Increased intratumoral androgens in human breast carcinoma following aromatase inhibitor exemestane treatment

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Abstract

Sex steroids play important roles in the development of many human breast carcinomas, and aromatase inhibitors are used for the anti-estrogen therapy. Recent studies have demonstrated that aromatase suppressed 5 α -dihydrotestosterone (DHT) synthesis in breast carcinoma cells, but intratumoral concentration of androgens and its significance have not been reported in the breast carcinoma patients treated with aromatase inhibitors. Therefore, we examined androgen concentrations in breast carcinoma tissues treated with exemestane, and further performed *in vitro* studies to characterize the significance of androgen actions. Intratumoral DHT concentration was significantly higher in breast carcinoma tissues following exemestane treatment ($n=9$) than those without the therapy ($n=7$), and 17 β -hydroxysteroid dehydrogenase type 2 (17 β HSD2) status was significantly altered to be positive after the treatment. Following *in vitro* studies showed that 17 β HSD2 expression was dose dependently induced by both DHT and exemestane in T-47D breast carcinoma cells, but these inductions were not additive. DHT-mediated induction of 17 β HSD2 expression was markedly suppressed by estradiol (E₂) in T-47D cells. E₂-mediated cell proliferation was significantly inhibited by DHT in T-47D cells, associated with an increment of 17 β HSD2 expression level. These findings suggest that intratumoral androgen actions are increased during exemestane treatment. 17 β HSD2 is a potent DHT-induced gene in human breast carcinoma, and may not only be involved in anti-proliferative effects of DHT on breast carcinoma cells but also serve as a potential marker for response to aromatase inhibitor in the breast carcinoma patients.

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Introduction

Breast carcinoma is one of the most common malignancies in women worldwide. Sex steroids play very important roles in the development of estrogen-responsive breast carcinoma, and estrogens contribute immensely to the development of the breast carcinoma through binding with estrogen receptor (ER). Circulating estrogens are mainly secreted from the ovaries in premenopausal women, but a majority of breast

carcinomas arise after menopause when ovaries ceased to be functional. Previous studies have demonstrated that biologically active estrogen, estradiol (E₂), was locally produced by estrogen-producing enzymes, such as aromatase (conversion from circulating androstenedione to estrone (E₁) or testosterone to E₂; Silva *et al.* 1989), steroid sulfatase (STS; hydrolysis of circulating E₁ sulfate to E₁; Evans *et al.* 1994), and 17 β -hydroxysteroid dehydrogenase type 1 (17 β HSD1;

conversion from E₁ to E₂; Speirs *et al.* 1998) in the breast carcinoma.

An intratumoral concentration of bioactive androgen, 5 α -dihydrotestosterone (DHT), was also reported to be significantly higher in the breast carcinoma than in plasma (Mistry *et al.* 1986, Recchione *et al.* 1995, Suzuki *et al.* 2007), and androgen-producing enzymes, e.g. 17 β HSD5 (conversion from circulating androstenedione to testosterone) and 5 α -reductase type 1 (5 α Red1; reduction of testosterone to DHT), were frequently expressed in the breast carcinoma (Suzuki *et al.* 2001). The potency of sex steroids is also regulated by sex steroid-metabolizing enzymes, such as estrogen sulfotransferase (EST; sulfonation of E₁ to E₁ sulfate) and 17 β HSD2 (oxidation of E₂ to E₁ or testosterone to androstenedione; Adams *et al.* 1979, Suzuki *et al.* 2000). However, expression of the sex steroid-metabolizing enzymes was frequently decreased in the breast carcinoma tissues, resulting in an accumulation of intratumoral sex steroids (Suzuki *et al.* 2003).

Among these sex steroid-related enzymes, intratumoral aromatase has been established as an important target for the anti-estrogen therapy in the hormone-dependent breast carcinoma in postmenopausal patients. Third-generation aromatase inhibitors are currently available, and these inhibitors are classified into two types: a steroidal aromatase inhibitor (e.g. exemestane), which interferes with the substrate-binding sites of aromatase as androgen analog; and non-steroidal aromatase inhibitors (e.g. anastrozole and letrozole), which block the electron transfer chain (Miller & Dixon 2002). These aromatase inhibitors were all significantly associated with improved disease-free survival and good tolerability in breast carcinoma patients (Goss *et al.* 2003, Baum 2004, Coombes *et al.* 2004, Howell *et al.* 2005). In addition, neoadjuvant aromatase inhibitor therapy is frequently considered to improve surgical outcomes for the breast carcinoma patients (Olson *et al.* 2009).

Recently, Suzuki *et al.* (2007) have reported that DHT synthesis in aromatase-negative MCF-7 breast carcinoma cells was significantly inhibited by co-culture with aromatase-positive stromal cells isolated from human breast carcinoma tissue, which was also reversed by an addition of steroidal aromatase inhibitor exemestane. These findings suggest that aromatase inhibitor therapy may cause increased androgen actions with estrogen deprivation. However, to the best of our knowledge, intratumoral concentration of androgens and its significance have not been reported in the breast carcinoma with

aromatase inhibitor treatment. Therefore, in this study, we first examined androgen concentrations in nine breast carcinoma tissues with exemestane treatment, and correlated these findings with immunohistochemical status of various sex steroid-related enzymes. These results demonstrated a strong association between exemestane treatment and intratumoral DHT concentration and 17 β HSD2 status. Therefore, we subsequently performed *in vitro* studies to further characterize the significance of 17 β HSD2 in the breast carcinoma.

Materials and methods

Patients and tissues

Two sets of tissue specimens were used in this study. As a first set, nine specimens of ER-positive breast carcinoma were obtained from postmenopausal women who underwent surgical treatment from 2006 to 2007 in Tohoku Kosai Hospital, Sendai, Japan. All the patients received oral exemestane (Aromasin; Pfizer Japan Inc. (Tokyo, Japan)), 25 mg daily for 2 weeks, before the surgery. The median age of these patients was 65 years (range 56–75). Specimens for steroid extraction were snap-frozen and stored at –80 °C until use, and those for immunohistochemistry were fixed with 10% formalin and embedded in paraffin wax. In all cases, the corresponding core needle biopsy (CNB) specimens before the exemestane treatment were available in the formalin-fixed and paraffin-embedded tissues.

