carrier family 7, member 5, TPD52L1; tumor protein D52-like 1, PR; progesterone receptor, TAL6; tumor-associated antigen L6, IGFBP-5; insulin-like growth factor-binding protein-5, SELENBP; selenium-binding protein-1, and RPL13A; ribosomal protein L 13a.
*Oligonucleotide primers for SLC7A5, PR and p21 were designed in the different exons in this study.
†The primers of PR recognize both PR-A and PR-B subtypes.

specific bands was measured by an LAS-1000 imaging system (Fuji Photo Film, Tokyo, Japan), and relative immunointensity of ERα, p21 or p27 was evaluated as a ratio (%) of β-actin immunointensity.

Microarray analysis

In this study, we used a custom-made microarray named EstrArray (InfoGenes, Tsukuba, Japan), which contains 175 estrogen-responsive genes identified in MCF-7 cells (Inoue *et al.* 2002, Hayashi *et al.* 2003). MCF-7 cells were cultured with phenol red-free RPMI 1640 medium containing 10% DCC-FBS for 3 days, and subsequently treated with estradiol (10 nM) with or without 15d-PGJ_2 (5 μM) for 72 h. The MCF-7 cells used as references were treated with the same volume of ethanol (final dilution – 0.1%) for 72 h.

Total RNA was isolated using TRIzol reagent (InVitrogen Life Technologies, Inc., Gaitherburg, MD, USA). Two micrograms of mRNA were reverse-transcribed with Cy3- or Cy5-dUTP (Amersham Biosciences, Bucks, UK) using a SUPERSCRIPT II Preamplification system (Gibco-BRL, Grand Island, NY, USA). Cy3- and Cy5-labeled cDNA probes were hybridized on the microarray slide for 16 h at 65 °C. The fluorescent signals were scanned by a GenePix 4000A (Axon Instruments, Foster City, CA, USA), and the ratio of Cy3 and Cy5 signal intensity of each spot was quantitatively calculated using GenePixPro 5.0 (Axon Instruments). The duplicated sets of values were averaged and normalized by subtracting the average of values for internal genes. The data from insufficient hybridization (signal areas below 100) were excluded from the analysis. Genes which showed a value of more than 2.0 or less than 0.5 were evaluated as significantly up-regulated or down-regulated respectively, in this study.

Real-time PCR

MCF-7 cells were cultured with phenol red-free RPMI 1640 medium containing 10% DCC-FBS for 3 days, and subsequently treated with the indicated concentration of estradiol and/or 15d-PGJ_2 for 72 h. As controls for the experiments, the cells were treated with the same volume of ethanol (final dilution – 0.1%) for 72 h. Total RNA was extracted using TRIzol reagent (InVitrogen Life Technologies, Inc.), and a reverse transcription kit (SUPERSCRIPT II Preamplification system (Gibco-BRL) was used in the synthesis of cDNA.

The Light Cycler System (Roche Diagnositics GmbH, Mannheim, Germany) was used to semi-quantify the mRNA expression levels by real-time PCR (Dumoulin *et al.* 2000). Characteristics of the primer sequences used in this study are summarized in Table 1 (Colombel *et al.* 1999, Schonherr *et al.* 2001, Vandesompele *et al.* 2002, Kao *et al.* 2003, Paruthiyil *et al.* 2004, Yoshida *et al.* 2004). Settings for the PCR thermal profile were: initial denaturation at 95 °C for 1 min followed by 40 amplification cycles of 95 °C for 1 s, annealing at 59 °C (PR), 60 °C (pS2, PDZ domain-containing protein (PDZK1), cathepsin D, selenium-binding protein 1 (SELENBP1), tumor protein D52-like 1 (TPD52L1), insulin-like growth

