Int. J. Cancer: **120**, 285–291 (2006)

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

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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.

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Received 29 March 2006; Accepted 11 August 2006
DOI 10.1002/ijc.22317
Published online 25 October 2006 in Wiley InterScience (www.interscience.wiley.com).

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.

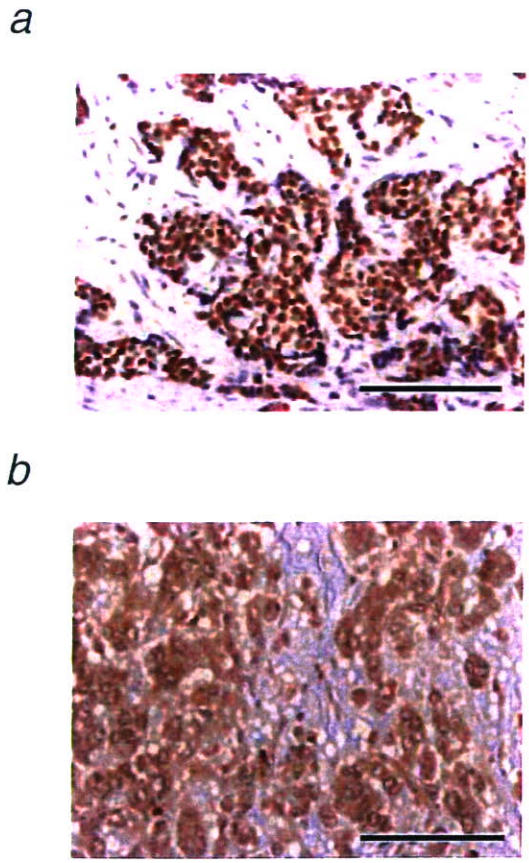

FIGURE 1 – Immunohistochemistry for AR (*a*) and 5αRed1 (*b*) in the invasive ductal carcinoma. Immunoreactivity for AR was detected in nuclei of carcinoma cells (*a*), and that of 5αRed1 was positive for the cytoplasm of these cells. Bar = 100 μm, respectively.

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.

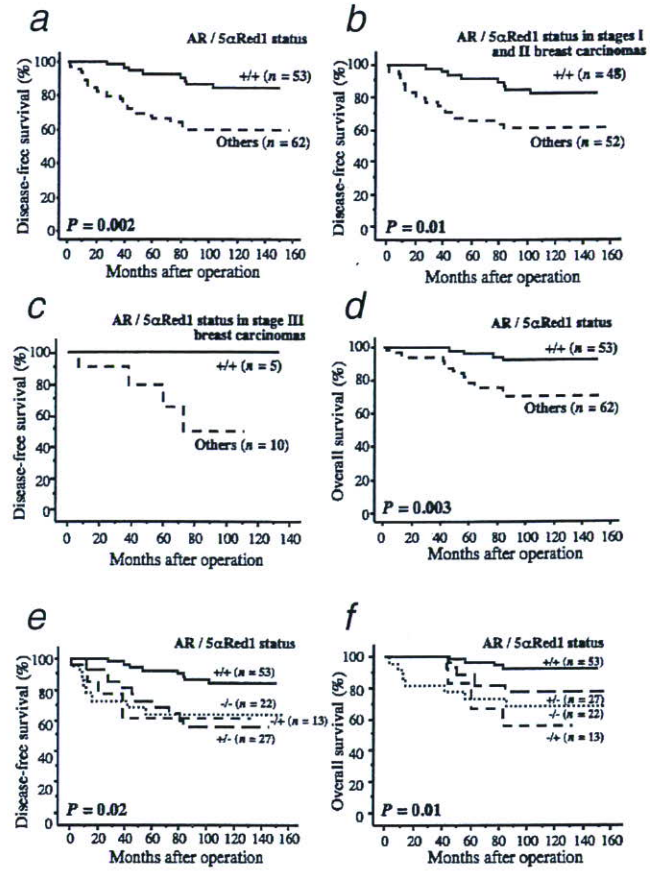

FIGURE 2 – Disease-free (*a–c*, *e*) and overall (*d*, *f*) survival curves of 115 patients with breast carcinoma according to AR and 5αRed1 status (Kaplan–Meier method). According to AR/5αRed1 status, breast carcinomas were classified into 2 groups (+/+, cases positive for both AR and 5αRed1, and others) (*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*).

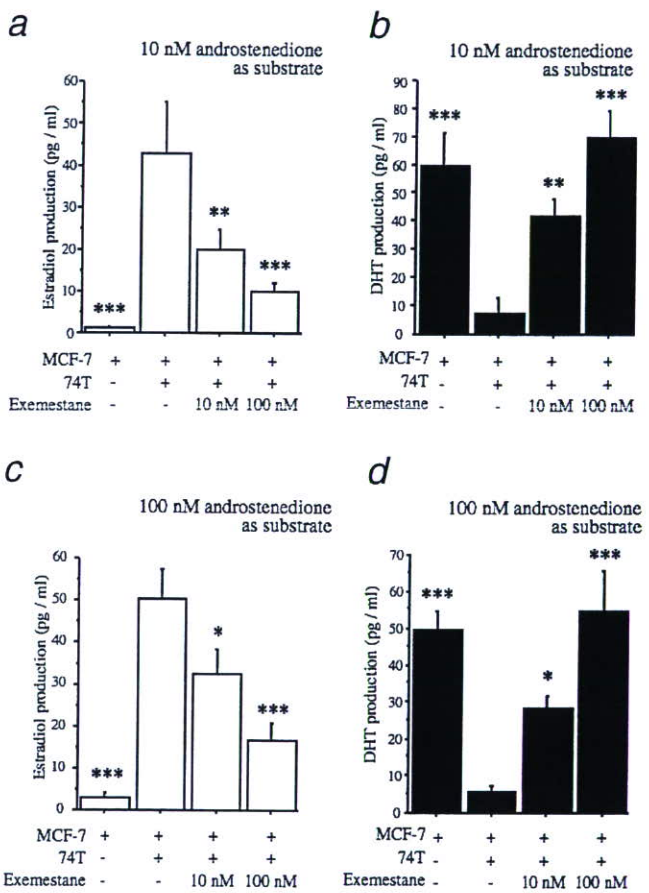

FIGURE 3 – Regulation of estradiol (*a*, *c*) and DHT (*b*, *d*) synthesis by aromatase in MCF-7 cells cocultured with aromatase-expressed 74T stromal cells. These cells were treated with androstenedione (10 nM (*a*, *b*) or 100 nM (*c*, *d*)) in absence or presence of aromatase inhibitor exemestane (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.

Acknowledgements

The authors appreciate the skillful technical assistance of Ms. Chika Kaneko, Mr. Katsuhiko Ono and Ms. Toshie Suzuki (Department of Pathology, Tohoku University School of Medicine, respectively).

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Aromatase Localization in Human Breast Cancer Tissues: Possible Interactions between Intratumoral Stromal and Parenchymal Cells

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Abstract

Aromatase is a key enzyme in intratumoral estrogen production required for the production of estrogens through the conversion of serum androgens in postmenopausal breast cancer patients. There have been, however, controversies regarding the intratumoral localization of aromatase in human breast carcinoma tissues. Therefore, we have first examined the intratumoral localization of aromatase mRNA/protein in 19 breast carcinomas using laser capture microdissection/quantitative reverse transcription-PCR (RT-PCR) and immunohistochemistry. Aromatase mRNA and protein were detected in both intratumoral stromal and parenchymal cells in breast carcinoma tissues. Subsequent microarray expression profiling and clustering analyses, in addition to quantitative RT-PCR studies, showed a significant positive correlation between aromatase and estrogen-related receptor α mRNA expression in isolated carcinoma cells. We further examined an interaction between stromal cells isolated from human breast carcinoma tissues and breast carcinoma cell lines using a coculture system to study the biological characteristic of aromatase expression in carcinoma cells. Aromatase mRNA and enzyme activity and 17β-hydroxysteroid dehydrogenase type 1 mRNA in breast carcinoma cell lines, including MCF-7 and SK-BR-3 cells, were up-regulated in the presence of patient-derived 32N or 74T intratumoral stromal cells. The results from steroid conversion assays were also consistent with the findings above. The results of our study also showed that aromatase inhibitors were more effective in inhibiting aromatization induced by coculture in MCF-7 than that in stromal 32N. The examination of the localization of aromatase and its regulation, including the interactions existing between different cell types in human breast carcinoma tissues, may provide important information as to achieving better clinical response to aromatase inhibitors in breast cancer patients. [Cancer Res 2007;67(8):3945–54]

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doi:10.1158/0008-5472.CAN-06-3105

Introduction

Estrogens play important roles in the growth and invasion of estrogen-dependent human breast carcinomas. While postmenopausal women have low levels of circulating plasma estrogens, the local synthesis or intratumoral production of estrogens that takes place in breast carcinoma tissue itself can lead to higher estrogen levels in the tumor (1, 2). Intratumoral production of estrogens occurs as a result of aromatization of C19 steroids such as androstenedione and testosterone into estrogens, and this is catalyzed by the cytochrome P450 aromatase enzyme (3–5). Previously, the localization of aromatase has been mostly examined using immunohistochemistry with the reported results demonstrating the presence of aromatase protein predominantly in tumoral stromal cells and adipocytes of breast carcinoma tissues (6, 7). However, there have been controversies regarding the cellular localization of intratumoral aromatase with other studies demonstrating aromatase immunoreactivity in carcinoma or parenchymal as well as stromal cells of human breast carcinoma tissues (8, 9).