As a second set, seven specimens of ER-positive breast carcinoma were also obtained from postmenopausal women who underwent surgical treatment from 2001 to 2002 in the Departments of Surgery at Tohoku University Hospital and in 2004 in the Tohoku Kosai Hospital, Sendai, Japan. These patients did not receive any neoadjuvant therapy including exemestane, and the median age was 57 years (range 50–69). Specimens for steroid extraction were snap-frozen, and those for immunohistochemistry were fixed with 10% formalin and embedded in paraffin wax. The clinicopathological characteristics of the breast carcinomas in these sets were summarized in Table 1. No statistical difference was detected in each parameter listed in Table 1, although these patient groups were treated at different periods of time (data not shown).

Research protocols for this study were approved by the ethics committee at Tohoku University School of Medicine and Tohoku Kosai Hospital.

Table 1 Clinicopathological characteristics of breast carcinomas used in this study

Value	Breast carcinoma treated with exemestane before surgery (n=9)	Breast carcinoma without any neoadjuvant therapy (n=7)
Patient age (years) ^a	65 (56–75)	57 (50–69)
Tumor size (mm) ^a	20 (10–35)	26 (10–50)
Lymph node metastasis		
Positive	1	3
Negative	8	4
Clinical stage		
I	5	2
II	4	5
III	0	0
Histological grade		
1	4	3
2	3	2
3	2	2
ER LI (%) ^a	72 (14–85)	81 (10–96)
PR LI (%) ^a	39 (0–76)	52 (8–75)
AR LI (%) ^a	50 (24–82)	35 (8–53)
HER2 status		
Positive	3	1
Negative	5	6

ER, estrogen receptor; PR, progesterone receptor; AR, androgen receptor; HER2, human epidermal growth factor 2; LI, labeling index.
^aData are represented as median (min–max). Other values are presented as the number of cases.

Liquid chromatography/electrospray tandem mass spectrometry

Concentrations of E₂, DHT, testosterone, and androstenedione were measured by liquid chromatography/electrospray tandem mass spectrometry (LC–MS/MS) analysis (ASKA Pharma Medical Co., Ltd, Kawasaki, Japan), as described previously (Miki *et al.* 2007, Suzuki *et al.* 2007, Yamashita *et al.* 2007, Shibuya *et al.* 2008). Briefly, breast carcinoma specimens (~100 mg for each sample) were homogenized in 1 ml of distilled water, and steroids were extracted with diethyl ether from the homogenate after the addition of 100 pg of E₂-¹³C₄, DHT-d3, testosterone-d3, and androstenedione-d7 as internal standard.

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, Mississauga, Ontario, Canada) operated with electron spray ionization in the positive ion mode, and the chromatographic separation was performed on Cadenza CD-C18 column (3×150 mm, 3.5 mm, Imtakt, Kyoto, Japan). The lower limit of quantification was 0.5 pg for E₂, 0.5 pg for DHT, 1 pg for testosterone, and 1 pg for androstenedione in this study.

Immunohistochemistry

The characteristics of primary antibodies for aromatase (Miki *et al.* 2007), STS (Suzuki *et al.* 2003), 17βHSD5 (Penning *et al.* 2006), 5αRed1 (Suzuki *et al.* 2001), and EST (Suzuki *et al.* 2003) were described previously. Rabbit monoclonal antibody for 17βHSD1 (EP1682Y) and rabbit polyclonal antibody for 17βHSD2 (10978-1-AP) were purchased from Epitomics Inc. (Burlingame, CA, USA) and Proteintech Group Inc. (Chicago, IL, USA) respectively. Monoclonal antibodies for ER (ER1D5), progesterone receptor (PR; MAB429), androgen receptor (AR; AR441), and Ki-67 (MIB1) were purchased from Immunotech (Marseille, France), Chemicon (Temecula, CA, USA), and DAKO (Carpinteria, CA, USA) respectively. Rabbit polyclonal antibody for human epidermal growth factor 2 (HER2; A0485) was obtained from DAKO.

A Histofine Kit (Nichirei, Tokyo, Japan), which employs the streptavidin–biotin amplification method, was used for immunohistochemistry in our study. 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.

Immunoreactivity of sex steroid-related enzymes was detected in the cytoplasm, and cases that had more than 10% of positive carcinoma cells were considered positive (Suzuki *et al.* 2007). Immunoreactivity of ER, 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). Cases with ER LI, PR LI, or AR LI of more than 10% were considered ER-, PR-, or AR-positive breast carcinoma, according to a report by Allred *et al.* (1998). HER2 immunoreactivity was evaluated according to a grading system proposed in HercepTest (DAKO), and moderately or strongly circumscribed membrane staining of HER2 in more than 10% carcinoma cells was considered positive.

Cell line and chemicals

T-47D human breast carcinoma cell line was provided from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan), and cultured in RPMI-1640 (Sigma–Aldrich) with 10% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA). T-47D cells were cultured with phenol red-free RPMI-1640 medium containing 10% dextran-coated charcoal (DCC)–FBS for 3 days before treatment in the experiment. DHT, E₂, and an AR

antagonist hydroxyflutamide were purchased from Wako Pure Chemical Industries (Osaka, Japan), Wako Pure Chemical Industries, and Toronto Research Chemicals (Downsview, Ontario, Canada) respectively. Exemestane was kindly provided from Pfizer Japan Inc.

Real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies Inc.), and cDNA was synthesized using a QuantiTect reverse transcription kit (Qiagen). Real-time PCR was carried out using the LightCycler System and FastStart DNA Master SYBR Green I (Roche Diagnostics). The PCR primer sequence of 17 β HSD2, 5 α Red1, and the ribosomal protein L13A (RPL13A) used in this study was as follows. 17 β HSD2 (NM_002153): forward 5'-CAAAGGGA-GGCTGGTGAA-3' and reverse 5'-TTGAGGACC-TCTGTGTATTT-3'; 5 α Red1 (NM_001047): forward 5'-TGGGAGGAGGAAAGCCTATG-3' and reverse 5'-GCCACACCACTCCATGATTTTC-3'; and RPL13A (NM_012423): forward 5'-CCTGGAGGAGAAGAG-GAAAGAGA-3' and reverse 5'-TTGAGGACCTCTG-TGTATTTGTCAA-3'. PCR products were purified and subjected to direct sequencing in order to verify amplification of the correct sequences. 17 β HSD2 and 5 α Red1 mRNA levels were summarized as the ratio of RPL13A mRNA level (%).