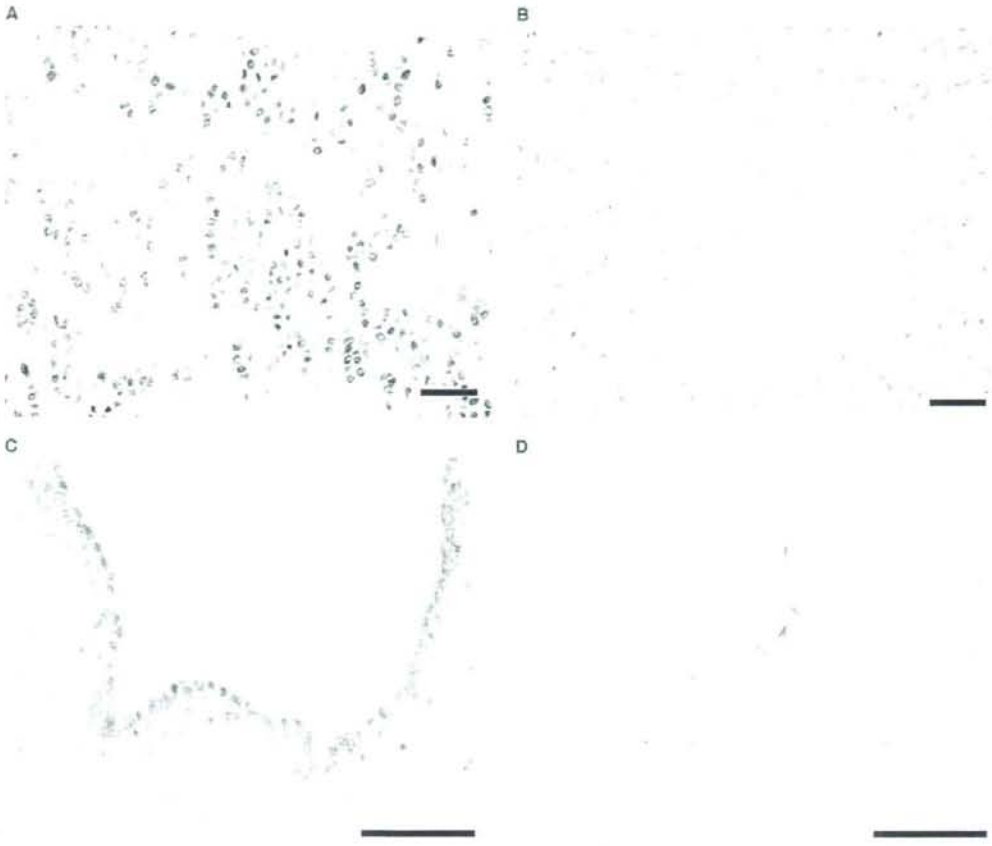

Figure 1 Immunohistochemistry for PPARγ in invasive ductal carcinoma. (A) PPARγ immunoreactivity was detected in the nuclei of carcinoma cells. (B) No significant immunoreactivity of PPARγ was detected in the sections where an immunohistochemical preabsorption test was performed as a negative control. (C) Immunoreactivity for PPARγ was detected in the nuclei of epithelial cells in morphologically normal mammary glands. (D) PPARγ immunoreactivity was positive in the nuclei of adipocytes. Bar = 50 μm.

factor-binding protein-5 (IGFBP-5), PDZ domain-containing-protein (PDZK1), and p21), 64 °C (cyclin D1, and tumor-associated antigen L6 (TAL6)), 66 °C (solute carrier family 7, member 5 (SLC7A5), and p27), or 68 °C (ribosomal protein L 13a (RPL13A)) for 15 s, and elongation at 72 °C for 15 s. To verify amplification of the correct sequences, PCR products were purified and subjected to direct sequencing. Negative control experiments lacked cDNA substrate to check for the possibility of exogenous contaminant DNA. The mRNA levels were summarized as a ratio of RPL13A, and subsequently evaluated as a ratio (%) compared with that of controls. The statistical analyses were performed using a one-way ANOVA and Bonferroni test.