Aromatase expression is well known to be regulated by various transcriptional factors, including nuclear receptors and their putative ligands in several types of human cells and tissues (10, 11). Both interleukins, such as interleukin (IL)-1, IL-6 and IL-11 released from carcinoma, and/or inflammatory cells have been shown to potently induce aromatase expression in adipose fibroblast cells (12). However, the correlation between nuclear receptors and aromatase in parenchymal or carcinoma cells of breast carcinoma tissues has remained largely unknown. In addition, there have been no studies reported examining whether the factors released from human intratumoral stromal cells affect aromatase expression of breast carcinoma or parenchymal cells.

Intratumoral aromatase has been considered a viable clinical target for the treatment of estrogen receptor–positive postmenopausal breast cancer patients (13). However, routine evaluation methods for the detection of intratumoral aromatase expression in clinical specimens have not been established. Therefore, in this study, we have first examined the localization of aromatase mRNA in 19 breast carcinoma tissues using laser capture microdissection (LCM), together with quantitative reverse transcription-PCR (RT-PCR), and then examined their correlation with clinicopathologic parameters of the patients. The expression and localization of the aromatase protein were also confirmed by immunohistochemistry using the aromatase monoclonal antibody termed 677 (14, 15). Microarray expression profiling and clustering analyses were also done on both isolated carcinoma and stromal cells obtained from the 19 breast carcinoma cases to identify possible

aromatase-regulating nuclear receptors in human breast carcinoma cells. We then examined the possible effects of isolated stromal cells from breast carcinoma tissues on both aromatase enzymatic activity and mRNA transcripts in breast carcinoma cell lines. Cocultured intratumoral stromal 32N or 74T cells, established from breast carcinoma tissues by primary culture, were used to evaluate the potential effects of carcinoma/stromal cell interactions on aromatase expression and enzyme activity in the carcinoma cells. Effects of coculture of MCF-7 cells with stromal 32N cells on MCF-7 cell proliferation and the inhibitory effects of the aromatase inhibitors on cell proliferation were subsequently investigated to further characterize the biological features of aromatase function in carcinoma or parenchymal cells.

Materials and Methods

Patients and tissue preparation. A total of 42 specimens of invasive ductal carcinoma of the breast were obtained from Japanese female patients from 2002 to 2005 at the Department of Surgery, Tohoku University Hospital and Tohoku Kosai Hospital (Sendai, Japan). The number of subjects examined in each experiment were as follows: 19 cases [54.2 years (range, 37–86; SD, 12.9)] for LCM/quantitative RT-PCR (qPCR) to investigate aromatase localization, 23 cases [55.0 years (range, 36–74; SD, 10.7)] for LCM/microarray studies, and 11 cases [53.1 years (range, 36–77; SD, 10.1)] for LCM/qPCR to validate results of microarray analysis. Nonpathologic breast and adipose tissues adjacent to the carcinoma were also available for examination in 12 out of 23 cases used in LCM/microarray analysis. Relevant clinical data were retrieved from the review of the patient's files. The histologic grade of each specimen was independently evaluated by three of the authors (T. Suzuki, T. Moriya, and H. Sasano), based on the modified methods of Bloom and Richardson (16), according to Elston and Ellis (17). The ethics committees at Tohoku University School of Medicine and Tohoku Kosai Hospital approved the research protocols (2004-144, 2005-068, and 2006-042, respectively), with informed consent being obtained from these patients before surgery in each institution.

Immunohistochemistry. For immunohistochemistry of aromatase, sequential frozen tissues, also used in the LCM analyses, were taken to examine the correlation between mRNA and protein in individual cellular compartments of the breast cancer tissues.

The aromatase monoclonal antibody 677 was raised against native recombinantly expressed human aromatase protein, with details of its characterization and utilization for immunohistochemistry being previously reported by the authors (14, 15). Tissue sections were immunostained by a biotin-streptavidin method with Histofine kit (Nichirei Co. Ltd., Tokyo, Japan). Breast carcinomas tissues were rapidly embedded in Tissue-Tek optimal cutting temperature compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and frozen materials sectioned at a thickness of 3 μm. The antigen-antibody complex was then visualized with 3.3′-diaminobenzidine solution and counterstained with hematoxylin. Evaluation of aromatase immunohistochemistry was done as previously reported using 10% formalin-fixed and paraffin-embedded tissue specimens (14). The approximate percentage of cells staining (proportion score) were classified into the following four groups: 0, <1%; 1, −25%; 2, −50%; and 3, >50% immunopositive cells. Relative intensity of aromatase immunopositive cells was classified as follows: 0, no immunoreactivity; 1, weak; 2, moderate; and 3, intense immunoreactivity. Aromatase immunoreactivity was evaluated as a total score composed of the proportion score + relative immunointensity score.

Other antibodies used in this study for characterizing clinicopathologic parameters of the cases are as follows: monoclonal antibodies: ERα (ER1D5; Immunotech S.A., Marseilles, France), progesterone receptor (MAB429; Chemicon International Inc., Temecula, CA), and Ki-67 (MIB1; DakoCytomation Co. Ltd., Kyoto, Japan); and rabbit polyclonal antibody: HER-2/*neu* (Ao485; DakoCytomation). The rabbit polyclonal antibody for 17β-HSD1, 17β-HSD5, and monoclonal antibody for steroid sulfatase (STS) were kindly provided by Dr. Poutanen (University of Oulu, Finland), Dr. Lu (Laval University Hospital Center, Québec, Canada) and Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan), respectively. These antibodies were used for immunohistochemistry in 10% formalin-fixed and paraffin-embedded tissue specimens of the cases. To score estrogen receptor (ER), progesteron receptor (PR), and Ki-67, more than 1,000 carcinoma cells from each case were counted independently by the same three authors as described above (Y.Miki, T. Suzuki, and H. Sasano), and the percentage of immunoreactivity as a labeling index (LI) was subsequently determined. The cases with <10% ERα LI or PR LI were designated ER- or PR-negative breast carcinomas according to the report by Allred et al. (18). For scoring of HER-2/*neu*, 17β-HSD1, 17β-HSD5, and STS (19–22), two groups were tentatively identified (0, no immunoreactivity and 1, positive carcinoma cells).

Total RNA extraction from breast tissues and cDNA synthesis. Total RNA was carefully extracted from 12 breast carcinoma specimens from both carcinomas and adipose compartments of the breast cancers in addition to nonpathologic breast tissues adjacent to the carcinoma using the TRIzol method (Invitrogen Corporation, San Diego, CA). A reverse transcription kit (Superscript II Preamplification system; Invitrogen) was used in the synthesis of cDNA.

Real-time RT-PCR. Real-time PCR was carried out using the LightCycler System (Roche Diagnostics GmbH, Mannheim, Germany). The primer sequences of aromatase, *ERRα*, and *RPL13A* were shown previously (23). Primer sets of *GCNF, HNF-4α, VDR, TRβ, TR4, HSD17B1, HSD17B2, HSD17B3, HSD17B4,* and *HSD17B5* were designed using OLIGO Primer Analysis Software (Takara Bio Inc., Shiga, Japan). cDNAs of known concentrations for target genes and the housekeeping gene, ribosomal protein L13a *(RPL13A)* were used to generate standard curves for real-time quantitative PCR to determine the quantity of target cDNA transcripts. The mRNA level in each case was represented as a ratio of *RPL13A* (%; refs. 20, 21, 23).

