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PRECLINICAL STUDY

Possible role of the aromatase-independent steroid metabolism pathways in hormone responsive primary breast cancers

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Abstract Aromatase inhibitors (AIs) exert antiproliferative effects by reducing local estrogen production from androgens in postmenopausal women with hormone-responsive breast cancer. Previous reports have shown that androgen metabolites generated by the aromatase-independent enzymes, 5α-androstane-3β, 17β-diol (3β-diol), androst-5-ene-3β, and 17β-diol (A-diol), also activate estrogen receptor (ER) α. Estradiol (E2) can also reportedly be generated from estrone sulfate (E1S) pooled in the plasma. Estrogenic steroid-producing aromatase-independent pathways have thus been proposed as a mechanism of AI resistance. However, it is unclear whether these pathways are functional in clinical breast cancer. To investigate this issue, we assessed the transcriptional activities of ER in 45 ER-positive human breast cancers using the adenovirus estrogen-response element-green fluorescent protein assay and mRNA expression levels of the ER target gene, progesterone receptor, as indicators of ex vivo and in vivo ER activity, respectively. We also determined mRNA expression levels of 5α-reductase type 1 (SRD5A1) and 3β-hydroxysteroid dehydrogenase type 1 (3β-HSD type 1; HSD3B1), which produce 3β-diol from androgens, and of steroid sulfatase (STS) and 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD type 1; HSD17B1), which produce E2 or A-diol from E1S or dehydroepiandrosterone sulfate. SRD5A1 and HSD3B1 expression levels were positively correlated with ex vivo and in vivo ER activities. STS and HSD17B1 expression levels were positively correlated with in vivo ER activity alone. Elevated expression levels of these steroid-metabolizing enzymes in association with high in vivo ER activity were particularly notable in postmenopausal patients. Analysis of the expression levels of steroid-metabolizing enzymes revealed positive correlations between SRD5A1 and HSD3B1, and STS and HSD17B1. These findings suggest that the SRD5A1-HSD3B1 as well as the STS-HSD17B pathways, could contributes to ER activation, especially postmenopause. These pathways might function as an alternative estrogenic steroid-producing, aromatase-independent pathways.

Keywords Breast cancer · Aromatase-inhibitor resistance · SRD5A1 · HSD3B1 · STS · HSD17B1

Abbreviations

AIs	Aromatase inhibitors
3β-diol	5α-Androstane-3β, 17β-diol

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A-diol	Androst-5-ene-3β, 17β-diol
ER	Estrogen receptor α
E2	Estradiol
E1S	Estrone sulfate
SRD5A1	5α-Reductase type 1 mRNA
3β-HSD type 1	3β-Hydroxysteroid dehydrogenase type 1 protein
HSD3B1	3β-Hydroxysteroid dehydrogenase type 1 mRNA
STS	Steroid sulfatase protein
STS	Steroid sulfatase mRNA
17β-HSD type 1	17β-Hydroxysteroid dehydrogenase type 1 protein
HSD17B1	17β-Hydroxysteroid dehydrogenase type 1 mRNA
DHEAS	Dehydroepiandrosterone sulfate
DHT	Dihydrotestosterone
TS	Testosterone
DHEA	Dehydroepiandrosterone
IHC	Immunohistochemical
FISH	Fluorescence in situ hybridization
GFP	Green fluorescent protein
Ds-Red	Fluorescent protein from *Discosoma*
FFPE	Formalin-fixed paraffin-embedded
E1	Estrone

Introduction

Estrogens are produced locally from circulating inactive steroids and play pivotal roles in the proliferation, and development of hormone-dependent breast cancer in postmenopausal women [1, 2]. In particular, aromatase plays a critical, rate-limiting step in intra-tumoral estrogen production in breast cancer [3–6]. Aromatase inhibitors (AIs) impair the growth of estrogen-dependent tumors by blocking the aromatase-mediated conversion of adrenal androgens to estrogens [4, 6]. The initial use of AIs provides substantial clinical benefit compared with the estrogen receptor antagonist tamoxifen, and they are now a standard treatment for postmenopausal breast cancer patients [6–8]. Nevertheless, approximately one-sixth of patients relapse because of primary or acquired resistance [6, 7]. Several hypotheses have been proposed to explain the mechanisms underlying AI resistance, including the activation of growth signaling pathways independent of estrogen and estrogen receptors (ERs) [9], constitutive ER activation caused by growth factor receptor pathways [10–12], and aromatase-independent sources of estrogenic steroids [13–16].