Cell proliferation assay and apoptosis analysis

The status of cell proliferation of MCF-7 cells was measured using a WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2-H-tetrazolium, monosodium salt) method (Cell Counting Kit-8; Dojindo Inc., Kumamoto, Japan)) (Isobe *et al.* 1999). We also examined apoptosis status of MCF-7 cells using an apoptosis screening kit (Wako, Osaka, Japan), which employed a modified TdT-mediated dUTP nick-end labeling (TUNEL) method (Gavrieli *et al.* 1992). Optical densities (OD = 450 nM for cell proliferation assay, and OD = 490 nM for

apoptosis analysis) were obtained with a Model 680 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). The cell number and apoptosis index were calculated according to the equation (Cell OD value after test material treatment)/(Vehicle control cell OD value), and subsequently evaluated as a ratio (%) compared with that of controls.

Results

Immunohistochemistry for PPARγ in breast carcinoma tissues

Immunoreactivity for PPARγ was detected in the nuclei of invasive ductal carcinoma cells (Fig. 1A and B). A mean value of PPARγ LI in 238 breast carcinoma cases examined was 15% (range 0–74%), and the number of PPARγ-positive breast carcinomas (i.e. PPARγ LI ≥ 10%) was 99 out of 238 cases (42%). Immunoreactivity of PPARγ was also detected in epithelia of morphologically normal mammary glands (Fig. 1C), and adipocytes (Fig. 1D).

Associations between PPARγ immunoreactivity and clinicopathological parameters in 238 breast carcinomas were summarized in Table 2. PPARγ immunoreactivity was significantly associated with ERα status ($P = 0.0003$), ERα LI ($P < 0.0001$), ERβ LI ($P = 0.0255$), PR LI ($P = 0.0012$), RXRα LI ($P = 0.0365$), RXRβ LI ($P < 0.0001$), RXRγ LI ($P = 0.0005$), p21 immunoreactivity ($P = 0.0057$) or p27 immunoreactivity ($P = 0.0019$). PPARγ immunoreactivity was negatively correlated with histological grade ($P = 0.0035$) or COX2 immunoreactivity ($P = 0.0178$). No significant association was detected between PPARα immunoreactivity and other clinicopathological parameters examined, including patient age, menopausal status, clinical stage, tumor size, lymph node status, HER2 status, Ki-67 LI, and c-Myc immunoreactivity in this study. The association between PPARγ immunoreactivity and RXRβ LI, RXRγ LI, COX2 immunoreactivity, p21 immunoreactivity or p27 immunoreactivity was significant regardless of ERα status of these cases (Table 3). PPARγ immunoreactivity was positively correlated with RXRα LI ($P = 0.0469$) and inversely with lymph node status ($P = 0.0303$) or Ki-67 LI ($P = 0.0485$) only in the ERα-positive group.

Influence of PPARγ immunoreactivity on association between ERα and estrogen-responsive genes in breast cancer tissues

pS2, cyclin D1, PR and cathepsin D are all well recognized as estrogen-responsive genes in human breast cancers. As shown in Table 4, a significant positive association was detected between ERα LI and the status of these immunoreactivity in the 238 breast cancer tissues examined ($P < 0.0001$), which is in good agreement with previous immunohistochemical studies

Table 2 Association between PPARγ immunoreactivity and clinicopathological parameters in 238 breast carcinomas