Laser Capture Microdissection. Nineteen breast carcinoma cases were frozen-sectioned at a thickness of 8 μm. Approximately 5,000 cells were laser-transferred from the carcinoma cells and the intratumoral stromal cells under light microscopic examination. For LCM/microarray expression profiling, after initial recovery and resuspension of the RNA pellet, a DNase digestion was done for 2 h at 37°C using 10 units of DNase (GenHunter, Nashville, TN) in the presence of 10 units of RNase inhibitor (Invitrogen), followed by extraction and precipitation. The pellet was resuspended in 27 μL of RNase-free H_2O and used for high-density cDNA array analysis.

Microarray analysis in isolated carcinoma cells. Twenty-three breast carcinomas were available for examination of gene expression patterns using microarray analysis following isolation by laser capture microscopy. Total RNA was extracted from ~5,000 carcinoma cells prepared by LCM procedures as described above. Sample preparation and processing were done essentially as described in the Affymetrix GeneChip Expression Analysis Manual (Affymetrix, Inc., Santa Clara, CA), with the exception that the labeled cRNA samples were hybridized to the complete human U133 GeneChip set (Affymetrix), including 22,215 and 22,577 genes. Relative levels of gene expression were calculated by global normalization. All gene expression data were clustered, and results were visualized using GeneSpring 7.2 (Agilent Technologies, Inc., Santa Clara, CA).

In this study, we focused on nuclear receptors that may modulate aromatase expression in carcinoma cells. Out of 44,792 genes, 88 genes were selected from the gene expression profiling for further analysis by reference to the web database of Nuclear Receptor.[11] Each case of breast carcinoma was ordered according to the level of aromatase gene expression determined by microarray and clustering analysis between each gene. Data from these categories and aromatase were subjected to hierarchical clustering analysis and visualization using the Cluster and TreeView programs (Stanford University, Palo Alto, CA; ref. 24) to generate tree structures based on the degree of similarity, as well as matrices comparing the levels of expression of individual genes in each sample examined. In addition, we further examined the correlations between the levels of aromatase mRNA expression and seven genes that were most closely

[11] NucleaRDB (April 2005, release 5.0); http://www.receptors.org/NR/index.html.

associated with aromatase in carcinoma cells isolated by LCM from 11 cases of human breast carcinoma.

Breast cancer cell lines and culture conditions. Human breast carcinoma cell lines MCF-7, T-47D, ZR-75-1, and SK-BR-3 and the human choriocarcinoma cell line BeWo were provided from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). Human breast carcinoma cell lines MDA-MB-231 and MDA-MB-468 and mouse preadipocyte 3T3-L1 cells were purchased from the American Type Culture Collection (Manassas, VA). The MCF-7, T-47D, ZR-75-1, and 3T3-L1 cell lines were maintained in RPMI 1640 (Sigma-Aldrich Co., St. Louis, MO) or Leivobitz's L-15 medium (Invitrogen) for the MDA-MB-231 and MDA-MB-468 cells and supplemented with 10% fetal bovine serum (FBS; SAFC Biosciences, Lenexa, KS). BeWo placental cells were maintained in Ham's F12 medium (Invitrogen) supplemented with 15% FBS. Primary stromal cells employed in this study were designated 74T and 32N and were isolated using collagenase treatment from human breast carcinoma tissues (25) and maintained in RPMI 1640 with 10% FBS.

Coculture system. For physical separation of stromal and carcinoma cell lines, transwell cultures were established in six-well plates or 100-mm dishes using Transwell Permeable Supports (0.4 μm pore; Corning, Incorporated, New York, NY). MCF-7 and SK-BR-3 cells were cultured in transwell chambers in the absence or presence of 32N, 74T, and 3T3-L1 cells and were cultivated on the bottom of the plates or dishes. After 24 h of cultivation using this coculture system, carcinoma and stromal cells were separated, and each component was examined in the aromatization assay, estrogen production assays, or by real-time RT-PCR. The cells after coculture with other cells were designated with subscript CO (i.e., MCF-7_{CO}, $32N_{CO}$). After these assays, viable cells were counted by the trypan blue exclusion (TBE) assay, and the total RNA was extracted using the TRIzol method described above.

Aromatization assay. Several previous studies have shown that the aromatase enzyme activity and/or mRNA levels were low or not detectable in the breast carcinoma cells such as MCF-7 (26–29). Therefore, in this study, we employed 6α-methylandrost-4-ene-3,17-dione (MeAD) assay as a quantitative evaluation of aromatization activity (30). The conversion of MeAD, an androgen analogue, into estrogen analogue (6α-methylestradiol) by aromatase was shown to be highly specific, and an evaluation of accurate aromatase activity could become possible with the measurement of its estrogen analogue produced (30). From the results of comparative studies using rat ovary tissue, the quantitative limit of detection of the MeAD assay was approximately 2-fold higher than that of the ^{3}H-water release assay (data not shown).

The aromatization assay using MeAD was done as described previously (30). MeAD and 6α-methylestradiol (MeE2) were generously provided by Dr. Numazawa (Tohoku Pharmaceutical College, Sendai, Japan). Estradiol-3-pentafluorobenzyl-17β-methylpyridinium ether (E2-PFBY) was synthesized at Teizo Medical Co., Ltd. (Kanagawa, Japan). We used LC-MS–grade water, methanol, and acetonitrile for liquid chromatography–tandem mass spectrometry (LC-MS/MS). The LC-MS/MS devices and measurement conditions were as follows: an API 4000 electrospray ionization mass spectrometer (MDS SCIEX; Applied Biosystems (Foster City, CA)) was used. For high-performance liquid chromatography (HPLC), an Agilent 1100 chromatograph (Agilent Technologies) was employed. HTC PAL autosampler (CTC Analytics AG Industriestrasse, Zwingen, Switzerland) and an X Terra MS C18 column (3.5 μm, 2.1 × 150 mm) were employed. Measurement was done in the positive ion mode. For the quantification of MeE2-PFBY, we used a product ion (m/z 353.2) produced from a precursor ion (m/z 558.3). On the other hand, product ion of ^{13}C-E2-PFBY was m/z 109.9 produced from precursor ion at m/z 548.2. For the quantification of MeE2, after estrone, $^{13}C_4$ (Hayashi Pure Chemical Industries, Inc., Osaka, Japan) was added to the medium, and the mixture was mixed. The extract was dissolved in methanol and reduced with 1% $NaBH_4$. The sample was dried and treated with pentafluorobenzyl bromide under 0.8% KOH ethanol. Then, the reactive solution was diluted with purified water, and a derivative was extracted with ether. The sample was dried under reduced pressure and mixed with 2% 2-fluoro-1-methylpyridinium-*p*-toluenesulfonate/dichloromethane and 10% triethylamine. E2-PFBY derivative was eluted with 10% formic acid solution/acetonitrile (2, 8). The elution vehicle was removed under reduced pressure, dissolved in 0.1 mL of the mobile phase, and used as a sample for LC-MS/MS.

Estrogen production assays. MCF-7 and MCF-7_{CO} cells were incubated at 37°C in FBS-free RPMI 1640 containing 10 nmol/L of androstenedione or testosterone as substrates for 24 h. Concentrations of estrone and estradiol were evaluated by LC-MS/MS analysis (31). All the cells treated with substrates were counted by TBE assay. After addition of 100 pg of androstenedione-$^2H_7$ (C/D/N Isotope Inc., Quebec, Canada), estrone-$^{13}C_4$, and estradiol-$^{13}C_4$ (Hayashi Pure Chemical Industries) as internal standards, steroids were extracted with diethyl ether from the media. The separated organic layer was evaporated and then dissolved in picolinoic anhydride in