Previous studies showed that the androgen metabolites 3β-diol and A-diol had substantial binding affinity for ER [17, 18], and were able to induce ER activation and growth [14, 19, 20] in MCF-7 breast cancer cells. The estrogen estradiol (E2) has also been shown to be generated from estrone sulfate (E1S) [1, 15, 19, 21], which is a biologically inactive form of estrogen. Moreover, intratumoral dihydrotestosterone (DHT) concentrations were significantly increased following AI treatment [22]. DHT is generated from its precursor testosterone by 5α-reductase, and further metabolized to 3β-diol by 3β-HSDs [14, 22–25]. E1S is a major circulating form of plasma estrogens in postmenopausal women, while dehydroepiandrosterone sulfate (DHEAS) is a major circulating form of plasma adrenal androgens [21, 26]. E1S and DHEAS are metabolized by steroid sulfatase (STS) to estrone (E1) and DHEA, respectively, and further metabolized to E2 or A-diol by 17β-HSD type 1 [2, 21, 27]. These aromatase-independent pathways responsible for the production of estrogenic steroids have been proposed to mediate AI therapy resistance [13–16]. In support of this hypothesis, we recently observed the increased expression of HSD3B1 in MCF-7 cell-derived AI-resistant models [28]. However, direct evidence for the involvement of these steroid-metabolism pathway in the regulation of ER activity through the production of estrogenic steroids is lacking.

To investigate this question, we assessed the transcriptional activities of ER in 45 ER-positive human breast cancers using the adenovirus estrogen-response element-green fluorescent protein (ERE--GFP) assay [29–31] and mRNA expression levels of the ER target gene, and progesterone receptor (PgR) [32], as the indicators of ex vivo and in vivo ER activity, respectively. In addition, mRNA expression levels of the nonaromatase steroid-metabolizing enzymes, SRD5A1, HSD3B1, STS, and HSD17B1, were assessed and compared with the estimated ER activity. We suggest that these enzymes could function in pathways responsible for the production of estrogenic steroids in an aromatase-independent manner, and thus could contribute to ER activation, especially in postmenopausal breast cancer.

Materials and methods

Tumor samples

All human breast cancer tissues were obtained after surgery at the Saitama Cancer Center Hospital (Saitama, Japan) after patient consent and with approval from the Saitama Cancer Center Ethics Committee. Tumor samples were obtained from 45 patients with untreated ER-positive breast cancer who had undergone surgery between 2005 and 2007. Clinicopathological data, including age, menopausal status and serum E2 concentrations, and

Table 1 Clinical and histopathological characteristics of 45 ER-positive breast cancer patients

	No. of patients (%)
Age (mean ± SD)	52.0 ± 12.2
Menopausal status	
Premenopausal	23 (51.1)
Postmenopausal	22 (48.9)
Invasive tumor size (mm: mean ± SD)	22.3 ± 13.6
Histological type	
IDC[a]	42 (93.3)
DCIS[b]	1 (2.2)
Others	2 (4.4)
Nuclear grade	
1	7 (15.6)
2	9 (20.0)
3	24 (53.3)
Unknown	5 (11.1)
Node status	
Positive	22 (48.9)
Negative	23 (51.1)
PgR status	
Positive	39 (86.7)
Negative	6 (13.3)
Lymphatic involvement	
Positive	25 (55.6)
Negative	20 (44.4)
HER2 over-expression	
Positive	4 (8.9)
Negative	30 (66.7)
Unknown	11 (24.4)

[a] Invasive ductal carcinoma

[b] Ductal carcinoma in situ

histopathological data were collected by reviewing patients' case records. At the time of surgery, patients who had not menstruated for more than 1 year were defined as postmenopausal. Other patients were defined as premenopausal. Serum E2 concentrations were measured with the chemiluminescence immunoassay (BML Inc., Tokyo, Japan) using blood samples collected before surgery, without consideration of menstrual cycle status. ER, PgR, and HER2 statuses were evaluated by immunohistochemical (IHC) staining. The cut-off value for ER and PgR positivity was set at ≥1 % [33]. Tumors were considered to overexpress HER2 if they were given a score of 3 during IHC staining, or if they showed >2.2-fold amplification of the HER2 gene, as assessed by fluorescence in situ hybridization (FISH) [34]. FISH testing was only carried out for tumors that scored 2 during IHC staining [34]. Patient characteristics are listed in Table 1.