	PPARγ immunoreactivity		
	Positive (n=99)	Negitive (n=139)	*P* value
Age (years)*	53.1±1.2	55.1±1.1	0.0850
Menopausal status			
Premenopausal	46 (19.3%)	54 (22.7%)	
Postmenopausal	53 (22.3%)	85 (35.7%)	0.2983
Stage			
I	30 (12.6%)	33 (13.9%)	
II	57 (24.0%)	75 (31.5%)	
III	12 (5.0%)	31 (13.0%)	0.1032
Tumor size (mm)*	32.6±0.4	29.4±0.2	0.3808
Lymph node status			
Positive	38 (16.0%)	68 (28.6%)	
Negative	61 (25.6%)	71 (29.8%)	0.1389
Histological grade			
1	35 (14.7%)	31 (13.0%)	
2	40 (16.8%)	46 (19.3%)	
3	24 (10.1%)	62 (26.1%)	**0.0035**
ERα status			
Positive	85 (35.7%)	89 (37.4%)	
Negative	14 (5.9%)	50 (21.0%)	**0.0003**
ERα LI*	54.5±3.1	34.8±2.9	**<0.0001**
ERβ LI*	19.6±2.4	13.4±1.6	**0.0255**
PR LI*	49.6±3.3	34.9±3.0	**0.0012**
RXRα LI*	27.1±3.5	18.4±2.4	**0.0365**
RXRβ LI*	36.0±4.7	10.5±2.2	**<0.0001**
RXRγ LI*	17.5±5.1	3.0±1.0	**0.0005**
COX2 immunoreactivity			
Positive	29 (12.2%)	72 (30.3%)	
Negative	70 (29.4%)	67 (28.2%)	**0.0178**
HER2 status			
Positive	46 (19.3%)	53 (22.7%)	
Negative	53 (22.7%)	85 (35.7%)	0.7836
Ki-67 LI*	22.7±1.6	26.7±1.6	0.0916
p21 immunoreactivity			
Positive	56 (23.5%)	45 (18.9%)	
Negative	43 (17.3%)	91 (38.2%)	**0.0057**
p27 immunoreactivity			
Positive	58 (24.4%)	47 (19.7%)	
Negative	41 (17.2%)	92 (38.7%)	**0.0019**
c-Myc immunoreactivity			
Positive	49 (20.6%)	62 (26.1%)	
Negative	50 (21.0%)	77 (32.4%)	0.5715

*Data are presented as means±95% confidence interval. All other values represent the number of cases and percentage. *P* values less than 0.05 were considered significant, and are in boldface.

(Horwitz & McGuire 1978, Barbareschi *et al.* 1997, Gillesby & Zacharewski 1999, Ioachim *et al.* 2003). When the breast cancers were classified into two groups according to their PPARγ status, no significant association was detected between ERα LI and pS2 ($P = 0.3785$) or cyclin D1 LI ($P = 0.1978$) in PPARγ-positive breast carcinomas, although significant association ($P < 0.0001$ for pS2, and $P = 0.0018$ for cyclin D1) was detected in PPARγ-negative breast carcinomas. On the other hand, ERα LI was significantly associated with PR LI ($P = 0.0008$ in PPARγ-positive cases, and $P < 0.0001$ in PPARγ-negative cases) or cathepsin D ($P = 0.0006$ in PPARγ-positive cases, and $P = 0.0003$ in PPARγ-negative cases) regardless of the PPARγ status in the breast carcinoma cases examined.

Table 3 Statistical association between PPARγ immunoreactivity and clinicopathological parameters according to the ERα status in 238 breast carcinomas

	PPARγ immunoreactivity (positive/negative)	
	ERα-positive group ($n = 173$)	ERα-negative group ($n = 64$)
Age	0.1107	0.1073
Menopausal status	0.6702	0.6616
Stage	0.1272	0.7195
Tumor size	0.6077	0.6063
Lymph node status	**0.0303**	0.7192
Histological grade	0.3691	0.2452
ERβ LI	0.0963	0.1541
PR LI	0.2826	0.5011
RXRα LI	**0.0469**	0.5403
RXRβ LI	**0.0005**	**0.0002**
RXRγ LI	**0.0108**	**0.0185**
COX2 immunoreactivity	**0.0205**	**0.0411**
HER2 status	0.7702	0.7616
Ki-67 LI	**0.0485**	0.7103
p21 immunoreactivity	**0.0113**	**0.0481**
p27 immunoreactivity	**0.0142**	**0.0205**
c-Myc immunoreactivity	0.3999	0.5404

Data are presented as *P* values. *P* values less than 0.05 were considered significant, and are in boldface.