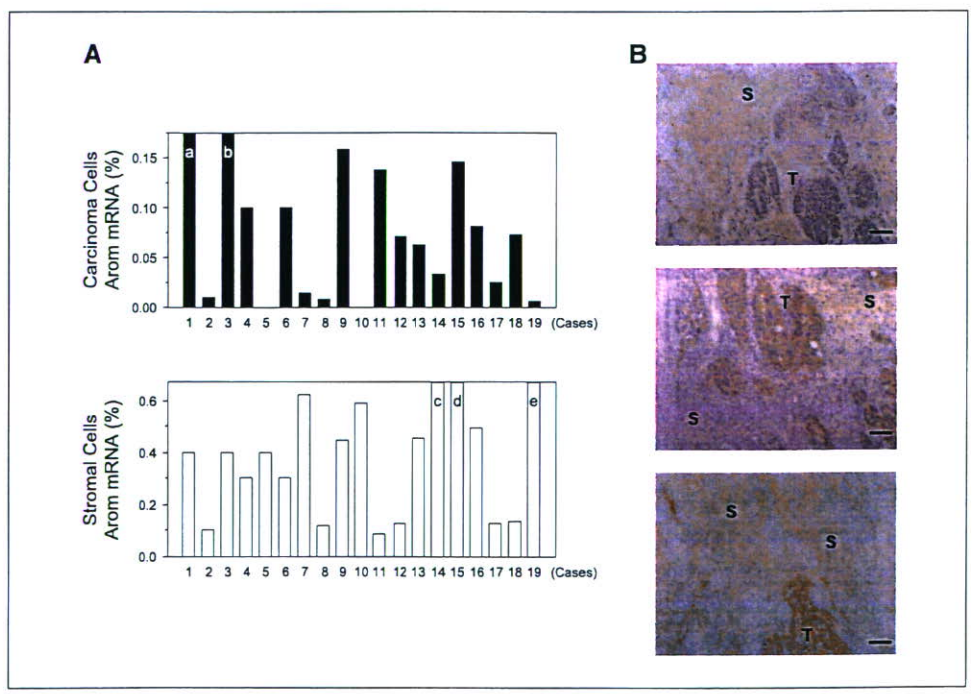

Figure 1. *A*, aromatase mRNA level in each case (1 to 19) of breast carcinoma (*top*) and stromal (*bottom*) cells. There were no detectable levels of aromatase in the breast carcinoma cells from two cases (5 and 10). For the following samples, the values of aromatase mRNA were *a* = 0.20%, *b* = 0.30%, *c* = 0.91%, *d* = 6.23%, and *e* = 1.02%. Aromatase immunoreactivity scores for each case (1 to 19) of breast carcinoma (*black*) and stromal (*white*) cells. There were no aromatase immunoreactivities observed in breast carcinoma cells from four cases (7, 12, 16, and 18). *B*, immunohistochemical localization of aromatase in human breast carcinoma tissues. Aromatase immunoreactivity was detected predominantly in carcinoma cells (*top*), only in stromal cells (*middle*), or in both carcinoma and stromal cells (*bottom*). *S*, stromal cells; *T*, carcinoma cells. *Bar*, 50 μm.

tetrahydrofran solution with triethylamine. After application to a Bond Elut C_{18} column, steroid derivatives were eluated with 80% acetonitrile solution. In this study, we used liquid chromatography (Agilent 1100; Agilent Technologies) coupled with an API 4000 triple-stage quadrupole mass spectrometer (Applied Biosystems) operated with electron spray isonization in the positive-ion mode, and the chromatographic separation was done on Cadenza CD-C_{18} columns (3 × 150 mm, 3.5 μm; Imtakt Corporation, Kyoto, Japan). Ion spray voltage was 4.5 kV, and turbo gas temperature was 450°C in ionization conditions. The estrogen levels in each case are presented as picograms per milliliter per 10^6 cells.

Cell proliferation assay. After coculture with stromal cells for 24 h, MCF-7 cells treated with 10^{-9} to 10^{-7} mol/L testosterone or androstenedione for 24 h were trypsinized and harvested in phenol red- and FBS-free medium in 96-well plates (3 × 10^4 cells/mL). Androstenedione and testosterone (10^{-9} to 10^{-7} mol/L) were added for 24 h. Cell proliferation was evaluated using the WST-8 method (Cell Counting Kit-8; Dojindo Inc., Kumamoto, Japan; ref. 32). We also examined the effects of aromatase inhibitors on cell proliferation by methods described above. Both steroidal (exemestane 10^{-8} mol/L; Pfizer Inc., New York, NY) and nonsteroidal (letrozole 10^{-8} mol/L; Novartis Pharma AG, Basel, Switzerland.) aromatase inhibitors were used in the androgen-treated MCF-7_{CO} and MCF-7 cells for 24 h.

Statistical analysis. Statistical analysis was done using the StatView 5.0 J software (SAS Institute Inc., Cary, NC). Values for patient's age, tumor size, Ki-67 LI, and mRNA levels for aromatase are represented as the mean ± SD. Simple regression analysis was employed to assess the correlations between aromatase and ERRα mRNA expression levels. An association between the degree of mRNA expression of aromatase and these parameters for each individual case was evaluated using one-way ANOVA and the Bonferroni test. Statistical differences between aromatase mRNA expression and ER status, PR status, menopausal status, stage, lymph node status, histologic grade and HER-2/*neu*, 17β-HSD1, and 17β-HSD5 immunoreactivities were all evaluated in a cross-table using the χ^2 test.

Results

Distributions of aromatase mRNA transcripts in breast tissues. The level of aromatase mRNA expression (mean ± SD; n = 12) was significantly higher in both carcinoma (1.327 ± 1.394%) and adipose tissues (2.103 ± 1.790%) than in non-neoplastic breast tissue (0.106 ± 0.095%) adjacent to carcinoma. The aromatase level in placenta tissues as a positive control was 80.770 ± 31.867% (mean ± SD; n = 3; data not shown).

Localization of intratumoral aromatase mRNA transcripts in breast carcinoma tissues. From 19 breast carcinoma cases, aromatase mRNA transcripts were detected in intratumoral stromal cells from all 19 cases and in the carcinoma cells from 17 of these cases (Fig. 1*A*). The mean value of aromatase mRNA transcript level was significantly higher (P = 0.009) in intratumoral stromal cells (0.348 ± 0.238) than in the carcinoma cells (0.112 ± 0.090). No significant correlation was detected in the aromatase mRNA level between stromal and carcinoma cells (r = 0.132, P = 0.592). Aromatase immunoreactivity was detected in the cytoplasmic compartment of both intratumoral stromal and carcinoma cells (Fig. 1*B* and *C*). Aromatase immunoreactivity was absent in the carcinoma cells of four cases.

Correlation between aromatase mRNA levels in intratumoral stromal and carcinoma cells and clinicopathologic status in breast carcinoma patients. An association between the intratumoral aromatase mRNA levels in stromal and/or parenchymal/carcinoma cells and clinicopathologic parameters in 19 breast carcinoma cases are summarized in Table 1. Two cases of aromatase-negative carcinoma cells were assigned as 0.000%. Aromatase mRNA levels in stromal cells were positively correlated with histologic grade (P = 0.032). Aromatase mRNA levels in

Table 1. Correlation between aromatase expression levels and clinicopathologic parameters in 19 breast carcinomas

Parameter	n	Carcinoma cells		Stromal cells	
		Mean ± SD	P	Mean ± SD	P
Histologic grade					
I + II	14	0.098 ± 0.085		0.342 ± 0.228	
III	5	0.014 ± 0.014	0.072	2.002 ± 2.847	0.032*
ER status					
+	11	0.116 ± 0.087		0.374 ± 0.239	
−	8	0.029 ± 0.031	0.015*	1.146 ± 2.080	0.234
PR status					
+	12	0.107 ± 0.089		0.337 ± 0.228	
−	7	0.033 ± 0.032	0.051	1.320 ± 2.190	0.134
17β-HSD1 status					
+	10	0.134 ± 0.090		0.413 ± 0.230	
−	9	0.028 ± 0.029	0.007*	1.101 ± 2.100	0.373
17β-HSD5 status					
+	11	0.096 ± 0.104		0.361 ± 0.164	
−	8	0.062 ± 0.052	0.437	1.266 ± 2.225	0.24
STS status					
+	12	0.074 ± 0.068		0.435 ± 0.292	
−	7	0.102 ± 0.135	0.592	1.723 ± 3.012	0.138
EST status					
+	8	0.068 ± 0.066		0.462 ± 0.337	
−	11	0.087 ± 0.094	0.683	0.891 ± 1.790	0.609

*Significantly different; in percent.