Isolation of primary tumor cells from breast cancer tissue

The isolation of breast tumor cells was performed as described previously [29–31]. In brief, fresh tumor samples were minced into 1 mm^3 pieces. Samples were then rinsed with phosphate-buffered saline (PBS) and digested with collagenase solution (2.5 mg/ml collagenase, 40 mg/ml bovine serum albumin, 2 mg/ml glucose, 1 × antibiotic–antimycotic liquid (Gibco BRL, Grand Island, NY, USA), and 50 mg/ml gentamicin in Hank's balanced salt solution) for 20–30 min at 37 °C. Tumor cells, including cancer cells and stromal cells, were washed several times with PBS, pelleted by centrifugation, and cultured in 24-well plates at 37 °C in a humidified atmosphere of 5 % CO_2 in air, with 400 µl of phenol red-free RPMI 1640 medium (Gibco BRL) containing 10 % fetal calf serum (Tissue Culture Biologicals, Tulare, CA, USA), which was stripped of steroids by absorption with dextran-coated charcoal.

Adenovirus ERE-GFP assay

To assess the transcriptional activityof ER, reporter assays were performed using an adenovirus vector carrying the ERE-tk-GFP gene (Ad-ERE-tk-GFP). Another adenovirus vector carrying the pCMV-fluorescent protein from *Discosoma* (DsRed) gene (Ad-CMV-DsRed) was used as a control vector to assess the infectivity of the adenovirus in primary tumor cells. Constructions of Ad-ERE-tk-GFP and Ad-CMV-DsRed were carried out as described previously [29]. Adenovirus infectivity in ERα-positive MCF-7 breast cancer cells was examined using an Ad-CMV-DsRed, and a minimum of 95 % of cells were found to be infected [29]. When MCF-7 cells were infected with Ad-ERE-tk-GFP, GFP expression was dose-dependently increased by the addition of E2, and this induced expression was strongly inhibited by the addition of the pure anti-estrogen, fulvestrant [29], indicating that the induction was mediated by ER and that the quantitative evaluation was possible.

Tumor cells isolated from fresh breast cancer tissues were infected either immediately or 1 day later with 2×10^9 plaque-forming units (PFU; in human kidney 293A cells) of Ad-ERE-tk-GFP. Initially, cells isolated from 36 cases were infected with 2×10^9 PFU of Ad-CMV-DsRed in separate wells to examine the infectivity of the adenovirus in tumor cells. After incubation for a further 3 days, tumor cells were harvested and GFP- and DsRed-expressing cells were then counted under a fluorescence microscope. Stromal cells that were included in the primary culture were excluded from the evaluation under the guidance of a pathologist specializing in breast cancer. Adenovirus infectivity in the initial 36 cases assessed using an Ad-CMV-DsRed was sufficiently high, with minimal