Correlation between PPARγ immunoreactivity and clinical outcome of the patients

No significant association was detected between PPARγ immunoreactivity and a risk of recurrence ($P = 0.8715$) (Fig. 2A). PPARγ immunoreactivity was significantly associated with a better clinical outcome of the 238 breast cancer patients ($P = 0.0257$) (Fig. 2B). This significant association was detected in the ERα-positive group ($P = 0.0057$) (Fig. 2C), but not in the ERα-negative group ($P = 0.6405$) (Fig. 2D). The significant correlation between PPARγ immunoreactivity and overall survival of ERα-positive breast cancer patients was not influenced by tamoxifen therapy after the surgery (Fig. 2E and F).

Utilizing a univariate analysis (Table 5), lymph node status ($P < 0.0001$), histological grade ($P < 0.0001$), tumor size ($P = 0.0002$), HER2 status ($P = 0.0029$), c-Myc immunoreactivity ($P = 0.0066$), and PPARγ immunoreactivity ($P = 0.0287$) turned out to be significant prognostic factors for overall survival in this study. Multivariate analysis revealed that only lymph node status ($P < 0.0001$) and c-Myc immunoreactivity ($P = 0.0024$) were independent prognostic factors with a relative risk over 1.0 (Table 5). When we examined a univariate analysis in the ERα-positive breast cancer patients ($n = 174$), lymph node status ($P < 0.0001$), histological grade ($P < 0.0001$), tumor size ($P < 0.0001$), HER2 status ($P = 0.0008$), PPARγ

Table 4 Correlation between ERα and estrogen-responsive gene immunoreactivity associated with PPARγ status in 238 breast carcinomas

	Total ($n = 238$)		PPARγ-positive ($n = 99$)		PPARγ-negative ($n = 139$)	
	ERα LI	*P* value	ERα LI	*P* value	ERα LI	*P* value
pS2						
Positive	53.2 ± 3.3		57.3 ± 4.4		52.4 ± 4.8	
Negative	31.8 ± 3.7	**< 0.0001**	49.0 ± 6.7	0.3785	22.0 ± 4.1	**< 0.0001**
Cyclin D1 LI*		**< 0.0001** ($r = 0.355$)		0.1978 ($r = 0.231$)		**0.0018** ($r = 0.313$)
PR LI*		**< 0.0001** ($r = 0.512$)		**0.0008** ($r = 0.402$)		**< 0.0001** ($r = 0.566$)
Cathepsin D						
Positive	54.2 ± 3.7		58.0 ± 4.4		51.2 ± 5.6	
Negative	17.5 ± 4.4	**< 0.0001**	24.8 ± 8.5	**0.0006**	12.2 ± 4.2	**0.0003**

P values less than 0.05 were considered significant, and are in boldface.
*The association was statistically evaluated utilizing a correlation coefficient (r) and regression equation.

