

Intratumoral concentration of sex steroids and expression of sex steroid-producing enzymes in ductal carcinoma *in situ* of human breast

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Abstract

It is well known that sex steroids play important roles in the development of invasive ductal carcinoma (IDC) of the human breast. However, biological significance of sex steroids remains largely unclear in ductal carcinoma *in situ* (DCIS), regarded as a precursor lesion of IDC, which is partly due to the fact that the intratumoral concentration of sex steroids has not been examined in DCIS. Therefore, in this study, we first examined the intratumoral concentrations of estradiol and 5 α -dihydrotestosterone (DHT) using liquid chromatography/electrospray tandem mass spectrometry in DCIS. Intratumoral concentrations of both estradiol and DHT were threefold higher in DCIS than non-neoplastic breast tissues and estrogen-producing enzymes (aromatase, steroid sulfatase, and 17 β -hydroxysteroid dehydrogenase type 1 (17 β HSD1)), and androgen-producing enzymes (17 β HSD5 and 5 α -reductase type 1 (5 α Red1)) were abundantly expressed in DCIS by real-time PCR and immunohistochemical analyses. The intratumoral concentration of DHT was significantly lower in IDC than DCIS, while the expression of aromatase mRNA in carcinoma cells and intratumoral stromal cells was significantly higher in IDC than those in DCIS. Immunohistochemistry for sex steroid-producing enzymes in DCIS demonstrated that 5 α Red1 immunoreactivity was positively correlated with Ki-67 labeling index and histological grade and was also associated with an increased risk of recurrence in patients with DCIS examined. Results of our study suggest that intratumoral concentrations of estradiol and DHT are increased in DCIS, which is possibly due to intratumoral production of these steroids. Therefore, estradiol and DHT may play important roles in the development of DCIS of the human breast.

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Introduction

Breast carcinoma is the most common malignant neoplasm in women worldwide. Among breast carcinomas, the incidence of ductal carcinoma *in situ* (DCIS) has been markedly increasing during the past two decades due to advancements in detection by mammographic screening (Li *et al.* 2005) such that DCIS now comprise ~20% of all human breast carcinomas diagnosed (Kepple *et al.* 2006, Tsikitis & Chung 2006). DCIS is regarded as a precursor lesion of invasive ductal carcinoma (IDC) and the risk of IDC developing was reported to be four to ten

times higher after the diagnosis of DCIS compared to women without DCIS (Franceschi *et al.* 1998, Warnberg *et al.* 2000). Therefore, it is very important to examine the biological features of DCIS in order to improve clinical outcome of breast carcinoma patients.

It is well known that breast tissue is a target for sex steroids. Among the sex steroids, estrogens greatly contribute to the growth of IDC (Vihko & Apter 1989) and anti-estrogens such as tamoxifen, aromatase inhibitors, or luteinizing hormone-releasing hormone (LH-RH) agonists are currently used in patients

with IDC to block the intratumoral estrogen actions. A great majority of DCIS cases express sex-steroid receptors, i.e., estrogen (ER), progesterone (PR), and androgen (AR) receptors (Selim et al. 2002, Baqai & Shousha 2003, Moinfar et al. 2003, Barnes et al. 2005, Rody et al. 2005, Kepple et al. 2006), which suggest an important role of sex steroids in both DCIS and IDC. Tamoxifen was reported to inhibit the growth of premalignant mammary lesions and the progression to invasive carcinoma in a transplantable mouse model of DCIS (Namba et al. 2005). The National Surgical Adjuvant Breast Project (NSABP) P-1 trial demonstrated that tamoxifen significantly reduced the risk of noninvasive breast cancer by 50% (Dunn et al. 2005) and results of the NSABP B-24 trial indicated that adjuvant tamoxifen therapy was clinically effective in ER-positive DCIS and reduced the recurrence of non-invasive breast carcinomas by 27% (Cuzick 2003).

The concentration of the biologically active estrogen, estradiol, is significantly higher in IDC than in the areas considered as morphologically normal (Chetrite et al. 2000), and estradiol is locally produced from circulating inactive steroids by estrogen-producing enzymes such as aromatase (conversion from circulating androstenedione to estrone or testosterone to estradiol), steroid sulfatase (STS; hydrolysis of circulating estrone sulfate to estrone), and 17 β -hydroxysteroid dehydrogenase type 1

(17 β HSD1; conversion of estrone to estradiol) in IDC (Suzuki et al. 2005a; Fig. 1). Intratumoral concentrations of the biologically active androgen, 5 α -dihydrotestosterone (DHT), were also significantly higher in IDC than in plasma (Recchione et al. 1995) and androgen-producing enzymes, such as 17 β HSD5 (conversion of circulating androstenedione to testosterone) and 5 α -reductase (5 α Red; reduction of testosterone to DHT) were frequently expressed in IDC (Suzuki et al. 2005a; Fig. 1). Immunolocalization of aromatase (Zhang et al. 2002, Oliveira et al. 2006) and 17 β HSD1 (Ariga et al. 2000) has been previously reported in DCIS, suggesting the possible importance of *in situ* production of sex steroids in DCIS. However, to the best of our knowledge, the intratumoral concentration of sex steroids has not been reported at all in DCIS and no information is available regarding the expression of androgen-producing enzymes in DCIS. Information on sex steroids is very limited in DCIS compared with that in IDC and so the clinical and/or biological significance of sex steroids in DCIS remains largely unclear. Therefore, in this study, we examined the intratumoral concentrations of estradiol and DHT and expression of sex steroid-producing enzymes in DCIS, and compared these findings with those in non-neoplastic breast and IDC tissues. In addition, we immunolocalized sex steroid-producing enzymes in 83 DCIS cases, and correlated these findings with various

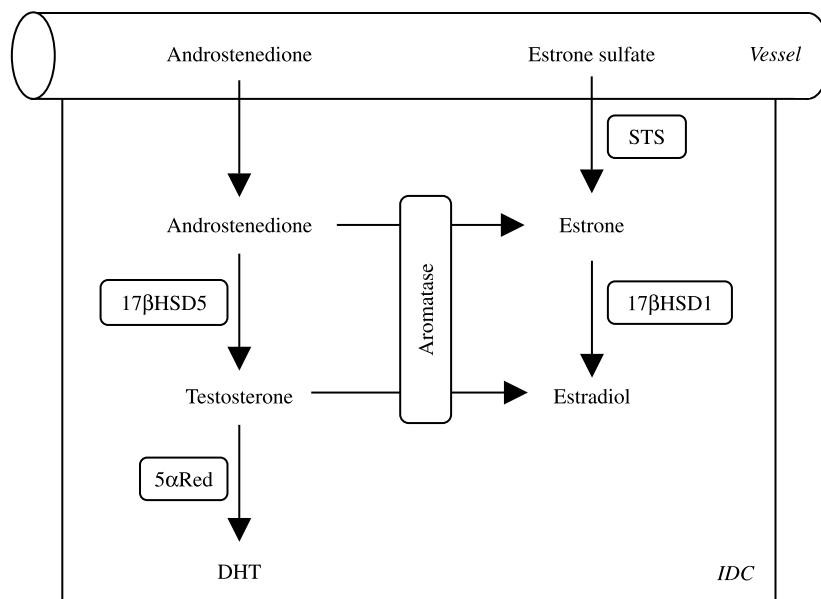


Figure 1 Schema representing intratumoral production of sex steroids in human invasive ductal carcinoma (IDC), which is currently postulated. STS, steroid sulfatase; 17 β HSD1, 17 β -hydroxysteroid dehydrogenase type 1; 17 β HSD5, 17 β -hydroxysteroid dehydrogenase type 5; and 5 α Red, 5 α -reductase.

clinicopathological parameters in order to further examine the significance of sex steroids in DCIS.