Immunoblotting

The cell protein was extracted using M-PER Mammalian Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL, USA) with Halt Protease Inhibitor Cocktail (Pierce Biotechnology). Twenty micrograms of the protein (whole cell extracts) were subjected to SDS-PAGE (10% acrylamide gel). Following SDS-PAGE, proteins were transferred onto Hybond P polyvinylidene difluoride membrane (GE Healthcare, Buckinghamshire, UK). The primary antibodies used in this study were 17 β HSD2 (10978-1-AP; Proteintech Group, Inc.) and β -actin (AC-15; Sigma-Aldrich). Antibody–protein complexes on the blots were detected using ECL Plus western blotting detection reagents (GE Healthcare), and the protein bands were visualized with LAS-1000 image analyzer (Fuji Photo Film Co., Tokyo, Japan).

Microarray analysis

Whole Human Genome Oligo Microarray (G4112F, ID 012391, Agilent Technologies), containing 41 000 unique probes, was used in this study. Total RNA was extracted from T-47D cells after the treatment of DHT (10 nM), exemestane (100 nM), E₂ (10 nM), or non-treatment for 3 days, and sample preparation and

processing were done according to the manufacturer's protocol. The relative levels of gene expression were calculated by global normalization, and scatter plot analysis of the microarray data was performed using GeneSpring 10.0.2 (Agilent Technologies).

Luciferase assay

The luciferase assay was performed according to a previous report (Sakamoto *et al.* 2002) with some modifications. Briefly, we used androgen-responsive reporter plasmids pPSAE-Luc, which contained KLK3 androgen-responsive element (ARE; kindly provided from ASKA Pharmaceutical Co., Ltd), and estrogen-responsive reporter plasmids ptk-estrogen-responsive element (ERE)-Luc, containing *Xenopus* vitellogenin A2 ERE (Saji *et al.* 2001), in this study. One microgram of pPSAE-Luc plasmids or ptk-ERE-Luc plasmids and 200 ng pRL-TK control plasmids (Promega) were used to measure the transcriptional activity of endogenous AR or ER. Transient transfections were carried out using TransIT-LT Transfection Reagents (TaKaRa, Tokyo, Japan) in T-47D cells, and the luciferase activity of lysates was measured using a Dual-Luciferase Reporter Assay system (Promega) and Luminescencer-PSN (AB-2200; Atto Co., Tokyo, Japan) after incubation with the indicated concentrations of DHT and/or E₂ for 24 h. The transfection efficiency was normalized against *Renilla* luciferase activity using pRL-TK control plasmids, and the luciferase activity for each sample was evaluated as a ratio (%) compared with that of controls.

Cell proliferation assay

T-47D cells were preincubated in phenol red-free RPMI-1640 medium containing 10% DCC-FBS with or without DHT (10 nM) for 3 days, and then seeded in 96-well plates (3000 cells/well). After the treatment with E₂ (100 pM) with or without DHT (10 nM) for 3 days, the status of cell proliferation of T-47D cells was measured using a WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2-H-tetrazolium, monosodium salt) method (Cell Counting Kit-8; Dojindo Inc., Kumamoto, Japan).

Results

Intratumoral concentration of androgens in breast carcinoma tissues treated with exemestane

We first examined tissue concentrations of sex steroids in breast carcinomas treated with exemestane using LC–MS/MS. Intratumoral E₂ concentration was

0.35-fold lower in the breast carcinoma tissues treated with exemestane (the median with min–max was 66 (33–176) pg/g) than those without the therapy (190 (16–534) pg/g), although P value did not reach a significant level ($P=0.56$; Fig. 1A). On the other hand, intratumoral DHT concentration was significantly higher (2.3-fold and $P=0.01$) in the breast carcinomas treated with exemestane (145 (91–4987) pg/g) than those without exemestane therapy (64 (4.6–119) pg/g; Fig. 1B), and the corresponding E₂:DHT ratio in each patient was significantly (0.08-fold and $P=0.01$) lower in a group of patients treated with exemestane therapy (Fig. 1C).

Intratumoral testosterone level was 1.4-fold higher in the group treated with exemestane (180 (110–427) pg/g) than that without exemestane treatment (133 (70–240) pg/g), although P value did not reach a statistical significance ($P=0.10$; data not shown). Intratumoral concentration of androstenedione demonstrated similar levels ($P=0.96$) regardless of the exemestane treatment (485 (153–2597) pg/g with exemestane and 561 (160–5785) pg/g without exemestane; Fig. 1D).

Intratumoral concentration of DHT was significantly associated with that of testosterone in the breast carcinoma ($P=0.02$, $r=0.57$), and DHT:testosterone ratio in each patient was similar regardless of the exemestane therapy ($P=0.28$; data not shown).

Immunolocalization of sex steroid-related enzymes in breast carcinoma tissues treated with exemestane

We then examined an association between intratumoral concentration of E₂ and DHT and immunohistochemical status of sex steroid-related enzymes in nine breast carcinomas treated with exemestane. The immunoreactivity was detected in six cases (67%) for aromatase, six cases (67%) for STS, six cases (67%) for 17 β HSD1, five cases (56%) for 17 β HSD5, five cases (56%) for 5 α Red1, six cases (67%) for EST, and four cases (44%) for 17 β HSD2 respectively. As shown in Table 2, intratumoral E₂ concentration was inversely

Figure 1 Intratumoral concentrations of E₂ (A), DHT (B), and androstenedione (D), and E₂:DHT ratio in each patient (C) in the breast carcinoma with or without exemestane treatment for 2 weeks using LC–MS/MS analysis. The median value is illustrated by a horizontal line in the box pot, and gray box denotes the 75th (upper margin) and 25th percentiles of the values (lower margin) respectively. The upper and lower bars indicate the 90th and 10th percentiles respectively. The statistical analyses were performed using a Mann–Whitney U test. P values <0.05 were considered significant, and are in bold.