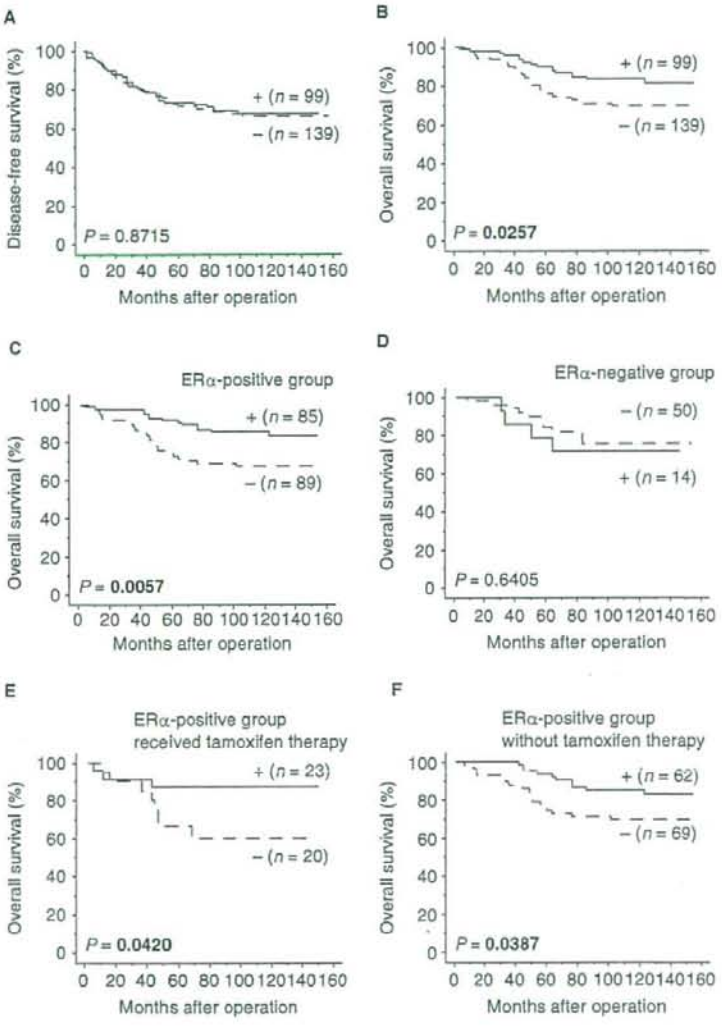

Figure 2 Disease-free (A) and overall (B–F) survival of 238 patients with breast carcinoma according to PPARγ immunoreactivity (Kaplan–Meier method). PPARγ immunoreactivity was not correlated with a risk of recurrence (P=0.8715 in log-rank test) (A), but significantly associated with an improved overall survival (P=0.0257 in log-rank test) (B). The significant association was detected in the ERα-positive group (n=174; P=0.0057 in log-rank test) (C), but not in the ERα-negative cases (n=64; P=0.6405 in log-rank test) (D). PPARγ immunoreactivity was significantly associated with an improved prognosis regardless of tamoxifen therapy after surgery in the ERα-positive breast cancer patients (E, F). P values less than 0.05 are in boldface.

immunoreactivity ($P = 0.0076$) and c-Myc immunoreactivity ($P = 0.0252$) were demonstrated as significant prognostic factors for overall survival (Table 6). A multivariate analysis revealed that lymph node status ($P < 0.0001$) and PPARγ immunoreactivity ($P = 0.0372$) were independent prognostic factors with relative risks over 1.0.

Effects of PPARγ activator 15d-PGJ$_2$ on estrogen-mediated transcription in MCF-7 cells in luciferase assay

Results of PPARγ immunoreactivity demonstrated a strong association between PPARγ and ERα in breast carcinoma tissues, suggesting a possible

Table 5 Univariate and multivariate analyses of overall survival in 238 breast cancer patients examined

Variable	Univariate *P*	Multivariate	
		P	Relative risk (95% CI)
Lymph node status (positive/negative)	**<0.0001**	**<0.0001**	12.319 (3.233–51.07)
Histological grade (3/1, 2)	**<0.0001**	0.1248	—
Tumor size (>20 mm/<20 mm)	**0.0002**	0.1248	—
HER2 status (positive/negative)	**0.0029**	0.4669	—
c-Myc immunoreactivity (positive/negative)	**0.0066**	**0.0024**	2.168 (1.079–4.356)
PPARγ immunoreactivity (negative/positive)	**0.0287**	0.2558	—
p27 immunoreactivity (negative/positive)	0.0797	—	—
Adjuvant chemotherapy (no/yes)	0.1458	—	—
Ki-67 LI (>10/<10)	0.2818	—	—
Tamoxifen therapy (no/yes)	0.5807	—	—
p21 immunoreactivity (negative/positive)	0.6751	—	—
ERα status (negative/positive)	0.9532	—	—

Data considered significant ($P<0.05$) in the univariate analyses are in boldface, and were examined in the multivariate analyses.