Materials and methods

Patients and tissues

Specimens of pure DCIS ($n = 12$) and IDC ($n = 12$) were obtained from female patients who underwent breast cancer surgical treatment from 2001 to 2004 in the Departments of Surgery at Tohoku University Hospital and Tohoku Kosai Hospital, Sendai, Japan. Non-neoplastic breast tissues were also obtained in 8 out of the 12 IDC patients, who underwent mastectomy and were examined in this study. Specimens for sex-steroid 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 Van Nuys classification (Silverstein *et al.* 1995) in DCIS and by the method of Elston & Ellis (1991) in IDC. Informed consent was obtained from all patients prior to their surgery and the examination of the specimens used in this study.

Eighty-three pure DCIS specimens were obtained by surgical excision from 1990 to 2005 in Department of Surgery, Tohoku University Hospital, Sendai, Japan. The mean age of the patients was 57.0 years (ranges 30–80 years). All of the patients did not receive irradiation, chemotherapy, or hormonal therapy prior to the surgery. Disease-free survival data were available in 78 patients, and the mean follow-up time was 54 months (ranges 8–117 months). All specimens were fixed with 10% formalin and embedded in paraffin wax.

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

Liquid chromatography/electrospray tandem mass spectrometry (LC–MS/MS)

Concentrations of estradiol and DHT were measured by LC–MS/MS analysis in Teizo Medical Co. (Kawasaki, Japan), as described previously (Miki *et al.* 2007, Suzuki *et al.* 2007, Yamashita *et al.* 2007). Briefly, the weights of the breast carcinoma specimens (32–89 mg for each sample) were measured by an electronic balance to a reasonable accuracy (AEX-200B (Shimadzu, Kyoto, Japan); readability, 0.1 mg; and capacity, 200 g), and these were then homogenized in 1 ml distilled water. After addition of 100 pg estradiol- $^{13}\text{C}_4$ (Hayashi Junyaku, Tokyo, Japan) or DHT- $^2\text{H}_3$ (CDN Isotope, Pointe-Claire Quebec, Canada) as internal standard, steroids were extracted

with diethyl ether from the homogenate. The separated organic layer was evaporated, and then dissolved in picolinic anhydride in tetrahydrofuran solution (100 μl) with triethylamine (20 μl). After application to a Bond Elut C18 column, steroid derivatives were eluted with 80% acetonitrile solution. The derivative estradiol and DHT fraction were dissolved in the elution solvent of LC.

In this study, we used an LC (Agilent 1100, Agilent Technologies, Waldbronn, Germany) coupled with an API 4000 triple-stage quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) operated with electrospray ionization in the positive-ion mode, and the chromatographic separation was performed on Cadenza CD-C18 column (3×150 mm, 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 a flow rate of 0.4 ml/min. Total run time was 10 min. We used a 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 times for the derived estradiol and DHT were 5.3 and 5.8 min respectively. 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 255.3 (I.S.: 258.3) as ion produced from 396.4 (I.S.: 399.4) and the m/z 262 (I.S.: 268) from 383.3 (I.S.: 487.2) respectively for estradiol and DHT derivatives.

In our present study, the lower limit of quantification (LLOQ) was 0.2 pg for both the estradiol and the DHT. It was determined by combination of determination validation, reproducibility, accuracy, and precision. The reproducibility was evaluated by intra- and inter-assays ($n = 3$) and their coefficient variations (CVs) were 11.8 and 2.4% for estradiol, and 15.0 and 13.4% for DHT respectively. The accuracy and precision were measured using five different concentrations (0.2, 0.5, 1.0, 10, and 50 pg) of estradiol or DHT and five determinations for each concentration. The LLOQ did not exceed 20% of the CV. The recovery for estradiol and DHT was 80–85%.

Real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies Inc.), and a reverse transcription kit (SUPERScript II Preamplification system (Gibco-BRL) was used in the synthesis of cDNA. The Light Cycler system (Roche Diagnostics GmbH)

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

cDNA (gene symbol; accession no.)	Sequence (position in cDNA)	Size (bp)	Reference
Aromatase ^a (aromatase; X13589)	FWD: 691–712 REV: 786–806	116	
STS ^a (STS; M16505)	FWD: 1550–1569 REV: 1683–1702	153	
17βHSD1 ^a (HSD17B1; NM000413)	FWD: 1300–1321 REV: 1604–1625	326	
17βHSD5 ^a (AKR1C3; NM003739)	FWD: 969–992 REV: 1052–1071	103	
5αRed1 ^a (SRD5A1; NM001047)	FWD: 658–677 REV: 796–815	158	
5αRed2 (SRD5A2; NM000348)	FWD: 500–520 REV: 794–814	315	Suzuki et al. (2001)
RPL13A (RPL13A; NM012423)	FWD: 487–509 REV: 588–612	125	Vandesompele et al. (2002)

^aOligonucleotide primers used in this study were designed in the different exons.

was used to semi-quantify the mRNA expression levels by real-time PCR (Dumoulin et al. 2000). Characteristics of the primer sequences used in this study were summarized in Table 1 (Suzuki et al. 2001, Vandesompele et al. 2002). Settings for the PCR thermal profile were as follows: initial denaturation at 95 °C for 10 min, followed by 40 amplification cycles of 95 °C for 10 s, annealing at 60 °C (17βHSD1), 64 °C (17βHSD5 and STS), 68 °C (aromatase, 5αRed1, 5αRed2 and ribosomal protein L 13a (RPL13A)) for 10 s, and elongation at 72 °C for 12 s. To verify amplification of the correct sequences, PCR products were purified and subjected to direct sequencing. Negative control experiments lacked cDNA substrate to check for the possibility of exogenous contaminant DNA. The mRNA level of the steroidogenic enzymes was summarized as a ratio (%) of that of RPL13A. The results of real-time PCR analyses were similar when we used other internal standards, such as glyceraldehyde-3-phosphate dehydrogenase (Suzuki et al. 2005b) and β-actin (Suzuki et al. 2001), instead of RPL13A as used in this study (data not shown).