Table 2 Primer and probe data used in real-time PCR analysis

Target mRNA	Accession no.		Sequence	Product (bp)
RPL13A	NM_012423.3	Forward	5′-CCTGGAGGAGAAGAGGAA AG-3′	126
		Reverse	5′-TTGAGGACCTCTGTGTATTT-3′	
PgR	NM_000926.4	Forward	5′-CGCCCTATCTCAACTACCT-3′	62
		Reverse	5′-GAAGCTGTATTGTGGGCTC-3′	
		Probe	5′-AGGCCGGATTCAGAAGCCAG-3′	
SRD5A1	NM_001047.2	Forward	5′-CCCAACTGCATCCTCCTG-3′	64
		Reverse	5′-ATGGGTAAATTAAGCACCGA-3′	
		Probe	5′-CCCGTAGTGGACGAGGAACATGG-3′	
HSD3B1	NM_000862.2	Forward	5′-GCTTGCTGAGAAGGCTGTACT-3′	70
		Reverse	5′-ACAAGTGTACAGGGTGCCG-3′	
		Probe	5′-CGTTTTTCAGATTCCACCCGTTAGCCGC-3′	
STS	NM_000351.4	Forward	5′-ACTGCAACGCCTACTTAAATG-3	63
		Reverse	5′-CCTTCCAGATGGATGTGCT-3′	
		Probe	5′-TGCGCTGGCACCCTCAGAAC-3′	
HSD17B1	NM_000413.2	Forward	5′-CCTTTGGGGTCCACTTG -3′	59
		Reverse	5′-CTCCATGAAGGCGGTGT-3′	
		Probe	5′-TGATCGAGTGCGGCCCAGTGCA-3′	

variability between samples (77.9 ± 5.2 %). The percentage of GFP-positive cells among all the epithelial cells was thus simply defined as the ex vivo ER activity without standardization by adenovirus infectivity, to avoid sample wastage.

Quantification of gene expression by real-time polymerase chain reaction

We selected formalin-fixed paraffin-embedded (FFPE) tissue blocks from each case, which were largely occupied by tumor. Total RNA was extracted from these samples using the RecoverAllTM Total Nucleic Acid Isolation Kit for FFPE (Ambion, Austin, TX, USA) according to the manufacturer's instructions. The extracted RNA (500 ng) was converted to first-strand cDNA primed with a random 9 mer in a 10 μl reaction volume using an RNA PCR kit (Takara Bio Inc., Otsu, Japan), and 2 μl was used as a template for real-time polymerase chain reaction (PCR).

Expression levels of the indicated mRNAs were assessed by real-time PCR, according to the standard protocol for SYBR® green or TaqMan® assay in the Applied Biosystems Step One real-time PCR system (Life Technologies Japan, Tokyo, Japan). Expression of the RPL13A internal control gene was measured by SYBR green assay, and the other genes were measured using the TaqMan® assay. Samples with CT-values <45 cycles were defined as being within the quantifiable range, and samples with CT-values ≥45 were defined as undetectable. RNA from FFPE samples has previously been reported to be fragmented and degraded by sample fixation or storage in paraffin [35, 36]. To increase detection sensitivity, we therefore chose primers and probes with the smallest possible amplicons. The primer and probe data are shown in Table 2. Expression levels of the target gene were normalized to the RPL13A internal control gene. In this study, mRNA expression levels of PgR, which is a well-known target gene of ER, were defined as in vivo ER activity.

Statistical analyses

Prior to statistical analysis, raw data for the expression levels of each mRNA were normalized by log2 transformation. Statistical analyses were performed using the StatFlex 6.0 software program (Artech Co., Ltd., Osaka, Japan). Relationships between two values were analyzed by Pearson's correlation. Unpaired two-tailed t tests were performed to assess the differences between two data-sets. Values of $p < 0.05$ were considered statistically significant. Actual p values are shown in the figures when the p value was between 0.05 and 0.10. Values of $p > 0.10$ are shown in figures as not significant (NS).

Results

ER activity is not always dependent on circulating estradiol

ER activity was determined both ex vivo and in vivo. Samples from 45 cases of ER-positive breast cancer were analyzed by adenovirus ERE-GFP assay and expression