Table 6 Univariate and multivariate analyses of overall survival in 174 ER α-positive breast cancer patients examined

Variable	Univariate *P*	Multivariate	
		P	Relative risk (95% CI)
Lymph node status (positive/negative)	**<0.0001**	**<0.0001**	19.006 (4.402–68.06)
Histological grade (3/1, 2)	**<0.0001**	0.0512	—
Tumor size (>20 mm/<20 mm)	**<0.0001**	0.0724	—
HER2 status (positive/negative)	**0.0008**	0.3899	—
PPARγ immunoreactivity (negative/positive)	**0.0076**	**0.0372**	2.799 (1.128–8.264)
c-Myc immunoreactivity (positive/negative)	**0.0252**	0.2891	—
p27 immunoreactivity (negative/positive)	0.0532	—	—
Adjuvant chemotherapy (no/yes)	0.1057	—	—
Ki-67 LI (>10/<10)	0.1123	—	—
Tamoxifen therapy (no/yes)	0.1497	—	—
p21 immunoreactivity (negative/positive)	0.2231	—	—

Data considered significant ($P<0.05$) in the univariate analyses are in boldface, and were examined in the multivariate analyses.

interaction of these two nuclear receptors in human breast carcinoma cells. Previously, Keller *et al.* (1995) reported that PPARα/RXRβ heterodimer can bind to ERE and possibly modulate the ER-signaling pathway, but this has not been examined in breast cancers.

In order to examine this hypothesis, we used MCF-7 breast cancer cells in the following *in vitro* experiments, because MCF-7 cells were associated with expression of ERα, PPARγ, and RXRα, β, γ (data not shown). When MCF-7 cells were transiently transfected with ptk-ERE-Luc plasmids and treated with 10 nM estradiol, the luciferase activity of the cells was 17-fold increased compared with their basal level (Fig. 3A). PPARγ activator 15d-PGJ_2 significantly inhibited ERE-dependent transactivation by estradiol in a dose-dependent manner, and the luciferase activity of MCF-7 cells treated with 10 nM estradiol and 5 μM 15d-PGJ_2 was decreased to 53% of that treated with 10 nM estradiol alone ($P<0.001$). 15d-PGJ_2 (5 μM) alone did not significantly change the luciferase activity compared with their basal level ($P=0.8837$). 15d-PGJ_2, however, did not significantly inhibit the ERE-dependent transactivation by estradiol, when these cells were treated with a potent PPARγ antagonist GW9662 (Leesnitzer *et al.* 2002).

The ERE-dependent transactivation by estradiol was also inhibited by other PPARγ agonists such as GW1929 and ciglitazone, in a dose-dependent manner ($P<0.001$, on addition of 5 μM GW1929 or ciglitazone), but was not altered by treatment with $PGF_{2\alpha}$, which does not activate PPARγ (Kliewer *et al.* 1995) (Fig. 3B). Results of immunoblotting analysis revealed that relative immunointensity of ERα was not significantly ($P=0.7749$) altered by the treatment with 15d-PGJ_2 in MCF-7 cells (Fig. 3C).