Laser capture microdissection (LCM)/real-time PCR for aromatase

Seven specimens of non-neoplastic breast, eight of DCIS and nine of IDC were available for LCM/real-time PCR analysis in this study. LCM was conducted using the Laser Scissors CRI-337 (Cell Robotics Inc., Albuquerque, NM, USA) and ~5000 epithelial cells or stromal cells were collected under the microscope from frozen sections of breast tissues. Total RNA was extracted according to a RNA microisolation protocol described by Niino et al. (2001). The real-time PCR protocol for aromatase was described above.

Immunohistochemistry

The characteristics of primary antibodies for steroidogenic enzymes, such as aromatase (Miki et al. 2007), STS (Suzuki et al. 2003), 17βHSD1 (Suzuki et al. 2000), 17βHSD5 (Suzuki et al. 2001), 5αRed1 (Suzuki et al. 2001), and 5αRed2 (Suzuki et al. 2001), used in this study were described previously. Monoclonal antibodies for ERα (ER1D5), PR (MAB429), AR (AR441), and Ki-67 (MIB1) were purchased from Immunotech (Marseille, France), Chemicon (Temecula, CA, USA), DAKO (Carpinteria, CA, USA) and DAKO respectively.

A Histofine Kit (Nichirei, Tokyo, Japan), which employs the streptavidin–biotin amplification method was used for immunohistochemistry in this study. Antigen retrieval for ERα, PR, AR, 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 this study was as follows: aromatase; 1/6000, STS; 1/9000, 17βHSD1; 1/500, 17βHSD5; 1/1000, 5αRed1; 1/1000, 5αRed2; 1/1000, ERα; 1/50, PR; 1/30, AR; 1/100, and Ki-67; 1/50. 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% H₂O₂) and counterstained with hematoxylin. As a negative control, normal rabbit or mouse immunoglobulin G (IgG) was used instead of the primary antibody.

Immunoreactivity of steroidogenic enzymes was detected in the cytoplasm and cases that had more than 10% of positive carcinoma cells staining were considered positive (Suzuki et al. 2007). Immunoreactivity of ERα, PR, AR, and Ki-67 was detected in the nucleus. These immunoreactivities were evaluated in more than 1000 carcinoma cells for each case

and subsequently the percentage of immunoreactivity, i.e., labeling index (LI), was determined (Suzuki *et al.* 2007).

Statistical analysis

The statistical analyses between two groups were performed using a Mann–Whitney *U* test, and *P* values <0.05 were considered significant. The relative ratio between two groups was evaluated by their median values. Disease-free survival curves were generated according to the Kaplan–Meier method and the statistical significance was calculated using a log-rank test.

Results

Intratumoral concentration of sex steroids in DCIS

We first examined the tissue concentration of sex steroids in the non-neoplastic breast, DCIS, and IDC tissues by LC–MS/MS. The median with min–max value of tissue concentration of estradiol was 16 (5–83) pg/g in non-neoplastic breast, 52 (10–494) pg/g in DCIS, and 206 (11–1586) pg/g in IDC. The median value in DCIS was 3.3-fold higher than that in non-neoplastic breast tissues (Fig. 2A). The intratumoral concentration of estradiol was 4.0-fold higher in IDC than DCIS, but no significant association was detected ($P=0.20$).

The median with min–max value of tissue concentration of DHT was 100 (63–128) pg/g in the non-neoplastic breast, 323 (140–1593) pg/g in DCIS, and 162 (41–990) pg/g in IDC. The tissue concentration of DHT was 3.2-fold higher in DCIS than non-neoplastic breast (Fig. 2B). The intratumoral concentration of DHT was significantly higher in DCIS than IDC ($P=0.04$ and 2.0-fold).

The intratumoral concentration of estradiol in DCIS was 2.2-fold higher in premenopausal women (93 (10–494) pg/g ($n=5$)) than in postmenopausal women (42 (13–70) pg/g ($n=7$)), but no significant association was detected ($P=0.46$). The median of the intratumoral concentration of DHT in DCIS was 260 (253–380) pg/g in premenopausal women and 326 (140–1593) pg/g in postmenopausal women ($P=0.52$).

mRNA expression of sex steroid-producing enzymes in DCIS

We next examined mRNA expression of sex steroid-producing enzymes in non-neoplastic breast, DCIS, and IDC using real-time PCR. As shown in Table 2, mRNA levels of aromatase, STS, 17 β HSD1, 17 β HSD5, and 5 α Red1 were significantly higher in DCIS than

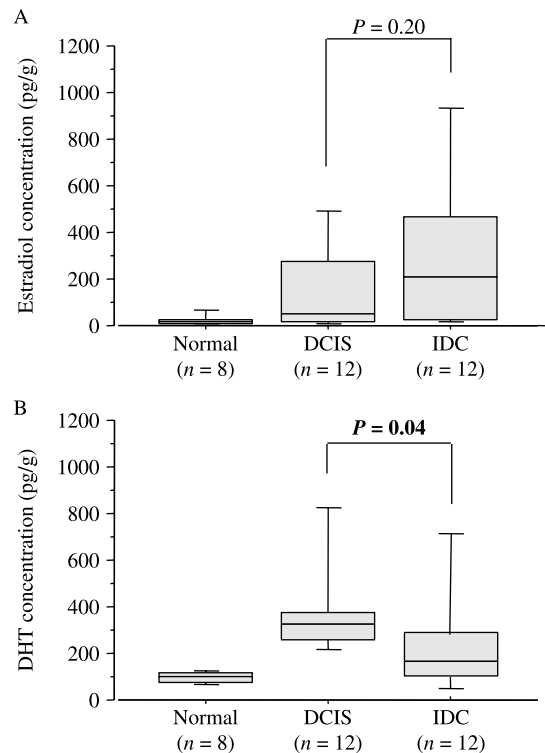


Figure 2 Tissue concentrations of (A) estradiol and (B) DHT in the non-neoplastic breast (normal), DCIS, and IDC tissues. Data are represented as box and whisker plots. The median value is shown by a horizontal line in the box plot and the gray box denotes the 75th (upper margin) and 25th percentiles of the values (lower margin). The upper and lower bars indicate the 90th and 10th percentiles respectively. The statistical analyses were performed between breast carcinoma (DCIS and IDC) groups by a Mann–Whitney *U* test. *P* values <0.05 were considered significant and are indicated in boldface.

non-neoplastic breast ($P=0.03$ and 4.0-fold in aromatase, $P=0.01$ and 9.5-fold in STS, $P=0.04$ (relative ratio could not be evaluated) in 17 β HSD1, $P=0.01$ and 18-fold in 17 β HSD5, and $P=0.02$ and 5.1-fold in 5 α Red1). The expression level of 5 α Red2 mRNA was negligible and no significant difference was detected between DCIS and non-neoplastic breast ($P=0.67$). The expression level of aromatase mRNA was significantly higher in IDC than DCIS ($P=0.046$ and 5.9-fold), but mRNA levels of other sex steroid-producing enzymes were not significantly changed between these two breast carcinoma groups.