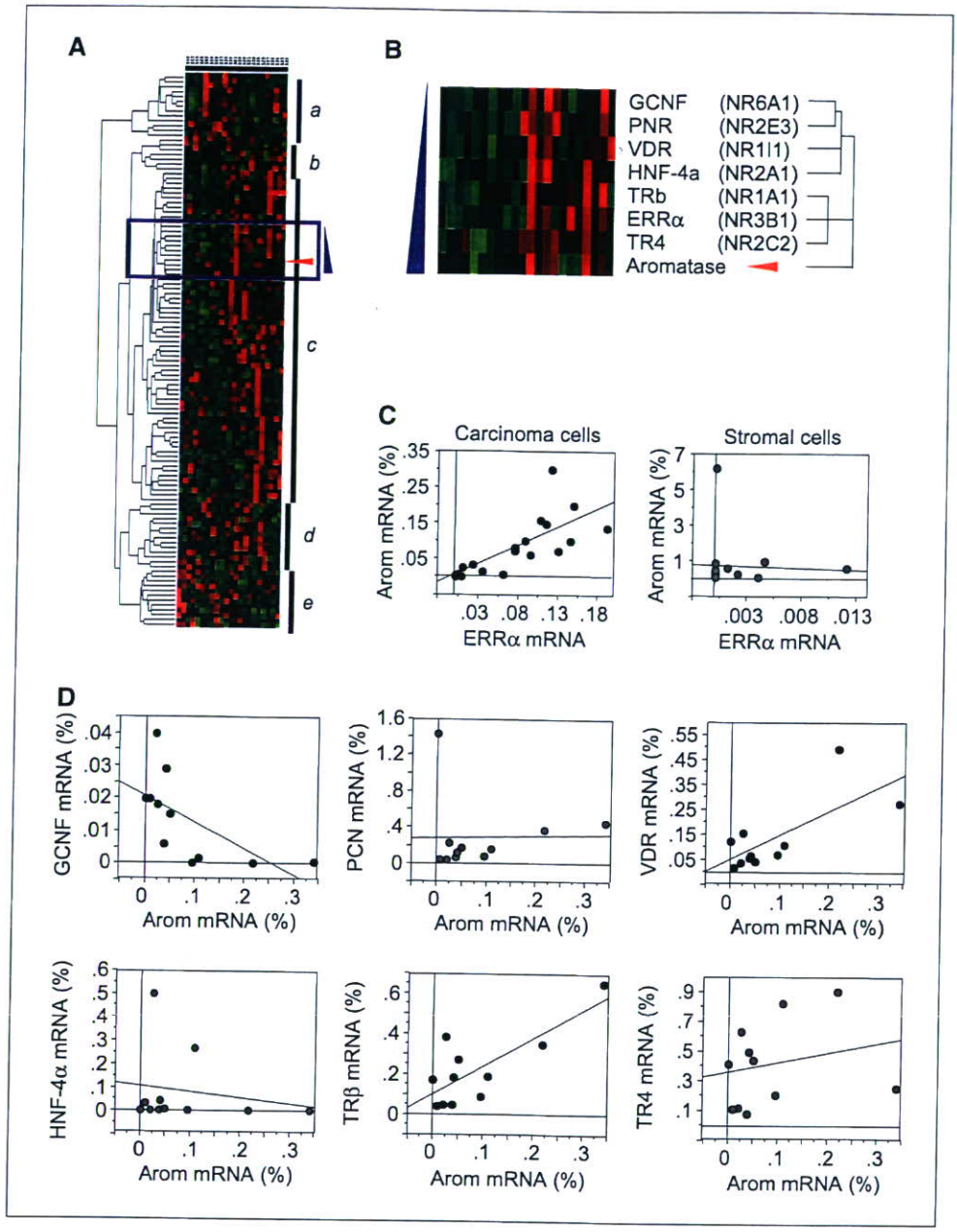

Figure 2. *A* and *B*, clustering analysis of microarray expression profiles of nuclear receptor genes. The cluster associated with the aromatase gene was composed of 59 genes (*A*, *group c*). More closely associated with the aromatase gene (*A*, *square*) were 12 genes (*B*). *C*, a statistically significant positive correlation was detected between aromatase and ERRα gene in 19 cases of (*left*) carcinoma cells ($r = 0.748$, $P = 0.002$), but not in (*right*) stromal cells ($r = 0.044$, $P = 0.860$). *D*, statistically significant correlations were detected between aromatase and ERRα ($r = 0.74$, $P = 0.01$; data not shown), VDR ($r = 0.73$, $P = 0.02$), TRβ ($r = 0.62$, $P = 0.04$), and GCNF ($r = -0.64$, $P = 0.03$) genes in 11 cases of carcinoma cells. No statistically significant correlations were detected between aromatase and PNR ($r = 0.02$, $P = 0.95$), HNF-4α ($r = -0.17$, $P = 0.61$), and TR4 ($r = 0.24$, $P = 0.48$) gene in 11 cases of carcinoma cells.

parenchymal/carcinoma cells were positively correlated with ER status ($P = 0.015$) and 17β-HSD1 status ($P = 0.007$), but not with histologic grade ($P = 0.072$). No significant association was detected between aromatase mRNA level in stromal or carcinoma cells and age, tumor size, lymph node status, PR status, HER-2/*neu* status, Ki-67 LI, 17β-HSD5 status, and STS status in this study.

Microarray analysis evaluated by hierarchical clustering. The results of focused clustering analysis subclassified 88 genes into five well-defined expression profiles or groups (Fig. 2*A*, *groups a–e*). The aromatase gene was included in group c (59 genes). The following seven genes were most closely associated with aromatase in group c (Fig. 2*B*): GCNF (NR6A1), PNR (NR2E3), VDR (NR1I1), HNF-4α (NR2A1), TRβ (NR1A1), ERRα (NR3B1), and TR4 (NR2C2).

Correlation between aromatase and nuclear receptors in isolated breast carcinoma cells. A statistically significant positive correlation was detected between aromatase and ERRα ($r = 0.74$, $P = 0.01$), VDR ($r = 0.73$, $P = 0.02$), and TRβ ($r = 0.62$, $P = 0.04$) in parenchymal/carcinoma cells of 11 human breast carcinoma cases (Fig. 2*D*). A statistically significant negative correlation was also detected between aromatase and GCNF ($r = -0.64$, $P = 0.03$) in these isolated parenchymal/carcinoma cells (Fig. 2*D*).

There were no significant increases or decreases of mRNA levels for the nuclear receptors GCNF, PNR, VDR, HNF-4α, TRβ, ERRα, and TR4 in MCF-7_{CO} compared with MCF-7 cells (data not present).

Correlation between aromatase and ERRα expressions in breast carcinoma cells. A statistically significant positive correlation was detected between aromatase and the ERRα gene (Fig. 2*C*) in 19 cases of parenchymal/carcinoma cells ($r = 0.748$, $P = 0.002$), but not in stromal cells ($r = 0.044$, $P = 0.860$).

In breast carcinoma cell lines, ERRα mRNA was detected in all cell lines examined in this study (Fig. 3*A*). Relatively low levels of

ERRα mRNA were detected in both stromal 32N and 74T cells. ERRα mRNA was also detected in placental BeWo cells.

Aromatase mRNA expression levels in human intratumoral stromal and carcinoma cells. Aromatase mRNA was detected in both stromal 32N and 74T cells isolated from human breast cancer tissues examined in this study, but the levels were lower than that of placental BeWo cells (Fig. 3*B*). Relatively low levels of aromatase mRNA was detected in mouse preadipocyte 3T3-L1 cells. Both 32N and 74T cells also exhibit aromatase enzyme activity, and there were no significant differences found between the aromatization levels in the 32N and 74T cells (P = 0.140).

Aromatase mRNA was detected in all breast carcinoma cell lines examined in this study (Fig. 3*B*). High levels of aromatase mRNA were detected in SK-BR-3 and MDA-MB-468 breast carcinoma cell lines, but the levels were lower than that of the 74T and 32N stromal cells and placental BeWo cells. We therefore used the higher aromatase mRNA expressing SK-BR-3 cells and the low aromatase mRNA expressing MCF-7 cells for further examinations. The aromatase mRNA level of native MCF-7 cells was significantly lower than that of native SK-BR-3 cells (P = 0.041). However, the levels in native SK-BR-3 cells were significantly lower than those of 32N (P = 0.032) or 74T cells (P = 0.044).

Effects of coculture on aromatase mRNA and activity levels in human intratumoral stromal and carcinoma cells. The results of the effects of coculture of breast cancer cell lines on the 32N and 74T cell aromatase activity are summarized in Fig. 3*C* to *E*. Aromatase mRNA level and activity in both 32N and 74T were significantly increased by cocultivation with MCF-7 but not with SK-BR-3 cells.

The results of the effects of coculture of stromal cells on MCF-7 and SK-BR-3 aromatase level are summarized in Fig. 3*D* and *E*. Both aromatase enzyme level (Fig. 3*D*) and mRNA level (Fig. 3*E*) in MCF-7_{CO} (after coculture with both 32N and 74T) were significantly higher than the levels found in monocultures of MCF-7 cells. There were no significant differences between native SK-BR-3 cells alone and SK-BR-3_{CO} (Fig. 3*D* and *E*). There were also no significant differences found in the aromatization levels between MCF-7_{CO} and SK-BR-3_{CO}. Aromatase activity/mRNA expression in both MCF-7_{CO} and 3T3-$L1_{CO}$, however, was not increased after coculture with MCF-7 and 3T3-L1 (Fig. 3*C–E*).