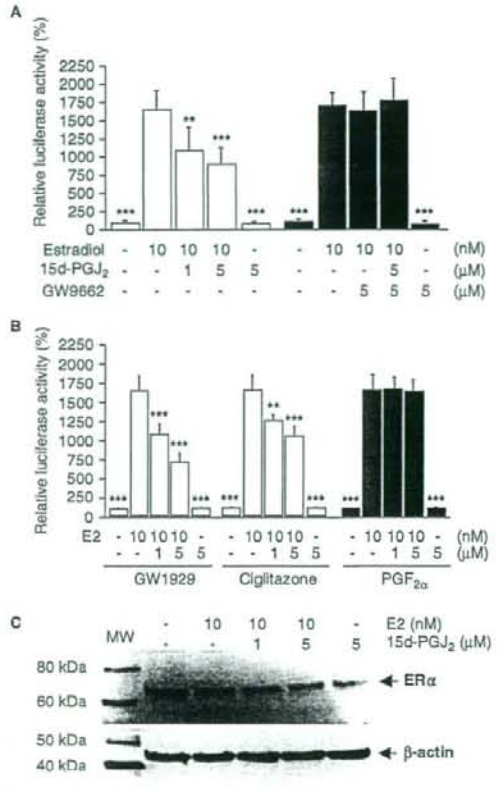

Figure 3 Effects of PPARγ activator 15d-PGJ_2 on ERE-dependent transactivation in MCF-7 cells. (A) MCF-7 cells were transiently transfected with pERE-Luc plasmids, and treated with the indicated concentrations of estradiol, 15d-PGJ_2 and GW9662 (PPARγ antagonist) for 24 h. The luciferase activity was evaluated as a ratio (%) compared with that of controls (treatment without estradiol, 15d-PGJ_2 or GW9662 for 24 h (left column)). Data are presented as means ± s.d. (n=3). $^{**}P<0.01$ and $^{***}P<0.001$ vs 10 nM estradiol alone. (B) MCF-7 cells transfected with pERE-Luc plasmids were treated with the indicated concentrations of estradiol and GW1929 (PPARγ agonist), ciglitazone (PPARγ agonist), or $PGF_{2\alpha}$ (no activator of PPARγ) for 24 h. Data are presented as means ± s.d. (n=3). $^{**}P<0.01$ and $^{***}P<0.001$ vs 10 nM estradiol alone respectively. (C) Immunoblotting for ERα in MCF-7 cells. ERα and β-actin immunoreactivities were detected as specific bands (approximately 66 and 42 kDa respectively); 20 μg of protein were loaded in each lane. Data of immunointensity ratio (ERα/β-actin) are presented as means ± s.d. (n=3). No significant association was detected.

Table 7 The number and percentage of genes classified into four groups according to the effects of 15d-PGJ_2 on the estrogen-mediated mRNA expression in the microarray analysis

	Regulation of mRNA expression by estradiol with 15d-PGJ_2		
	Not significant	Significant	Total
Estrogen up-regulated genes	Group A n=9 (18%)	Group B n=23 (47%)	— n=32 (65%)
Estrogen down-regulated genes	Group C n=7 (14%)	Group D n=10 (20%)	— n=17 (35%)
Total	n=16 (33%)	n=33 (67%)	n=49 (100%)

A custom-made microarray (EstrArray; InfoGenes, Co. Ltd, Tsukuba, Japan), which contains 175 estrogen-responsive genes (Inoue *et al.* 2002, Hayashi *et al.* 2003), was used in this study.
MCF-7 cells were treated with estradiol (10 nM) with or without 15d-PGJ_2 (5 μM) for 72 h. Genes showing the value of more than 2.0- or less than 0.5-fold compared with the control (treated without estradiol or 15d-PGJ_2 for 72 h) were regarded as significantly regulated.

Effects of 15d-PGJ_2 on estrogen-mediated transcription in MCF-7 cells in microarray analysis

We further examined the effects of 15d-PGJ_2 on estrogen-mediated transcription in MCF-7 cells using a custom-made microarray. Significant alterations of mRNA expression by estradiol treatment (10 nM for 72 h) were detected in 49 genes in this study, and these genes were classified into the following four groups (Table 7): Group A; mRNA expression was significantly up-regulated by estradiol, but the significance vanished on addition of 15d-PGJ_2 (5 μM) (nine genes; Table 8), Group B; mRNA expression was significantly up-regulated by estradiol with or without 15d-PGJ_2 (23 genes; Table 9), Group C; mRNA expression was significantly down-regulated by estradiol, but the significance vanished on addition of 15d-PGJ_2 (seven genes; Table 10), and Group D; mRNA expression was significantly down-regulated by estradiol with or without 15d-PGJ_2 (ten genes; Table 11).

Effects of 15d-PGJ_2 on estrogen-mediated transcription in MCF-7 cells using real-time PCR analysis

In order to confirm the results from microarray analysis, we performed real-time PCR analyses for the representative 11 genes in MCF-7 cells (Fig. 4A–K).