Previous studies demonstrated that aromatase was immunolocalized in carcinoma and stromal cells in human breast carcinoma (Zhang *et al.* 2002, Suzuki *et al.* 2005a). Therefore, we further examined the expression of aromatase mRNA according to cell type in the non-neoplastic breast, DCIS, and IDC using LCM/real-time PCR. In the epithelial cells, the median

Table 2 mRNA expression of sex steroid-producing enzymes in non-neoplastic breast, ductal carcinoma *in situ* (DCIS), and invasive ductal carcinoma (IDC) tissues

Enzyme	mRNA expression level (median (min–max)) × (%)			P value	
	Normal (n=8)	DCIS (n=12)	IDC (n=12)	DCIS versus normal	DCIS versus IDC
Estrogen-producing enzymes					
Aromatase	3.5 (0.0–8.0)	14.1 (1.5–113.3)	82.5 (0.0–528.5)	0.03	0.046
STS	0.4 (0.0–1.5)	3.8 (0.0–93.1)	13.6 (0.3–241.3)	0.01	0.13
17βHSD1	0.0 (0.0–0.5)	0.6 (0.0–4.0)	1.5 (0.0–3.9)	0.04	0.07
Androgen-producing enzymes					
17βHSD5	0.6 (0.0–3.1)	10.8 (1.0–57.3)	10.9 (0.0–39.6)	0.01	0.91
5αRed1	11.5 (0.0–19.8)	58.6 (3.0–223.6)	34.2 (0.0–438.4)	0.02	0.60
5αRed2	0.0 (0.0–15.9)	0.2 (0.0–49.0)	0.2 (0.0–58.8)	0.67	0.83

Normal; non-neoplastic breast tissues. The statistical analyses between two groups were performed using a Mann–Whitney's *U* test. *P* values <0.05 were considered significant and are indicated in boldface. The mRNA level of the steroidogenic enzymes was summarized as a ratio (%) of that of RPL13A.

with min–max value of aromatase mRNA was 0.00 (0.00–0.30) × 10⁻²% in epithelial cells of non-neoplastic breast, 0.06 (0.00–0.29) × 10⁻²% in carcinoma cells of DCIS, and 1.00 (0.01–3.00) × 10⁻²% in carcinoma cells of IDC and its expression level was significantly higher in IDC than non-neoplastic breast (*P*=0.01 and relative ratio could not be evaluated) or DCIS (*P*=0.03 and 17-fold; Fig. 3A). The expression level of aromatase mRNA in the stromal cells was 0.00 (0.00–0.04) × 10⁻²% in non-neoplastic breast, 0.04 (0.00–0.10) × 10⁻²% in DCIS, and 4.00 (1.00–6.23) × 10⁻²% in IDC, and the expression level was significantly higher in IDC than non-neoplastic breast (*P*=0.001, and relative ratio could not be evaluated) or DCIS (*P*=0.001, and 100-fold; Fig. 3B).

Immunoreactivity of sex steroid-producing enzymes in DCIS

Aromatase immunoreactivity was detected in the cytoplasm of carcinoma cells in 45 out of 83 DCIS cases (54%; Fig. 4A) and was also detected in some intratumoral stromal cells (Fig. 4B). Immunoreactivity of other sex steroid-producing enzymes was detected in the cytoplasm of carcinoma cells and the number of positive cases was as follows: STS; 45/83 (54%; Fig. 4C), 17βHSD1; 54/83 (65%; Fig. 4D), 17βHSD5; 59/83 (71%; Fig. 4E), 5αRed1; 52/83 (63%; Fig. 4F), and 5αRed2; 13/83 (16%). Associations between immunoreactivity of sex steroid-producing enzymes and clinicopathological parameters in the 83 DCIS cases are summarized in Tables 3 and 4. Among the estrogen-producing enzymes, STS immunoreactivity was significantly associated with the histological grade (Van Nuys classification; *P*=0.01), while no significant association was detected between aromatase or

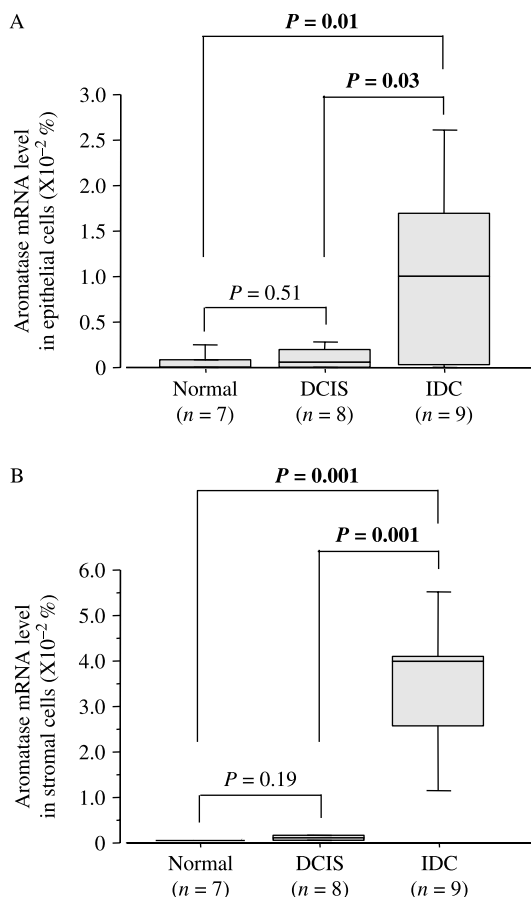


Figure 3 Expression of aromatase mRNA in (A) epithelial cells or (B) stromal cells in non-neoplastic breast (normal), DCIS, and IDC tissues. Epithelial cells and the adjacent stromal cells in non-neoplastic breast tissues, or carcinoma cells and intratumoral stromal cells in DCIS and IDC were collected separately by LCM. Data are represented as box and whisker plots. The statistical analyses were performed using a Mann–Whitney *U* test between indicated two groups. *P* values <0.05 were considered significant and are indicated in boldface.