Table 2 Association between intratumoral concentration of sex steroids and the status of sex steroid-related enzymes in nine breast carcinomas with exemestane treatment

Enzyme	<i>n</i>	E ₂ concentration (pg/g)	<i>P</i> value	DHT concentration (pg/g)	<i>P</i> value
<i>Estrogen-producing enzyme</i>					
Aromatase					
Positive	6	60 (33–133)	0.44	167 (91–4897)	0.80
Negative	3	66 (59–176)		118 (117–317)	
STS					
Positive	6	57 (33–176)	0.30	117 (91–4897)	0.12
Negative	3	76 (59–133)		317 (190–345)	
17βHSD1					
Positive	6	62 (45–76)	0.44	167 (109–897)	0.44
Negative	3	133 (33–176)		117 (91–345)	
<i>Androgen-producing enzyme</i>					
17βHSD5					
Positive	5	66 (45–76)	> 0.99	118 (109–4897)	0.62
Negative	4	68 (33–133)		253 (91–133)	
5αRed1					
Positive	5	47 (33–73)	0.03	145 (91–4897)	0.81
Negative	4	105 (66–176)		154 (117–345)	
<i>Sex steroid-metabolizing enzyme</i>					
EST					
Positive	6	75 (33–176)	0.30	167 (91–4897)	0.61
Negative	3	59 (45–66)		118 (109–317)	
17βHSD2					
Positive	4	46 (33–59)	0.01	213 (91–4897)	0.81
Negative	5	76 (66–176)		145 (117–345)	

Status of each sex steroid-related enzyme was evaluated by immunohistochemistry. Data are presented as the median with min–max. The statistical analyses were performed using a Mann–Whitney *U* test. *P* values <0.05 were considered significant, and described as boldface.

associated with the status of 5αRed1 (*P*=0.03) and 17βHSD2 immunoreactivity (*P*=0.01), while no significant association was detected between intratumoral DHT concentration and any of sex steroid-related enzyme immunoreactivity status in our present study. All four cases positive for 17βHSD2 were also positive for 5αRed1, and a significant positive association (*P*=0.047) was detected between 17βHSD2 and 5αRed1 status determined by immunohistochemistry.

We also evaluated an immunohistochemical status of these enzymes in the corresponding nine CNB specimens obtained before exemestane therapy. As shown in Table 3, 17βHSD2 status became increased (*P*=0.046) after the exemestane treatment (Fig. 2), but no significant association was detected in the other six sex steroid-related enzymes examined in this study.

Ki-67 LI in carcinoma cells was significantly (*P*=0.01) decreased after the exemestane therapy (19 (7–35) % before the therapy and 10 (2–23) % after the therapy), as previously reported in exemestane (Miller & Dixon 2002), anastrozole (Dowsett et al.

2005b), and letrozole (Ellis et al. 2003) neoadjuvant therapy treatment. PR LI was also significantly (*P*=0.046) decreased after the exemestane therapy (39 (0–76) % before the therapy and 20 (0–67) % after the therapy), as previously reported in the anastrozole treatment (Dowsett et al. 2005a). On the other hand, ER LI, AR LI, and HER2 status were not significantly different in each case between before and after the exemestane therapy in this study (*P*=0.42, *P*=0.16, and *P*=0.56 respectively).

17βHSD2 as a DHT-induced gene in breast carcinoma cells

In our LC–MS/MS analysis in human breast carcinoma tissues, intratumoral DHT concentration was significantly higher in the breast carcinoma treated with exemestane (Fig. 1B), and 17βHSD2 status significantly increased after the treatment (Table 3). These results suggest an induction of 17βHSD2 by DHT and/or exemestane in the breast carcinoma cells, but such findings have not been reported yet to the best of

Table 3 Association of sex steroid-related enzyme status in nine paired breast carcinoma tissues obtained before and after the exemestane treatment

Enzyme	Before the treatment (CNB specimens)	After the treatment (surgical specimens)	P value
<i>Estrogen-producing enzyme</i>			
Aromatase			
Positive	6	6	
Negative	3	3	>0.99
STS			
Positive	6	6	
Negative	3	3	0.56
17 β HSD1			
Positive	5	6	
Negative	4	3	>0.99
<i>Androgen-producing enzyme</i>			
17 β HSD5			
Positive	3	5	
Negative	6	4	0.16
5 α Red1			
Positive	5	5	
Negative	4	4	>0.99
<i>Sex steroid-metabolizing enzyme</i>			
EST			
Positive	5	6	
Negative	4	3	0.56
17 β HSD2			
Positive	0	4	
Negative	9	5	0.046

Status of each steroid-related enzyme was evaluated by immunohistochemistry. Data are presented as the number of cases. The statistical analyses were performed using a Wilcoxon signed rank test. *P* values <0.05 were considered significant, and described as boldface.

our knowledge. Therefore, we used T-47D breast carcinoma cells, which expressed both ER and AR, to further analyze this aspect (Migliaccio *et al.* 2000).

As shown in the upper panel of Fig. 3A, expression level of 17 β HSD2 mRNA was increased by DHT in a dose-dependent manner in T-47D cells, and this increment became significant from 100 pM ($P<0.001$) compared to the basal level (non-treatment). DHT did not significantly change the 17 β HSD2 mRNA expression level when the cells were treated together with DHT and a potent AR antagonist hydroxyflutamide (Lee *et al.* 2002; $P=0.07$; Fig. 3A). Hydroxyflutamide alone did not significantly ($P=0.32$) change the 17 β HSD2 mRNA level in T-47D cells (data not shown). DHT-mediated induction of 17 β HSD2 expression was also confirmed at protein levels by immunoblotting in T-47D cells treated under the same condition (lower panels in Fig. 3A). Induction of

17 β HSD2 mRNA expression by DHT occurred in a time-dependent manner, and when T-47D cells were treated with 10 nM DHT, it became significant ($P=0.01$) from 24 h after the treatment (data not shown). On the other hand, DHT treatment (10 nM for 3 days) did not significantly (1.0-fold and $P=0.83$) change the 5 α Red1 mRNA level in T-47D cells, although our immunohistochemical results showed a positive association between 5 α Red1 and 17 β HSD2 status in breast carcinomas treated with exemestane.