Effects of aromatase inhibitors on MCF-7 or stromal cells. Both 10^{-8} mol/L exemestane and 10^{-8} mol/L letrozole inhibited the increase in aromatization activity of the MCF-7_{CO} (Fig. 4*A*). Both 10^{-8} mol/L exemestane and 10^{-8} mol/L letrozole also inhibited the increase in aromatase activity of $32N_{CO}$ compared with the aromatase activity level of the 32N cells alone (Fig. 4*A*). The aromatization activity levels of MCF-7_{CO} following treatment with the aromatase inhibitors in MCF-7_{CO} were significantly lower than the aromatase activity level found in native MCF-7 cells (Fig. 4*A*).

The results of the cell proliferation assays are summarized in Fig. 4*B* to *D*. In monocultures of MCF-7 cells, there were no changes in the number of cells after 24 h incubation with 10^{-7} to 10^{-9} mol/L androstenedione, whereas 10^{-7} mol/L testosterone significantly increased the number of cells after 24 h. In MCF-7_{CO}, there were significant increments in the number of cells after 24 h treatment with 10^{-7} mol/L androstenedione and 10^{-8} to 10^{-7} mol/L testosterone. The cell numbers of MCF-7_{CO} treated with 10^{-8} mol/L androstenedione and 10^{-8} mol/L testosterone were significantly higher than those found in monocultures of MCF-7 cells. All of these increases of MCF-7_{CO} cell proliferation were inhibited following treatment with 10^{-8} mol/L exemestane (Fig. 4*C*) or 10^{-8} mol/L letrozole (Fig. 4*D*).

Estrogen production and expression levels of 17β-hydroxysteroid dehydrogenases in MCF-7. The results of estrogen production assays are summarized in Table 2*A* and *B*. Following the treatment with androstenedione (10^{-9} mol/L; 286.4 mg/mL) as the aromatase substrate, the rate of conversion into both estrone and estradiol in MCF-7_{CO} was higher than that in MCF-7 cells alone. The rate of conversion into estradiol but not into estrone in

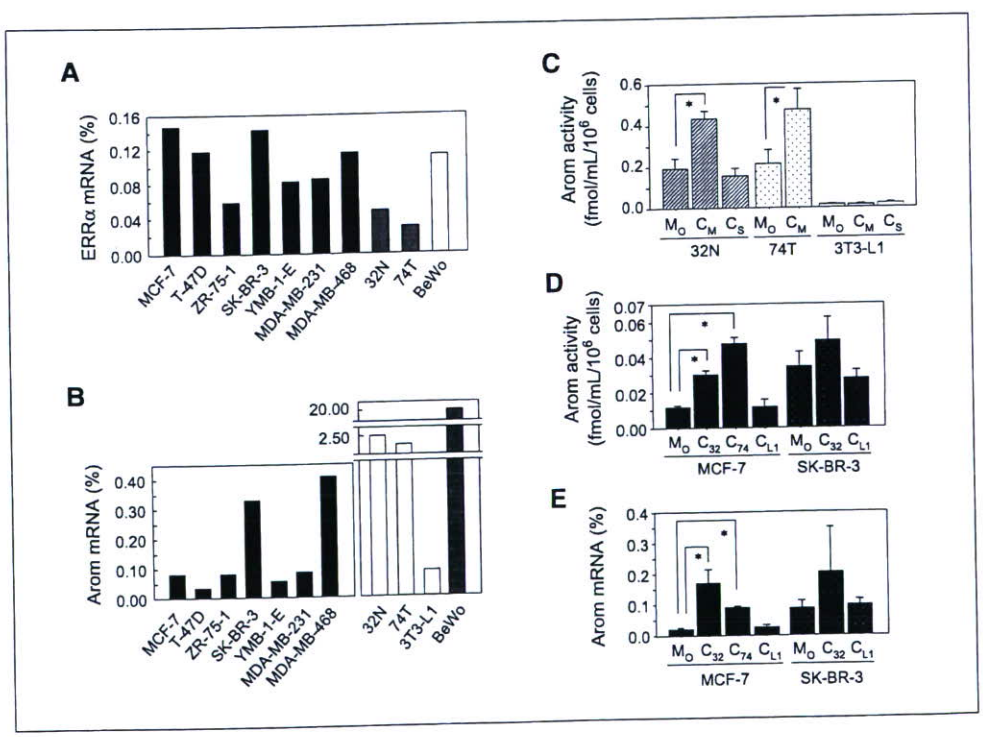

Figure 3. *A*, ERRα mRNA was detected in all breast carcinoma cell lines examined in this study, stromal 32N and 74T, and BeWo (in percent of RPL13A). *B*, aromatase levels in breast carcinoma cell lines and breast cancer-derived stromal 32N and 74T cells, mouse preadipocyte 3T3-L1 cells, and placental BeWo cells. *C–E*, the effects of coculture on aromatase mRNA level and enzyme activity in breast carcinoma and stromal cells. *C*, aromatase mRNA level in 32N, 74T, and 3T3-L1 cells; *D*, aromatase enzyme activity in MCF-7 and SK-BR-3 cells; *E*, aromatase mRNA level in MCF-7 or SK-BR-3 cells; M_O, monoculture; *C*, after coculture with 32N (C_{32}), 74T (C_{74}), 3T3-L1 (C_{L1}), MCF-7 (C_M), or SK-BR-3 (C_S) cells.

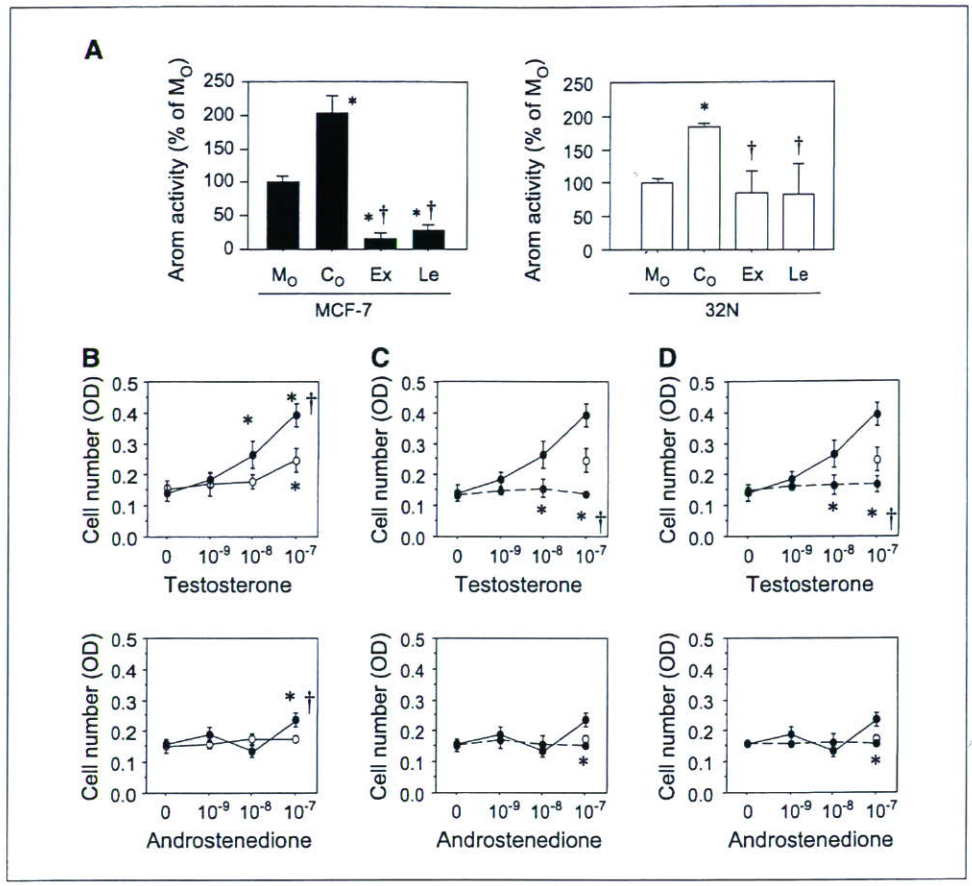

Figure 4. *A*, the effects of aromatase inhibitors on aromatase enzyme activities in MCF-7 (*left*) and 32N cells (*right*). *Ex*, treatment with 10^{-8} mol/L exemestane; *Le*, treatment with 10^{-8} mol/L letrozole; *, $P < 0.05$ versus M_O; †, $P < 0.05$ versus C_{32} C_{32} or C_M; in percent of RPL13A (aromatase mRNA level) or femtomoles per milliliter per 10^6 cells (aromatase enzyme activity). *B*, cell proliferation of MCF-7_{CO} and MCF-7 cells treated with testosterone (*top*) and androstenedione (*bottom*). ●, MCF-7_{CO}; ○, MCF-7 cells; *, $P < 0.05$ versus vehicle control (0 nmol/L); †, $P < 0.05$ versus MCF-7 treated with 10 or 100 nmol/L androgens. *C* and *D*, cell proliferation following treatment with the aromatase inhibitors, (*C*) exemestane and (*D*) letrozole. ●, *solid line*, MCF-7_{CO}; ●, *dashed line*, MCF-7_{CO} treated with aromatase inhibitor; ○, MCF-7; *, $P < 0.05$ versus MCF-7_{CO}; †, $P < 0.05$ versus MCF-7.