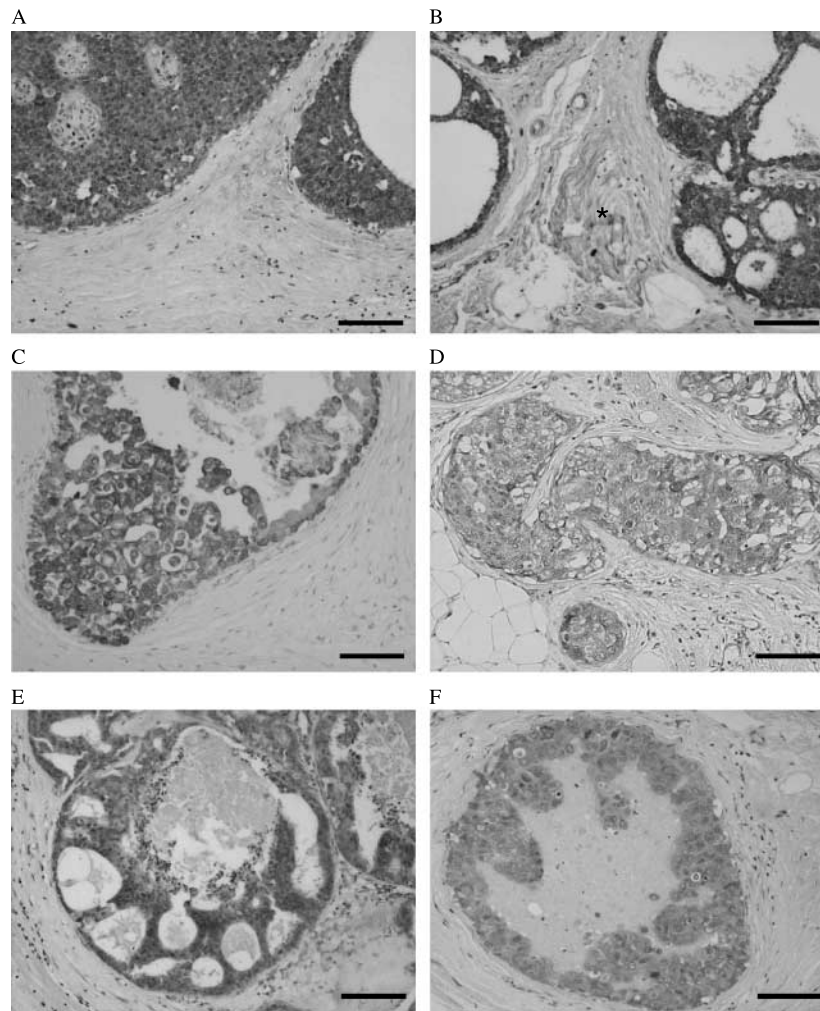


Figure 4 (A and B) Immunohistochemistry for aromatase, (C) STS, (D) 17 β HSD1, (E) 17 β HSD5, and (F) 5 α Red1 in DCIS. Aromatase immunoreactivity was mainly detected in the cytoplasm of carcinoma cells in (A) DCIS, but it was also positive in some intratumoral stromal cells (*) (B) STS, 17 β HSD1, 17 β HSD5, and 5 α Red1 immunoreactivities were detected in the cytoplasm of carcinoma cells in DCIS. Bar = 100 μ m.

17 β HSD1 immunoreactivity and the clinicopathological factors examined (Table 3).

Among the androgen-producing enzymes, 5 α Red1 immunoreactivity was positively associated with the Van Nuys classification ($P=0.001$) or Ki-67 LI ($P=0.02$), but 17 β HSD5 immunoreactivity was not significantly correlated with the clinicopathological parameters examined (Table 4). No significant association was detected between 5 α Red2 immunoreactivity and the clinicopathological parameters examined (data not shown). There was no significant association among the immunoreactivity of these five sex steroid-producing enzymes in DCIS. The significant correlations described above were confirmed in increased rankings of the positivity to three groups (0–9, 10–49, and 50–100%

positive cells; STS and Van Nuys classification, $P=0.04$; 5 α Red1 and Van Nuys classification, $P=0.01$; and 5 α Red1 and Ki-67 LI, $P=0.04$).

As summarized in Fig. 5, the status of 5 α Red1 immunoreactivity was associated with an increased risk of recurrence in 78 DCIS patients examined, although P value was not available because no patients were associated with clinical recurrence in a group of 5 α Red1-negative breast carcinomas. On the other hand, no significant association was detected between the status of other steroidogenic enzyme immunoreactivity and risk of recurrence in these DCIS patients (aromatase, $P=0.87$; STS, $P=0.47$; 17 β HSD1, $P=0.83$; 17 β HSD5, $P=0.98$; and 5 α Red2, $P=0.45$).

Table 3 Association between immunoreactivity of estrogen-producing enzymes and clinicopathological parameters in 83 ductal carcinoma *in situ* (DCIS) tissues

Value	Aromatase immunoreactivity			STS immunoreactivity			17βHSD1 immunoreactivity		
	+	–	P value	+	–	P value	+	–	P value
Age ^a (years)	60 (34–80)	56 (30–77)	0.37	54 (34–77)	61 (30–80)	0.43	57 (30–80)	61 (34–77)	0.70
Menopausal status									
Premenopausal	13 (16%)	14 (17%)		16 (19%)	11 (13%)		14 (17%)	13 (16%)	
Postmenopausal	32 (39%)	24 (29%)	0.59	29 (35%)	27 (33%)	0.68	40 (48%)	16 (19%)	0.08
Van Nuys classification									
Group 1	14 (17%)	13 (16%)		8 (10%)	19 (23%)		17 (20%)	10 (12%)	
Group 2	21 (25%)	19 (23%)		25 (30%)	15 (18%)		26 (31%)	14 (17%)	
Group 3	10 (12%)	6 (7%)	0.75	12 (14%)	4 (5%)	0.01	11 (13%)	5 (6%)	0.93
ERα LI ^a	69 (0–97)	82 (0–97)	0.20	67 (0–96)	80 (0–97)	0.13	78 (0–97)	66 (4–94)	0.13
PR LI ^a	52 (0–92)	33 (0–91)	0.12	33 (6–92)	47 (0–93)	0.63	38 (0–93)	31 (0–92)	0.60
AR LI ^a	56 (0–97)	55 (5–94)	0.54	53 (0–93)	59 (4–97)	0.39	56 (2–97)	55 (0–93)	0.86
Ki-67 LI ^a	16 (2–35)	13 (2–35)	0.22	17 (2–35)	12 (2–32)	0.23	13 (2–35)	17 (3–33)	0.45

^aData are presented as median with min–max values and were evaluated by a Mann–Whitney *U* test. All other values represent the number of cases and percentage.

Discussion

To the best of our knowledge, this is a first report that demonstrates intratumoral concentrations of sex steroids in DCIS. Median values of both estradiol and DHT concentrations were (3.3-fold in estradiol and 3.2-fold in DHT) higher in DCIS than those in non-neoplastic breast tissues. Results of our present study also demonstrated that mRNA expression of both estrogen (aromatase, STS, and 17βHSD1)- and androgen (17βHSD5 and 5αRed1)-producing enzymes was significantly higher in DCIS than the non-neoplastic breast tissues. Previous studies demonstrated that aromatase immunoreactivity was detected

in the carcinoma and stromal cells (Zhang *et al.* 2002) in 70% of DCIS tissues (Oliveira *et al.* 2006) and 17βHSD1 immunoreactivity was positive in the carcinoma cells in 63% of DCIS cases (Ariga *et al.* 2000). In our present study, we detected aromatase and 17βHSD1 immunoreactivities in 54 and 65% of DCIS cases and these frequencies and localization were consistent with the previous reports. On the other hand, expression of STS, 17βHSD5, and 5αRed has not been reported in DCIS. Our present results showed that immunoreactivity for STS, 17βHSD5, 5αRed1, and 5αRed2 was positive in 54, 71, 63, and 16% of DCIS cases respectively and these frequencies were similar