Exemestane also induced 17 β HSD2 mRNA expression in T-47D cells in a dose-dependent manner at a significant level from 1 nM of exemestane ($P<0.05$ versus the non-treatment; Fig. 3B). Exemestane did not significantly alter the 17 β HSD2 mRNA expression level when the T-47D cells were treated together with hydroxyflutamide ($P=0.48$). A similar tendency was confirmed at protein levels by immunoblotting (lower panels in Fig. 3B). However, 17 β HSD2

Figure 2 Immunohistochemistry for 17 β HSD2 in the breast carcinoma tissue before (A) and after (B) the exemestane therapy in the same case. 17 β HSD2 immunoreactivity was negative in (A), but was detected in the cytoplasm of breast carcinoma cells in (B). Bar=100 μ m respectively.

mRNA expression was not significantly (1.1-fold and $P=0.46$) changed in T-47D cells treated with non-steroidal aromatase inhibitor letrozole (100 nM) for 3 days (data not shown). The exemestane-mediated induction of 17βHSD2 mRNA was not detected in T-47D cells treated with 10 nM DHT ($P=0.15$ between DHT (10 nM) alone versus DHT (10 nM) with exemestane (100 nM); Fig. 3C).

We further examined effects of DHT and exemestane on gene expressions in T-47D cells using microarray analysis. After the treatment with DHT (10 nM) or exemestane (100 nM) for 3 days, genes which demonstrated more than 2.5-fold increase compared to the basal level (non-treatment) were evaluated as 'an induced gene' in this study (Kannan et al. 2001). The number of DHT-induced genes identified was 337, while that of exemestane-induced genes was 308 (Table 4). Among these genes, 160 DHT-induced genes and 159 exemestane-induced genes were present in the gene ontology (GO) depth of 4 by FatiGO analysis (<http://babelomics.bioinfo.cipf.es/EntryPoint?loadForm=fatigo>), and these were frequently associated with 'metabolic process' (Table 4). The number of genes induced by DHT and/or exemestane was 477 in total in this study, and we subsequently compared these gene expression profiles by a scatter plot. As shown in Fig. 3D, 48 genes (10%) were predominantly (more than 2.0-fold) induced by DHT (group A), while 53

Figure 3 Induction of 17βHSD2 expression by DHT or exemestane in T-47D breast carcinoma cells. (A and B) Effects of DHT (A) or exemestane (B) on 17βHSD2 mRNA expression demonstrated by real-time PCR analysis. T-47D cells were treated with indicated concentrations of DHT (A) or exemestane (B) with or without an AR blocker hydroxyflutamide (10 μM) for 3 days. 17βHSD2 mRNA was evaluated as the ratio of *RPL13A* mRNA level, and subsequently relative 17βHSD2 mRNA level was summarized as a ratio (%) compared with the basal level (non-treatment). Data are presented as mean ± s.d. ($n=3$). * $P<0.05$ and *** $P<0.001$ versus non-treatment (left column). The statistical analyses were performed using a one-way ANOVA and Bonferroni test. The induction of 17βHSD2 expression was confirmed by immunoblotting under the same condition (lower panels). Immunoblotting for β-actin was performed as an internal standard of the experiment. (C) Effects of exemestane on DHT-mediated 17βHSD2 mRNA by real-time PCR analysis. T-47D cells were treated with indicated concentrations of exemestane with DHT (10 nM) for 3 days. Relative 17βHSD2 mRNA level was summarized as a ratio (%) compared with the non-treatment. Data are presented as mean ± s.d. ($n=3$). (D) Scatter plot analysis of microarray data for the induced gene expression profile by DHT or exemestane. Four hundred and seventy-seven genes, those that were more than 2.5-fold induced by DHT (10 nM) or exemestane (100 nM) treatment for 3 days, were plotted on the logarithmic graph. Genes, those that were more than 2.0-fold higher in the DHT or exemestane treatment, were located outside of the diagonal line, and classified as group A or group B respectively. Genes <2.0-fold changes were plotted inside of these two lines, and classified as group C. The location of 17βHSD2 was marked in this figure.

Table 4 Representative genes up-regulated by DHT or exemestane in T-47D cells and corresponding GO terms at level 4

Induced by DHT			Induced by exemestane		
Fold	Common name	Gene symbol	Fold	Common name	Gene symbol
(A) Representative genes up-regulated by DHT or exemestane in T-47D cells by microarray analysis					
24.4	NM_023938	C1orf116	16.5	AK127378	AK127378
23.7	BQ706262	BQ706262	16.1	NM_002776	KLK10
18.9	NM_002776	KLK10	15.5	BQ706262	BQ706262
18.5	NM_001185	AZGP1	14.6	NM_001185	AZGP1
16.6	AK127378	AK127378	13.5	NM_023938	C1orf116
14.4	NM_145000	RANBP3L	13.3	DT220604	DT220604
12.4	NM_199328	CLDN8	12.0	NM_005253	FOSL2
11.4	DT220604	DT220604	10.4	NM_145000	RANBP3L
11.2	NM_006006	ZBTB16	9.1	NM_033226	ABCC12
10.7	XR_017216	LOC646282	9.1	NM_001880	ATF2
10.4	NM_004925	AQP3	8.4	NM_002867	RAB3B
10.1	NM_005627	SGK	7.6	NM_199328	CLDN8
10.0	NM_002867	RAB3B	7.3	XR_017216	LOC646282
8.7	NM_033226	ABCC12	7.1	NM_006006	ZBTB16
8.6	NM_001880	ATF2	6.7	NM_012429	SEC14L2
8.2	NM_024508	BED2	6.6	NM_144682	SLFN13
8.0	NM_012429	SEC14L2	6.2	THC2688670	THC2688670
8.0	NM_000063	C2	6.2	A_24_P558141	A_24_P558141
7.8	BC002830	ATG3	6.0	THC2611204	THC2611204
7.8	NM_013989	DIO2	6.0	NM_002153	HSD17B2
7.5	NM_002272	KRT4	5.9	NM_004925	AQP3
6.8	NM_144682	SLFN13	5.8	NM_005627	SGK
6.7	BC043381	GJE1	5.7	NM_003714	STC2
6.6	NM_002153	HSD17B2	5.5	BC043381	GJE1
6.4	NM_001030059	PPAPDC1A	5.4	NM_002272	KRT4
6.2	A_24_P290087	A_24_P290087	5.4	NM_024508	ZBED2
6.0	NM_014141	CNTNAP2	5.2	THC2677796	THC2677796
5.9	NM_024307	GDPD3	5.2	NM_014141	CNTNAP2
5.7	NM_007253	CYP4F8	5.1	NM_198794	MAP4K5
5.5	NM_005980	S100P	5.0	BC039117	OVOS2