MCF7_{CO} was also higher than that observed with MCF-7 cells alone, following the treatment with testosterone (10^{-9} mol/L; 288.4 mg/mL) as the aromatase substrate.

The results of 17β-hydroxysteroid dehydrogenases mRNA levels in cocultures and monocultures of MCF-7 cells are summarized in Table 2*C*. The 17β-HSD1 mRNA level in MCF-7_{CO} was significantly higher than that found in MCF-7 cells. The 17β-HSD2 mRNA level in MCF7_{CO} was significantly lower than that found in MCF-7 cells. There were no significant increases or decreases of other types of 17β-HSD types such as types 3, 4, and 5 in MCF-7_{CO}.

Discussion

Lu et al. (33) previously reported localization of aromatase protein and mRNA using immunohistochemistry and mRNA *in situ* hybridization, respectively, in the same breast cancer specimens. They showed that the aromatase protein and mRNA expression was predominantly detected in parenchymal cells (33). Further studies have shown the localization of aromatase protein using immunohistochemistry in human breast tissues (6–9), but the reported results have been markedly different between the different laboratories. These discrepancies in the cellular localization of intratumoral aromatase expression in human breast carcinomas may be due to the different aromatase antibodies and probe sequences employed in these different studies. This is the first study to show aromatase mRNA expression in the different cellular compartments of human breast carcinoma tissues following isolation using laser capture microscopy and subsequent qPCR analysis. The results of the combined LCM/qPCR study in our study showed that intratumoral aromatase in human breast cancer is expressed in both stromal and carcinoma or parenchymal components of the tissue. This finding confirms results of previous immunohistochemical study using the monoclonal antibody 677 done in 10% formalin-fixed and paraffin-embedded materials. We have also done immunohistochemistry using this monoclonal antibody 677 in frozen tissue sections adjacent to those in which LCM/qPCR analysis was conducted and have evaluated the immunoreactivity using the scoring system developed on 10% formalin-fixed and paraffin-embedded tissue specimens (14). Parenchymal/carcinoma cells are the major cell types of breast cancer tissues, and estrogens produced *in situ* by carcinoma cells could effectively activate the ER in the nuclei of carcinoma cells via an autocrine mechanism. Stromal cells also express aromatase, and thus, this source of intratumoral estrogen biosynthesis and subsequent estrogen-dependent cell proliferation is considered significant. An important aspect is the potential interplay that may exist between the carcinoma and stromal cell compartments. Therefore, we did further characterization of the potential regulation of aromatase in parenchymal or carcinoma cells in human breast cancer tissues.

Aromatase mRNA in adipose stromal cells was shown to be increased by coculture with MCF-7 cells (27). The results of previously reported studies all showed that various aromatase-stimulating factors (ASF), such as IL-1, IL-6, IL-11, IL-6 soluble receptor, tumor necrosis factor-α and prostaglandin E_2, etc. (10, 34, 35), are released from parenchymal or carcinoma cells, which resulted in the up-regulation of aromatase expression in stromal cells (including adipostromal cells, preadipocytes, or fibroblasts) within

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.

Acknowledgments

Received 8/22/2006; revised 12/24/2006; accepted 2/2/2007.

Grant support: The Yasuda Medical Research Foundation, Osaka, Japan.

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We thank Katsuhiko Ono and Toshie Suzuki (Department of Pathology, Tohoku University School of Medicine, Sendai, Japan) for skillful technical assistances.

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Review

Controversies of aromatase localization in human breast cancer—Stromal versus parenchymal cells☆

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Abstract

Aromatase is a key enzyme of estrogen production through conversion from serum androgens in estrogen-dependent postmenopausal breast cancer. Aromatase has been reported to be predominantly located in intratumoral stromal cells and adipocytes but not in parenchymal or carcinoma cells in breast cancer tissue. It is, however, true that there have been controversies regarding intratumoral localization of aromatase in human breast carcinoma, especially whether intratumoral production of estrogens through aromatase occurs in parenchymal or stromal cells. Results of several studies suggested that aromatase present in parenchymal carcinoma cells plays more important roles in the growth and invasion of breast carcinomas than that in stromal cells through providing higher levels of estrogens to carcinoma cells. Aromatase inhibitors are increasingly being used in place of tamoxifen after results of various clinical trials demonstrated that aromatase inhibitors are more effective in increasing survival and recurrence of estrogen-dependent breast cancer patients. Therefore, it is important to clarify the estrogen supplying pathway by aromatase inside of breast carcinoma tissues in order to evaluate the possible efficacy of aromatase inhibitor treatment. In this review, the controversies regarding these intratumoral localization patterns in human breast carcinoma will be briefly summarized.

Keywords: Aromatase; Breast carcinoma; Estrogen; Localization

Contents

1. Introduction

The aromatase P450 gene is located at chromosome 15 (15q21.2). This enzyme complex consists of two components such as aromatase cytochrome P450 (aromatase) and NADPH-cytochrome P450 reductase (reductase). Both aromatase and reductase are located in the endoplasmic reticulum [1]. However, human placental aromatase was reported to be associated with both the mitochondrial and microsomal compartments [2]. Aromatase is a key enzyme in the estrogen synthesis involved in aromatization of C19 steroids such as androstenedione and testosterone into estrogens. In vertebrates, aromatase is expressed primarily in gonads such as ovary, whereas human aromatase was detected in numerous other peripheral tissues including adipose tissue [3], bone [4], muscle [3], skin [5], aorta [6] and placenta [7]. In addition, it is also well known that pathological tissues such as breast

☆ Presented at the VIII International Aromatase Conference: 'Aromatase 2006' (Baltimore, Maryland, USA, 18–20 September 2006).

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0960-0760/$ – see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2007.05.007

[8,9] and endometrial cancer [9,10] also express increased amounts of intratumoral aromatase. For example, in human breast carcinoma, 60–70% of the specimens had aromatase activity comparable with or greater than that found in other tissues. In addition, Miller et al. [11] also detected estrogen biosynthesis in all (247/247) breast adipose tissue specimens obtained from the patients with breast cancer. Therefore, aromatase has been established as a potential target for treatment of postmenopausal breast cancer patients. Several clinical trials demonstrated that aromatase inhibitors were better than tamoxifen in postmenopausal women diagnosed with estrogen receptor and/or progesterone receptor positive breast cancer cases [12–14]. However, it is also true that the methods of evaluating intratumoral aromatase applicable in clinical specimens have not necessary been standardized and therefore, it is not known that aromatase inhibitors exerted their effects on which types of cells in human breast carcinoma tissues.

2. Controversies in aromatase localization in human breast cancer

Aromatase localization has been mostly examined by immunohistochemistry and mRNA *in situ* hybridization in previous studies. Results from these published reports demonstrated the presence of aromatase protein predominantly in tumoral stromal cells and adipocytes of breast carcinoma tissues using rabbit polyclonal antibody (from Dr. N. Harada) [8,15]. However, it is true that there have been controversies regarding intratumoral aromatase localization. Results of previous immunohistochemical studies of aromatase in human breast cancer are summarized in Table 1 [8,15–24]. Esteban et al. [16] and Lu et al. [18] reported aromatase expression in breast carcinoma cells using another rabbit polyclonal antibody and monoclonal antibody (from Dr. E. Simpson). Furthermore, Zhang et al. [22] demonstrated that aromatase immunoreactivity (using polyclonal antibody from Dr. N. Harada described above) was detected not only in stromal cell but also carcinoma cells, in both the ductal carcinoma *in situ* and invasive ductal carcinoma specimens. Recently, Sasano et al. [23,24] developed the new mouse monoclonal antibody for aromatase raised against from native aromatase protein (#677), and demonstrated that aromatase immunoreactivities were detected in various types of cells such as carcinoma cells, stromal cells, and adipocytes (Fig. 1). Furthermore, there is a statistically significant correlation between enzymatic activity of aromatase and #677 immunoreactivity in parenchymal carcinoma cells but not in stromal cells [24]. Lu et al. [18] also examined localization of aromatase mRNA using mRNA *in situ* hybridization in breast cancer specimens. They demonstrated that aromatase mRNA expression was predominantly detected in parenchymal cells [18]. These discrepancies regarding intratumoral localization of aromatase in human breast cancer tissues may be due to the different anti-human aromatase antibodies employed [25] and probe positions employed in these studies in the cases of mRNA *in situ* hybridization. These discrepancies may be therefore, easily resolved if carcinoma and stromal cells could be separated in human breast carcinoma specimens. Laser capture microdissection (LCM) can provide researches with the ability to accurately procure nearly pure population of target cells from the regions of breast carcinoma specimens [26,27]. The results of combined LCM and RT-PCR analysis of aromatase demonstrated that aromatase mRNA transcripts were detected in breast carcinoma or parenchymal cells as well as stromal cells (Fig. 2A). Therefore, it is reasonably postulated that at this juncture, intratumoral