Table 4 Association between immunoreactivity of androgen-producing enzymes and clinicopathological parameters in 83 ductal carcinoma *in situ* (DCIS) tissues

Value	17βHSD5 immunoreactivity		P value	5αRed1 immunoreactivity		P value
	+	–		+	–	
Age ^a (years)	53 (30–80)	61 (42–69)	0.19	55 (30–80)	61 (39–77)	0.43
Menopausal status						
Premenopausal	23 (28%)	5 (6%)		19 (23%)	8 (10%)	
Postmenopausal	36 (43%)	19 (23%)	0.18	33 (40%)	23 (28%)	0.44
Van Nuys classification						
Group 1	20 (24%)	7 (8%)		11 (13%)	16 (19%)	
Group 2	26 (3%)	14 (17%)		26 (31%)	14 (17%)	
Group 3	13 (16%)	3 (4%)	0.43	15 (18%)	1 (1%)	0.001
ERα LI ^a	71 (0–97)	87 (0–94)	0.12	73 (0–97)	79 (0–97)	0.48
PR LI ^a	53 (0–92)	57 (12–87)	0.96	32 (0–92)	43 (0–93)	0.14
AR LI ^a	73 (16–97)	83 (0–93)	0.10	54 (2–93)	62 (0–97)	0.12
Ki-67 LI ^a	20 (2–35)	13 (7–24)	0.07	18 (3–35)	11 (2–32)	0.02

P values <0.05 were considered significant and are indicated in boldface.

^aData are presented as median with min–max values and were evaluated by a Mann–Whitney *U* test. All other values represent the number of cases and percentage and were statistically analyzed using a cross-table using the χ^2 test.

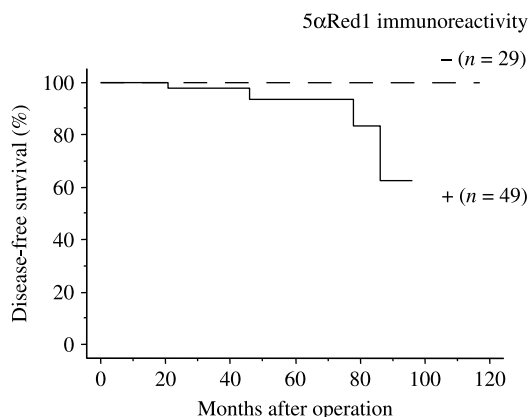


Figure 5 Disease-free survival curve of 78 DCIS patients according to 5 α Red1 immunoreactivity (Kaplan–Meier method).

to those reported in IDC (59–88, 53, 58, and 15% respectively; Suzuki *et al.* 2005a). 5 α Red activity is considered to be mainly mediated by 5 α Red1 in IDC, because 5 α Red2 expression was shown to be very low (Suzuki *et al.* 2005a). Results of our present study all suggest that both the estradiol and the DHT are locally produced from circulating inactive steroids by sex steroid-producing enzymes, which results in increased intratumoral concentrations of these steroids in DCIS in a similar manner to IDC. Recently, Faratian *et al.* (2005) reported that proliferation of DCIS was reduced by aromatase inhibitors, such as letrozole and anastrozole. Therefore, endocrine therapies, such as anti-estrogens, aromatase inhibitors, and/or LH-RH agonists, may be clinically effective in a selective group of DCIS patients.

In our present study, aromatase mRNA was detected in both carcinoma and intratumoral stromal cells and the expression level was significantly higher in IDC than DCIS in these two cellular components (17- and 100-fold respectively). Previous *in vitro* studies demonstrated that breast carcinoma cells secrete various factors that induce aromatase expression in adipose fibroblasts (Zhou *et al.* 2001), including prostaglandin E2 (Zhao *et al.* 1996), interleukin (IL)-1, IL-6, IL-11, and tumor necrosis factor α (Reed & Purohit 2001, Simpson & Davis 2001). On the other hand, it has been also reported that exogenous growth factors such as epidermal growth factor (Ryde *et al.* 1992), transforming growth factor (Ryde *et al.* 1992), and keratinocyte growth factor (Zhang *et al.* 1998) stimulated aromatase activity in MCF-7 breast carcinoma cells. Very recently, Miki *et al.* (2007) reported that mRNA level and enzymatic activity of aromatase in MCF-7 breast carcinoma cells were

significantly increased on coculture with primary stromal cells isolated from human breast carcinoma tissue. Therefore, aromatase expression is suggested to be, at least in a part, regulated by tumor–stromal interactions in breast carcinoma tissues, which may be promoted by invasion of the carcinoma cells into the stroma.

Intratumoral DHT level is associated with the testosterone level in IDC (Mistry *et al.* 1986, Recchione *et al.* 1995) and is considered to be mainly determined by amounts of the precursor. Aromatase catalyzes the conversion of androstenedione and testosterone, which are precursors of DHT, to estrone and estradiol respectively (Fig. 1). Spinola *et al.* (1988) previously showed that treatment with an aromatase inhibitor (4-hydroxyandrostenedione) markedly elevated intratumoral testosterone concentrations in dimethylbenz(a)anthracene-induced rat mammary tumors. In addition, Sonne-Hansen & Lykkesfeldt (2005) reported that aromatase preferred testosterone as a substrate in MCF-7 cells. Very recently, Suzuki *et al.* (2007) demonstrated that aromatase expression was inversely associated with intratumoral DHT concentrations in IDC and aromatase inhibitors suppressed the DHT synthesis from androstenedione in coculture experiments. These findings all suggest that aromatase is a negative regulator of local DHT production in human breast carcinoma. In this study, the intratumoral DHT concentration was significantly lower in IDC than DCIS. On the other hand, aromatase expression was significantly higher in IDC than DCIS, while expression levels of androgen-producing enzymes were not significantly different between these two groups. Therefore, higher expression of aromatase in IDC may increase the conversion of androgens into estrogens with a subsequent decrease of intratumoral DHT concentrations.