GO ID	Term	Number of genes
(B) Representative GO terms at depth 4 in the DHT- or exemestane-induced genes		
DHT-induced genes		
GO:0007165	Signal transduction	43
GO:0050794	Regulation of cellular process	40
GO:0043283	Biopolymer metabolic process	40
GO:0019538	Protein metabolic process	32
GO:0044260	Cellular macromolecule metabolic process	32
GO:0006810	Transport	28
GO:0019222	Regulation of metabolic process	24
GO:0006139	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	24
GO:0048731	System development	17
GO:0048518	Positive regulation of biological process	16
Exemestane-induced genes		
GO:0050794	Regulation of cellular process	40
GO:0043283	Biopolymer metabolic process	39
GO:0007165	Signal transduction	35
GO:0019538	Protein metabolic process	32
GO:0006810	Transport	32

Table 4 continued

GO ID	Term	Number of genes
GO:0044260	Cellular macromolecule metabolic process	31
GO:0019222	Regulation of metabolic process	25
GO:0006139	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	25
GO:0030154	Cell differentiation	25
GO:0048731	System development	21

(A) Top 30 genes were listed according to the fold change in each group. Genes that were induced by both DHT and exemestane are in bold. (B) Depth of GO terms was classified by FatiGO analysis (<http://babelomics.bioinfo.cipf.es/EntryPoint?loadForm=fatigo>), and top ten GO terms at depth 4 are listed according to the number of genes belonged. The GO terms listed in both DHT- and exemestane-induced genes are in bold.

genes (11%) were predominantly induced by exemestane (group B). However, a great majority (376 genes; 79%) of the genes were induced by DHT or exemestane (group C) in a similar manner, and *17βHSD2* was classified in this group.

Suppression of 17βHSD2 expression by E₂ in breast carcinoma cells

We have demonstrated that expression of *17βHSD2* was induced by DHT in the breast carcinoma cells. However, it is also true that *17βHSD2* expression was almost negligible in the breast carcinoma, although intratumoral DHT concentration was at a significant level (Recchione et al. 1995, Suzuki et al. 2000). In order to further explore these inconsistent findings, we examined effects of estrogens on *17βHSD2* expression in T-47D cells.

When T-47D cells were transiently transfected with pPSAE-Luc plasmids and treated with 10 nM DHT, the luciferase activity of the cells was significantly (7.4-fold and *P*<0.001) increased compared to the basal level (non-treatment; Fig. 4A). E₂ inhibited ARE-dependent transactivation by DHT in a dose-dependent manner, and the luciferase activities of T-47D cells treated with 10 nM DHT and 10 nM E₂ were significantly decreased to 0.35-fold of that in cells treated with 10 nM DHT alone (*P*<0.001). E₂ alone (10 nM) did not significantly alter the luciferase activity (*P*=0.58). DHT (10 nM), however, did not significantly affect the ERE-dependent transactivation at both 10 pM and 10 nM E₂ treatment (Fig. 4B). DHT-mediated induction of *17βHSD2* mRNA was significantly inhibited by E₂ in a dose-dependent manner (Fig. 4C). *17βHSD2* mRNA level in T-47D cells treated with both 10 nM DHT and 1 nM E₂ was decreased to 0.25-fold of that in cells treated with 10 nM DHT alone (*P*<0.001), and it was a similar level compared to the basal level (non-treatment with DHT; *P*=0.32; Fig. 4C).

In the microarray analysis in T-47D cells, we identified 810 genes as E₂-induced genes (treatment with 10 nM of E₂ for 3 days). The number of DHT- and/or E₂-induced genes was 1029 in total. As shown in Fig. 4D, 144 genes (14%) were predominantly induced by DHT (group D), 412 genes (40%) were predominantly induced by E₂ (group E), and 473 genes (46%) were induced by DHT or E₂ in a similar manner (group F). The 144 genes in group D were all DHT-induced genes but not E₂-induced genes, and *17βHSD2* was classified in this group.

When T-47D cells were treated with E₂ (100 pM) for 3 days, the number of cells increased 1.4-fold in this study (Fig. 4E). The E₂-mediated cell proliferation was significantly (0.87-fold and *P*<0.05) inhibited by the addition of DHT (10 nM), which was enhanced (0.76-fold and *P*<0.01) by pretreatment with DHT (10 nM) for 3 days before the treatment with E₂ and DHT. However, under the same conditions of the experiment, expression level of *17βHSD2* mRNA was significantly higher (1.7-fold and *P*<0.001) following the treatment with E₂ and DHT, and the highest (2.0-fold and *P*<0.001) by the pretreatment with DHT, compared to the cells treated with E₂ alone. The cell proliferation activity was not significantly altered (*P*=0.48) in T-47D cells between the treatment with DHT (10 nM) alone and non-treatment (data not shown).