Table 1
Summary of previous immunohistochemical analysis of aromatase in human breast carcinoma tissues

References	Antibody	Source	Tissue	Localization
Esteban et al. [16]	Rb polyclonal	P. Hall	IDC	CA
Sasano et al. [8]	Rb polyclonal	N. Harada	IDC	ST
Santen et al. [15]	Rb polyclonal	N. Harada	IDC	ST > CA and NB
Berstein et al. [17]	Rb polyclonal	E. Simpson	IDC	CA > NB
Lu et al. [18]	Ms monoclonal	E. Simpson	IDC	CA > ST
Shenton et al. [19]	Rb polyclonal	N. Harada	IDC	ST
	Ms monoclonal	E. Simpson	IDC	CA and ST
Brodie et al. [20]	Ms monoclonal	E. Simpson	IDC	CA
Tsunoda et al. [21]	Rb polyclonal (PAbR-9)	Y. Osawa	FNA	CA
			IDC*	CA
			Cell	MCF-7, SK-BR-3
Zhang et al. [22]	Rb polyclonal	N. Harada	IDC	CA and ST
			DCIS	CA and ST
Sasano et al. [23]	Ms monoclonal (F2)	Original	IDC	ST and CA and NB
Sasano et al. [24]	Ms monoclonal (#677)			

Rb: rabbit; Ms: mouse; IDC: invasive ductal carcinoma; FNA: fine-needle aspiration sample; DCIC: ductal carcinoma *in situ*; CA: carcinoma cells; ST: stromal cells; NB: normal (non-malignant) breast epithelium; *: same patients tissues with FNA.

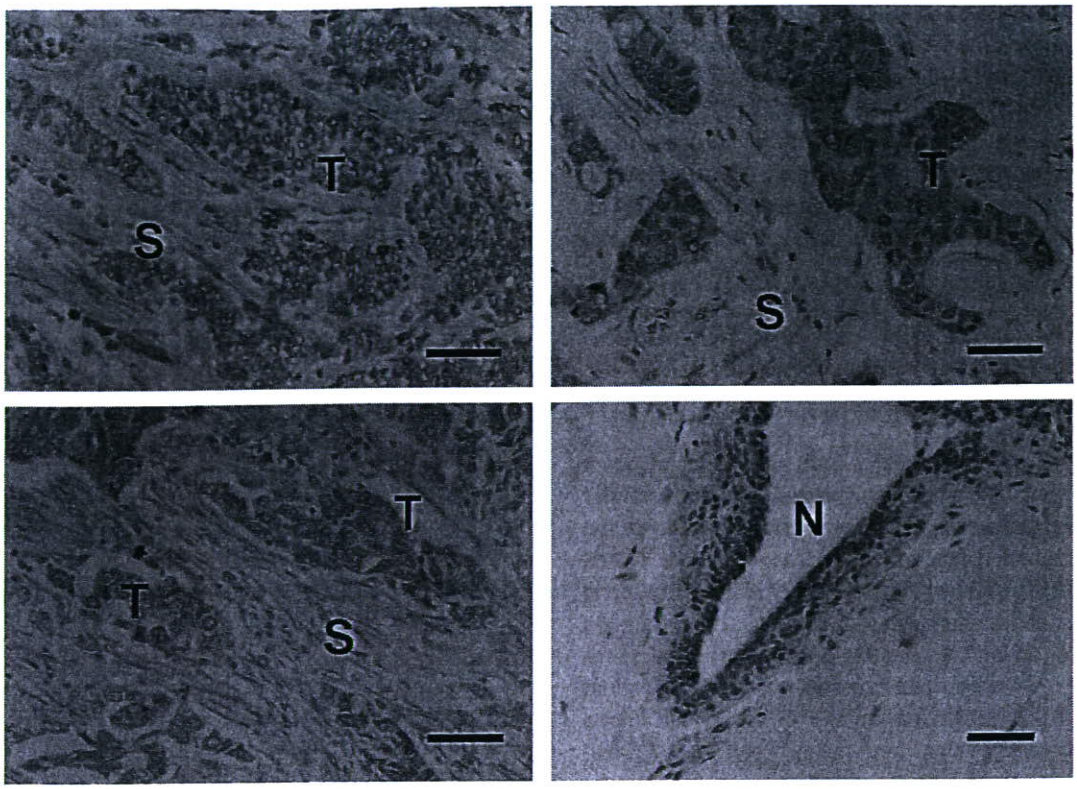

Fig. 1. Immunolocalization of aromatase in breast carcinoma tissues. Aromatase immunoreactivity was detected in the cytoplasm of stromal (S, top left), parenchymal carcinoma cells (T, top right), or both parenchymal and stromal cells (bottom left). Aromatase immunoreactivity was also detected in non-malignant duct epithelial cells (N, bottom right); bar: 50 μm.

aromatization occurs in various components of breast carcinoma tissues.

3. Aromatase in human breast carcinoma cell lines

There have also been controversies regarding aromatase in human breast carcinoma cell lines. It is well known that aromatase mRNA expression/enzymatic activity is below the detectable level in the great majority of breast cancer cell lines, MCF-7 and T-47D [28–30]. Therefore, stable aromatase expressing cell lines such as MCF-7aro and T-47Daro have been developed by some investigators [31]. However, several studies demonstrated native aromatase mRNA and enzymatic activity in breast carcinoma cell lines including MCF-7 [28–34]. Furthermore, Yang et al. [35] demonstrated that estrogen-related receptor α was a positive regulator of aromatase mRNA expression in SK-BR-3 breast carcinoma cell line. Mu et al. [36] reported that the combined treatment of two types of retinoids, TTNPB for RAR and LG100268 for RXR synergistically stimulate aromatase enzymatic activity in MCF-7. These nuclear receptors such as ERRα, RAR, and RXR were all detected in parenchymal carcinoma cells but not in stromal nor in normal epithelial cells of breast carcinoma tissues [37–39]. In RT-PCR analysis of aromatase (Fig. 2B), aromatase mRNA transcripts were detected in breast carcinoma cell lines such as MCF-7, ZR-75-1, SK-BR-3, MDA-MB-231, and MDA-MB-468 but not T-47D and YMB-1-E. Relatively high level of aromatase mRNA was detected in SK-BR-3 and MDA-MB-468 (Fig. 2B). In previous immunocytochemical analysis using polyclonal antibody (from Dr. Y. Osawa), MCF-7 and SK-BR-3 cells also showed aromatase immunoreactivity in their cytoplasm [21]. It was also reported that non-steroidal aromatase inhibitor, letrozole directly inhibited growth of the

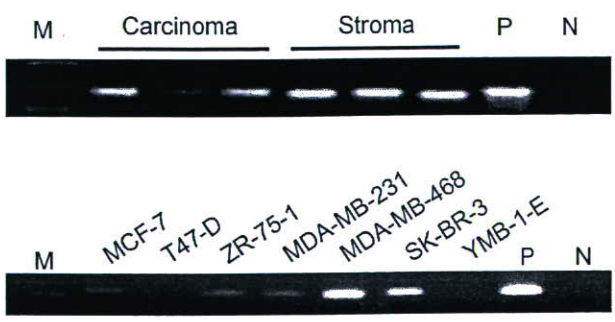

Fig. 2. (A) Localization of aromatase mRNA in breast carcinoma tissues. Aromatase mRNA was detected both in parenchymal and stromal cells collected by laser capture microdissection. (B) Expression of aromatase mRNA in breast carcinoma cell lines. M: molecular marker; Pc: positive control (placenta); Nc: negative control (H_2O).