Various *in vitro* studies have shown that DHT inhibits the cell proliferation of breast carcinoma cells (de Launoit *et al.* 1991, Lapointe & Labrie 2001, Ando *et al.* 2002) and the proapoptotic effect of DHT was also reported in breast carcinoma cells (Kandouz *et al.* 1999). DHT treatment resulted in a rapid fall in tumor volume of ZR75-1 cells injected into athymic mice (Dauvois *et al.* 1991). However, it is also true that some divergent findings have been reported. For instance, Birrell *et al.* (1995) showed that both DHT and the synthetic non-metabolizable androgen, mibolerone, increased the cell proliferation of MCF-7 and MDA-MB-453 cells. In addition, Zhang *et al.* (2004) demonstrated that DHT-benzoate (DHT-B) induced growth of mouse mammary ductal cells, although it is much weaker than estradiol and

treatment with both estradiol and DHT-B caused more pronounced hyperplasia of mammary ducts and alveoli, compared with the treatment with each hormone alone. In our present study, 5 α Red1 immunoreactivity was significantly associated with Ki-67 LI and the Van Nuys classification in 83 DCIS cases and it was also associated with an increased risk of recurrence in the 78 DCIS patients. The Ki-67 antibody recognizes cells in all phases of the cell cycle except the G0 (resting) phase and the Ki-67 LI is closely correlated with the S-phase fraction and mitotic index (Vandesompele et al. 2002). The Van Nuys classification is known as a powerful prognostic classification for DCIS and Silverstein et al. (1995) reported that the incidence of local recurrence after breast-conservation surgery for DCIS in 238 patients was 4% in Group 1 (non-high-grade DCIS without comedo-type necrosis), 11% in Group 2 (non-high-grade DCIS with comedo-type necrosis), and 27% in Group 3 (high-grade DCIS with or without comedo-type necrosis). ER and PR status in DCIS was inversely associated with the histological differentiation or nuclear grade (Selim et al. 2002, Baqai & Shousha 2003). However, AR status was not correlated with ER status in DCIS (Rody et al. 2005) and a significant number of poorly differentiated DCIS was reported ER-negative, PR-negative, but AR-positive (Moinfar et al. 2003). Results of these previous and our present studies are indicative that DHT may be involved in the development of DCIS. However, no information is currently available on the effects of androgens in DCIS to our knowledge and so further examinations are required to clarify the significance of androgens in human DCIS.

In summary, intratumoral concentrations of estradiol and DHT were higher in DCIS than non-neoplastic breast tissues and estrogen-producing enzymes (aromatase, STS, and 17 β HSD1) and androgen-producing enzymes (17 β HSD5 and 5 α Red1) were highly expressed in DCIS. The intratumoral concentration of DHT was significantly lower in IDC than DCIS and the expression of aromatase mRNA was significantly higher in IDC. Results of immunohistochemistry for the sex steroid-producing enzymes demonstrated that 5 α Red1 immunoreactivity was associated with Ki-67 LI, histological grade, and increased risk of recurrence in DCIS patients. Results of our present study suggest that intratumoral concentrations of estradiol and DHT are increased in DCIS, which is possibly due to intratumoral production of these steroids. DCIS frequently expresses ER and/or AR in the carcinoma cells and therefore, both estradiol and DHT may play important roles in the development of DCIS.

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References

- Ando S, De Amicis F, Rago V, Carpino A, Maggiolini M, Panno ML & Lanzino M 2002 Breast cancer: from estrogen to androgen receptor. *Molecular and Cellular Endocrinology* **193** 121–128.
- Ariga N, Moriya T, Suzuki T, Kimura M, Ohuchi N, Satomi S & Sasano H 2000 17 β -Hydroxysteroid dehydrogenase type 1 and type 2 in ductal carcinoma *in situ* and intraductal proliferative lesions of the human breast. *Anticancer Research* **20** 1101–1108.
- Baqai T & Shousha S 2003 Oestrogen receptor negativity as a marker for high-grade ductal carcinoma *in situ* of the breast. *Histopathology* **42** 440–447.
- Barnes NL, Boland GP, Davenport A, Knox WF & Bundred NJ 2005 Relationship between hormone receptor status and tumour size, grade and comedo necrosis in ductal carcinoma *in situ*. *British Journal of Surgery* **92** 429–434.
- Birrell SN, Bentel JM, Hickey TE et al. 1995 Androgens induce divergent proliferative responses in human breast cancer cell lines. *Journal of Steroid Biochemistry and Molecular Biology* **52** 459–467.
- Chetrite GS, Cortes-Prieto J, Philippe JC, Wright F & Pasqualini JR 2000 Comparison of estrogen concentrations, estrone sulfate and aromatase activities in normal, and in cancerous, human breast tissues. *Journal of Steroid Biochemistry and Molecular Biology* **72** 23–27.
- Cuzick J 2003 Treatment of DCIS—results from clinical trials. *Surgical Oncology* **12** 213–219.
- Dauvois S, Geng CS, Levesque C, Merand Y & Labrie F 1991 Additive inhibitory effects of an androgen and the antiestrogen EM-170 on estradiol-stimulated growth of human ZR-75-1 breast tumors in athymic mice. *Cancer Research* **51** 3131–3135.
- Dumoulin FL, Nischalke HD, Leifeld L et al. 2000 Semi-quantification of human C–C chemokine mRNAs with reverse transcription/real-time PCR using multi-specific standards. *Journal of Immunological Methods* **241** 109–119.
- Dunn BK, Wickerham DL & Ford LG 2005 Prevention of hormone-related cancers: breast cancer. *Journal of Clinical Oncology* **23** 357–367.