Discussion

This is the first report to evaluate intratumoral androgen concentrations in the breast carcinoma treated with aromatase inhibitor. In our present study, intratumoral DHT concentrations were significantly (2.3-fold) higher in the breast carcinomas treated with exemestane than those not treated with exemestane (Fig. 1B). Our present results also demonstrated that E₂:DHT ratio in each patient was significantly (0.08-fold) lower in the breast carcinomas treated with exemestane than those with non-treated cases

(Fig. 1C), suggesting that DHT level is relatively higher than E₂ level in each breast carcinoma tissue treated with exemestane. Moreover, intratumoral concentration of DHT was associated with that of testosterone in this study. DHT is locally produced from testosterone in human breast carcinoma tissues (Suzuki *et al.* 2007), and the intratumoral DHT level is considered to be mainly determined by amounts of the precursor testosterone (Mistry *et al.* 1986, Recchione *et al.* 1995). Aromatase catalyzes the conversion of androstenedione and testosterone to E₁ and E₂ respectively. Previously, Spinola *et al.* (1988) 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 & Lykkesfeldt (2005) showed that aromatase preferred testosterone as a substrate in MCF-7 breast carcinoma cells. Recently, Suzuki *et al.* (2007) have demonstrated that aromatase expression was inversely associated with intratumoral DHT concentrations in the breast carcinomas without neoadjuvant therapy, and aromatase suppressed DHT synthesis from androstenedione in the co-culture

Figure 4 Interaction of ER and AR functions in T-47D cells. (A and B) Effect of E₂ on DHT-dependent transactivation (A) or that of DHT on E₂-dependent transactivation (B) by luciferase analysis. T-47D cells were treated with indicated concentrations of DHT and E₂ for 24 h. Relative luciferase activity was evaluated as the ratio (%) compared with the basal level (non-treatment). Data are presented as mean ± s.d. (n=3). *P<0.05 and ***P<0.001 versus non-treatment (left column) respectively. The statistical analyses were performed using a one-way ANOVA and Bonferroni test. (C) Effects of E₂ on DHT-mediated 17βHSD2 mRNA expression by real-time PCR analysis. T-47D cells were treated with DHT (10 nM) and indicated concentrations of E₂ for 3 days. Relative 17βHSD2 mRNA level was summarized as a ratio (%) compared with the basal level (non-treatment). Data are presented as mean ± s.d. (n=3). **P<0.01 and ***P<0.001 versus treatment with DHT alone (left column). (D) Scatter plot analysis of microarray data for the induced gene expression profile by DHT or E₂. One thousand and twenty-nine genes, those that were more than 2.5-fold induced by DHT (10 nM) or E₂ (10 nM) treatment for 3 days, were plotted on the logarithmic graph. Genes, those that were more than 2.0-fold higher in the DHT or E₂ treatment, were located outside of the diagonal line, and classified as group D or group E respectively. Genes <2.0-fold changes were plotted inside of these two lines, and classified as group F. The location of 17βHSD2 was marked in this figure. (E) Effects of DHT on E₂-mediated proliferation of T-47D cells by proliferation assay. T-47D cells were pretreated with or without DHT (10 nM) for 3 days, and then treated with E₂ (100 pM) with or without DHT (10 nM) for 3 days. The number of the cells was evaluated as a ratio (%) compared with that at day 0 after the treatment. The data are presented as gray bars and as mean ± s.d. (n=3). Expression levels of 17βHSD2 mRNA were also evaluated by real-time PCR analysis under the same condition, and represented as closed bars and as mean ± s.d. (n=3). *P<0.05, **P<0.01, and ***P<0.001 versus treatment with E₂ alone (left column) respectively.

experiments. Results of these studies all indicate that aromatase is a negative regulator of intratumoral DHT production in the breast carcinoma by mainly reducing concentration of the precursor testosterone. On the other hand, our present results showed that DHT:testosterone ratio in each patient, which suggests 5α Red activity, was at a similar level regardless of the exemestane therapy, and DHT did not change the 5α Red1 mRNA level in T-47D cells. Therefore, neoadjuvant aromatase inhibitor therapy is considered to accompany additional effects through increasing local DHT concentration mainly by the inhibition of aromatase activity with estrogen deprivation.

Previous studies demonstrated that intratumoral E_2 concentration was markedly suppressed in breast carcinoma tissues treated with non-steroidal aromatase inhibitors, such as anastrozole (89% suppression for 15 weeks (Geisler *et al.* 2001)) and letrozole (98% for 16 weeks (Geisler *et al.* 2006)). In our present study, intratumoral concentration of E_2 in a group who received exemestane treatment for 2 weeks was 35% of that in a group without this mode of therapy (Fig. 1A). Although no data are currently available on the influence of steroidal aromatase inhibitor in intratumoral concentrations of E_2 to the best of our knowledge, results of our present study are in good agreement with the previous results of non-steroidal aromatase inhibitors. These results suggest that intratumoral E_2 concentration is deprived in breast carcinoma tissues by aromatase inhibitors regardless of the types of inhibitors used. However, it is also true that change of the E_2 concentration was not significant ($P=0.56$) in this study, different from the DHT concentration ($P=0.01$). It may be partly due to the fact that two separate sets of patients who were treated at two periods of times were used. However, considering that E_2 is locally produced in the breast carcinoma tissue by several estrogen-producing enzymes such as aromatase, STS, and 17β HSD1, while DHT is synthesized by 5α Red1, it may be possible to speculate that STS and/or 17β HSD1 interrupt the rapid decrement of E_2 level in the breast carcinoma tissue treated with exemestane. It awaits further examinations.

In our present study, we demonstrated that 17β HSD2 was induced by DHT in T-47D breast carcinoma cells, which was significantly inhibited by the addition of a potent AR blocker hydroxyflutamide (Fig. 3A). Several potential AREs were identified in the upstream region from -5 to -7 kbp of 17β HSD2 gene using Transcription Element Search System (<http://www.cbil.upenn.edu/cgi-bin/tess/tess>), and Wang & Tuohimaa (2007) reported an induction of

17β HSD2 mRNA expression by DHT in a prostate cancer cell line (LNCaP). Therefore, 17β HSD2 is considered a DHT-induced gene in the breast carcinoma cells, although androgen-responsive genes are not currently characterized in the breast carcinoma, in contrast to the estrogen-induced genes. In our present study, exemestane directly caused androgen actions as a chemical in T-47D cells, including induction of 17β HSD2 expression, and a great majority of exemestane-induced genes were overlapped with the DHT-induced genes (Fig. 3B and D; Table 4). Such findings have not been reported to the best of our knowledge, but these are considered reasonable because a steroidal aromatase inhibitor exemestane interferes with the substrate-binding sites of aromatase as an androgen analog (Miller & Dixon 2002). Considering the fact that expression of 17β HSD2 mRNA was not additively induced by DHT and exemestane (Fig. 3C), induction of 17β HSD2 mRNA by these agents may be directly mediated by the same mechanisms through AR as an androgen or androgen analog. Therefore, 17β HSD2 expression might be induced in a similar manner by other non-steroidal aromatase inhibitors through increasing the local DHT levels, but it awaits further investigations for clarification.