- Elston CW & Ellis IO 1991 Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer. Experience from a large study with long-term follow-up. *Histopathology* **19** 403–410.
- Faratian D, White S, Murray J *et al.* 2005 Effects of letrozole and anastrozole on ductal carcinoma *in situ* (DCIS): results from a randomised trial. *Breast Cancer Research and Treatment* **94** (Suppl 1) S262 (abstract 6041).
- Franceschi S, Levi F, La VC, Randimbison L & Te VC 1998 Second cancers following *in situ* carcinoma of the breast. *International Journal of Cancer* **77** 392–395.
- Kandouz M, Lombet A, Perrot JY *et al.* 1999 Proapoptotic effects of antiestrogens, progestins and androgen in breast cancer cells. *Journal of Steroid Biochemistry and Molecular Biology* **69** 463–471.
- Kepple J, Henry-Tillman RS, Klimberg VS *et al.* 2006 The receptor expression pattern in ductal carcinoma *in situ* predicts recurrence. *American Journal of Surgery* **192** 68–71.
- Lapointe J & Labrie C 2001 Role of the cyclin-dependent kinase inhibitor p27(Kip1) in androgen-induced inhibition of CAMA-1 breast cancer cell proliferation. *Endocrinology* **142** 4331–4338.
- de Launoit Y, Dauvois S, Dufour M, Simard J & Labrie F 1991 Inhibition of cell cycle kinetics and proliferation by the androgen 5 alpha-dihydrotestosterone and anti-estrogen *N,n*-butyl-*N*-methyl-11-[16' alpha-chloro-3', 17 beta-dihydroxy-estra-1',3',5'-(10')triene-7' alpha-yl] undecanamide in human breast cancer ZR-75-1 cells. *Cancer Research* **51** 2797–2802.
- Li CI, Daling JR & Malone KE 2005 Age specific incidence rates of *in situ* breast carcinomas by histologic type, 1980 to 2001. *Cancer Epidemiology, Biomarkers and Prevention* **14** 1008–1011.
- Miki Y, Suzuki T, Tazawa C, Yamaguchi Y, Kitada K, Honma S, Moriya T, Hirakawa H, Evans DB, Hayashi S *et al.* 2007 Aromatase localization in human breast cancer tissues: possible interactions between intratumoral stromal and parenchymal cells. *Cancer Research* **67** 3945–3954.
- Mistry P, Griffiths K & Maynard PV 1986 Endogenous C19-steroids and oestradiol levels in human primary breast tumour tissues and their correlation with androgen and oestrogen receptors. *Journal of Steroid Biochemistry* **24** 1117–1125.
- Moinfar F, Okcu M, Tsybrovskyy O *et al.* 2003 Androgen receptors frequently are expressed in breast carcinomas: potential relevance to new therapeutic strategies. *Cancer* **98** 703–711.
- Namba R, Young LJ, Maglione JE *et al.* 2005 Selective estrogen receptor modulators inhibit growth and progression of premalignant lesions in a mouse model of ductal carcinoma *in situ*. *Breast Cancer Research* **7** R881–R889.
- Niino YS, Irie T, Takaishi M *et al.* 2001 PKC θ II, a new isoform of protein kinase C specifically expressed in the seminiferous tubules of mouse testis. *Journal of Biological Chemistry* **276** 36711–36717.
- Oliveira VM, Piato S & Silva MA 2006 Correlation of cyclooxygenase-2 and aromatase immunohistochemical expression in invasive ductal carcinoma, ductal carcinoma *in situ*, and adjacent normal epithelium. *Breast Cancer Research and Treatment* **95** 235–241.
- Recchione C, Venturelli E, Manzari A, Cavalleri A, Martinetti A & Secreto G 1995 Testosterone, dihydrotestosterone and oestradiol levels in postmenopausal breast cancer tissues. *Journal of Steroid Biochemistry and Molecular Biology* **52** 541–546.
- Reed MJ & Purohit A 2001 Aromatase regulation and breast cancer. *Clinical Endocrinology* **54** 563–571.
- Rody A, Diallo R, Poremba C *et al.* 2005 Androgen receptor expression in ductal carcinoma *in situ* of the breast: not a helpful marker for classification such as estrogen receptor alpha and progesterone receptor. *Applied Immunohistochemistry and Molecular Morphology* **13** 25–29.
- Ryde CM, Nicholls JE & Dowsett M 1992 Steroid and growth factor modulation of aromatase activity in MCF7 and T47D breast carcinoma cell lines. *Cancer Research* **52** 1411–1415.
- Selim AG, El-Ayat G & Wells CA 2002 Androgen receptor expression in ductal carcinoma *in situ* of the breast: relation to oestrogen and progesterone receptors. *Journal of Clinical Pathology* **55** 14–16.
- Silverstein MJ, Poller DN & Waisman JR 1995 Prognostic classification of breast ductal carcinoma *in situ*. *Lancet* **345** 1154–1157.
- Simpson ER & Davis SR 2001 Minireview: aromatase and the regulation of estrogen biosynthesis – some new perspectives. *Endocrinology* **142** 4589–4594.
- Sonne-Hansen K & Lykkesfeldt AE 2005 Endogenous aromatization of testosterone results in growth stimulation of the human MCF-7 breast cancer cell line. *Journal of Steroid Biochemistry and Molecular Biology* **93** 25–34.
- Spinola PG, Marchetti B, Merand Y, Belanger A & Labrie F 1988 Effects of the aromatase inhibitor 4-hydroxyandrostenedione and the antiandrogen flutamide on growth and steroid levels in DMBA-induced rat mammary tumors. *Breast Cancer Research and Treatment* **12** 287–296.
- Suzuki T, Moriya T, Ariga N, Kaneko C, Kanazawa M & Sasano H 2000 17 β -Hydroxysteroid dehydrogenase type 1 and type 2 in human breast carcinoma: a correlation to clinicopathological parameters. *British Journal of Cancer* **82** 518–523.
- Suzuki T, Darnel AD, Akahira JI *et al.* 2001 5 α -Reductases in human breast carcinoma: possible modulator of *in situ* androgenic actions. *Journal of Clinical Endocrinology and Metabolism* **86** 2250–2257.
- Suzuki T, Nakata T, Miki Y *et al.* 2003 Estrogen sulfotransferase and steroid sulfatase in human breast carcinoma. *Cancer Research* **63** 2762–2770.
- Suzuki T, Miki Y, Nakamura Y *et al.* 2005a Sex steroid-producing enzymes in human breast cancer. *Endocrine-Related Cancer* **12** 701–720.
- Suzuki T, Urano T, Tsukui T, Horie-Inoue K, Moriya T, Ishida T, Muramatsu M, Ouchi Y, Sasano H & Inoue S

- 2005b Estrogen-responsive finger protein as a new potential biomarker for breast cancer. *Clinical Cancer Research* **11** 6148–6154.
- Suzuki T, Miki Y, Moriya T *et al.* 2007 5α -Reductase type 1 and aromatase in breast carcinoma as regulators of *in situ* androgen production. *International Journal of Cancer* **120** 285–291.
- Tsikitis VL & Chung MA 2006 Biology of ductal carcinoma *in situ* classification based on biologic potential. *American Journal of Clinical Oncology* **29** 305–310.
- Vandesompele J, De Preter K, Pattyn F *et al.* 2002 Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* **3** RESEARCH0034.
- Vihko R & Apter D 1989 Endogenous steroids in the pathophysiology of breast cancer. *Critical Reviews in Oncology/Hematology* **9** 1–16.
- Warnberg F, Yuen J & Holmberg L 2000 Risk of subsequent invasive breast cancer after breast carcinoma *in situ*. *Lancet* **355** 724–725.
- Yamashita K, Okuyama M, Watanabe Y, Honma S, Kobayashi S & Numazawa M 2007 Highly sensitive determination of estrone and estradiol in human serum by liquid chromatography–electrospray ionization mass spectrometry. *Steroids* **72** 819–827.
- Zhang Y, Kulp SK, Sugimoto Y, Farrar WB, Brueggemeier RW & Lin YC 1998 Keratinocyte growth factor (KGF) induces aromatase activity in cultured MCF-7 human breast cancer cells. *Anticancer Research* **18** 2541–2546.
- Zhang Z, Yamashita H, Toyama T *et al.* 2002 Semi-quantitative immunohistochemical analysis of aromatase expression in ductal carcinoma *in situ* of the breast. *Breast Cancer Research and Treatment* **74** 47–53.
- Zhang J, Sun Y, Liu Y, Sun Y & Liao DJ 2004 Synergistic effects of androgen and estrogen on the mouse uterus and mammary gland. *Oncology Reports* **12** 709–716.
- Zhao Y, Agarwal VR, Mendelson CR & Simpson ER 1996 Estrogen biosynthesis proximal to a breast tumor is stimulated by PGE2 via cyclic AMP, leading to activation of promoter II of the CYP19 (aromatase) gene. *Endocrinology* **137** 5739–5742.
- Zhou J, Gurates B, Yang S, Sebastian S & Bulun SE 2001 Malignant breast epithelial cells stimulate aromatase expression via promoter II in human adipose fibroblasts: an epithelial–stromal interaction in breast tumors mediated by CCAAT/enhancer binding protein beta. *Cancer Research* **61** 2328–2334.