Previous *in vitro* studies demonstrated that DHT predominantly exerted anti-proliferative effects on mitogenic effects of estrogens in breast carcinoma cells (Poulin *et al.* 1988, Lapointe & Labrie 2001), although some divergent or inconsistent findings have been reported in the literature (Ortmann *et al.* 2002, Somboonporn & Davis 2004). This inhibitory effect was associated with an increment of a proportion of cells in G_0/G_1 phase or increased levels of p21 and/or p27 (Lapointe & Labrie 2001, Greeve *et al.* 2004). In our present study, E_2 -mediated proliferation of T-47D cells was significantly inhibited by DHT, which was also associated with an increment of 17β HSD2 expression level (Fig. 4E). 17β HSD2 catalyzes the oxidation of E_2 to E_1 (Wu *et al.* 1993), and intratumoral E_2 concentration was inversely associated with 17β HSD2 status in the breast carcinoma with exemestane in our present study (Table 2). Therefore, DHT is considered to inhibit an E_2 -mediated proliferation of breast carcinoma cells, at least in part, through decreasing local E_2 concentration by 17β HSD2.

It is known that oxidative 17β HSD2 activity is a preferential direction in normal breast tissues (Miettinen *et al.* 1999), but the reductive 17β HSD1 pathway has been reported to be dominant in actual human breast carcinoma tissues (Speirs *et al.* 1998, Miettinen *et al.* 1999). We previously reported

Figure 5 Scheme representing possible effects of aromatase inhibitor treatment on androgens in the breast carcinoma tissue, which is postulated from the results of our present study. DHT is locally produced in the breast carcinoma tissue, but its actions are possibly suppressed by predominant estrogen actions. The DHT level is increased in the breast carcinoma tissue by aromatase inhibitor treatment, causing induction of various DHT-induced genes. *17βHSD2* is identified as a DHT-induced gene in the breast carcinoma in this study, and it may be, at least in part, involved in the anti-proliferative effects of DHT by further decreasing intratumoral *E₂* concentration.

no *17βHSD2* immunoreactivity in 111 breast carcinoma tissues examined (Suzuki *et al.* 2000), and Gunnarsson *et al.* (2001) also reported that *17βHSD2* mRNA expression was detected only in 12 out of 84 (14%) breast carcinomas. Recently, Han *et al.* (2008) have demonstrated that *17βHSD2* immunoreactivity was detected in 10 out of 50 (20%) breast carcinomas, while its level of expression was 83% of the adjacent non-neoplastic mammary tissues. Although it might be partly due to the loss of heterozygosity of chromosome 16 in which *17βHSD2* gene is located (Casey *et al.* 1994, Cleton-Jansen *et al.* 2001), it is unclear why *17βHSD2* expression was so suppressed in human breast carcinoma.

Results of our present study demonstrated that ARE-dependent transactivation by DHT was markedly

suppressed by *E₂* in T-47D cells (Fig. 4A), and DHT-mediated induction of *17βHSD2* expression was also inhibited by *E₂* in a dose-dependent manner (Fig. 4C). Possible interaction of ER and AR functions was proposed by several groups. For instances, Panet-Raymond *et al.* (2000) reported that coexpression of ER with AR decreased AR transactivation by 35%, and demonstrated that both AR and ER can interact directly using the yeast and mammalian two-hybrid systems. In addition, Lanzino *et al.* (2005) showed that an AR-specific coactivator ARA70 also increased the ER transcriptional activity and modulated the functional ER/AR interplay in MCF-7 breast carcinoma cells. These results suggest that androgen actions are, in general, suppressed in breast carcinoma by predominant estrogen actions, even if the carcinoma cells expressed AR and intratumoral DHT reached a significant level. In addition, expression of an androgen-induced gene *17βHSD2* may reflect intratumoral DHT actions in breast carcinoma more precisely than AR status. Gunnarsson *et al.* (2005) reported a significant association between *17βHSD2* mRNA and better recurrence-free survival in the breast carcinoma. Therefore, *17βHSD2*-positive breast carcinoma after a neoadjuvant aromatase inhibitor therapy possibly may grow more slowly by increased intratumoral DHT actions and/or further decreased estrogen actions by *17βHSD2* (Fig. 5). Thus, *17βHSD2* status may be a potent marker for response to neoadjuvant aromatase inhibitor therapy in the breast carcinoma, but it awaits further examinations to clarify the clinical significance of *17βHSD2* in the breast carcinoma.

In summary, intratumoral DHT concentration was significantly higher in the breast carcinomas treated with exemestane compared to those without the therapy, and *17βHSD2* immunoreactivity was significantly increased by the treatment. Subsequent *in vitro* studies demonstrated that *17βHSD2* expression was induced by DHT in T-47D breast carcinoma cells in a dose-dependent manner, but the DHT-mediated induction was markedly suppressed by the addition of *E₂*. *E₂*-mediated cell proliferation was significantly inhibited by DHT in T-47D cells, which was also associated with an increment of the *17βHSD2* expression level. These results suggest that intratumoral DHT actions are increased during a neoadjuvant aromatase inhibitor therapy. *17βHSD2* is identified as a potent DHT-induced gene in the breast carcinoma, and may be not only involved in the anti-proliferative effects of DHT on the breast carcinoma cells but also serve as a potential marker for response to a neoadjuvant aromatase inhibitor therapy in the breast carcinoma patient.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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