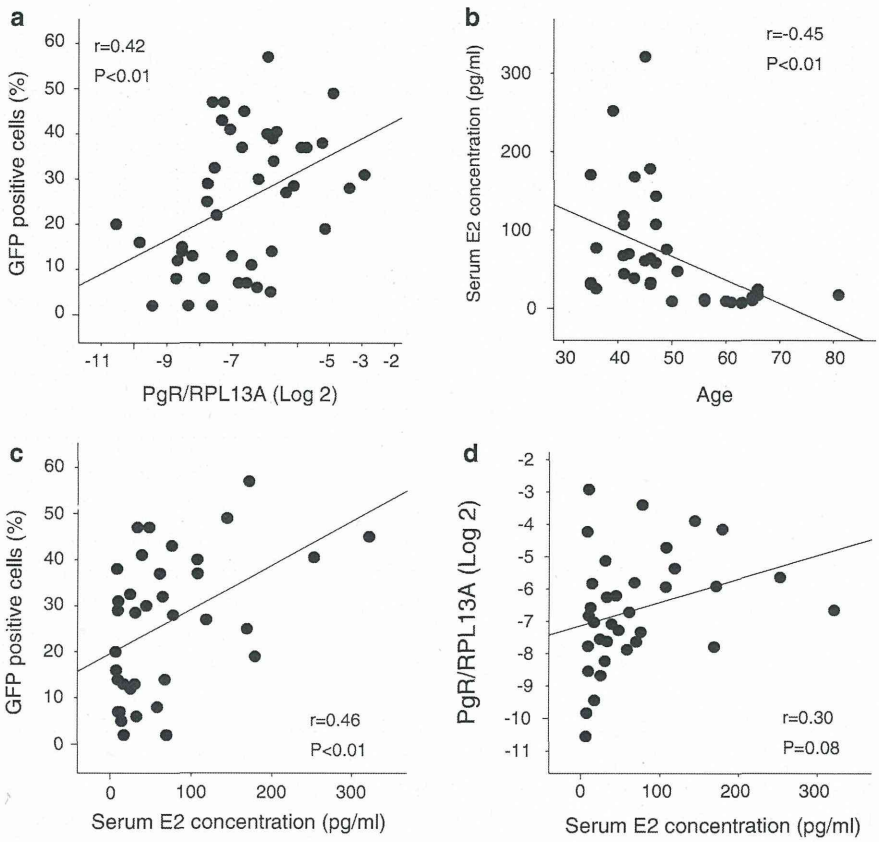

Fig. 1 Relationship between serum E2 concentration and ER activity in breast cancer tissue. **a** ER activity was assessed by adenovirus ERE-GFP assay [GFP-positive cells (%)] and real-time PCR for PgR expression levels [PgR/RPL 13A (log2)] in tumor cells isolated from ER-positive human breast cancers. The relationship between these two values were analyzed by Pearson's correlation. **b** Patient serum E2 concentrations were measured by chemiluminescence immunoassay. The relationship between age and serum E2 concentrations were analyzed by Pearson's correlation. **c**, and **d** Relationships between serum E2 concentration and ex vivo [GFP-positive cells (%)] (**c**) and in vivo [PgR/RPL13A (log2)] (**d**) ER activities were analyzed by Pearson's correlation. Pearson's correlation coefficients (r), and p values are indicated

levels of PgR were quantified in 43 cases (2 cases were undetectable). *Ex vivo* and in vivo ER activities were moderately correlated with each other, indicating that the quantitative evaluation of ER activity by adenovirus ERE-GFP assay was also possible in clinical samples (Fig. 1a). Serum E2 concentrations were reduced in patients over the age of 55, which generally corresponds with postmenopause in Japanese women (Fig. 1b). Ex vivo ER activity showed a moderate positive correlation with serum E2 concentrations (Fig. 1c). In vivo ER activity also tended to be positively correlated with serum E2 concentrations, though the relationship was not statistically significant (Fig. 1d). Notably, in vivo ER activity remained relatively high in some cases with low serum E2 concentration, suggesting that factors other than serum E2 concentration could be involved in the maintenance of ER activity in the presence of low serum E2, such as in postmenopausal patients.

Non-aromatase steroid-metabolizing enzymes are involved in the activation of ER in the postmenopausal state

one of the 45 cases examined, SRD5A1 and STS expression levels fell within the quantitative range in all cases, while the expression levels of HSD3B1 and HSD17B1 were within the quantitative range of 44 and 18 cases, respectively. We analyzed the relationships between those steroid-metabolizing enzymes and ER activities (Fig. 2a, b). Expression levels of SRD5A1 and HSD3B1 were positively correlated with both ex vivo and in vivo ER activities. Notably, the correlation coefficient for ex vivo ER activity was higher than that for in vivo activity. Expression levels of STS and HSD17B1, were positively correlated with in vivo ER activity, although the correlation coefficient for STS was low. These results indicate that HSD3B1 and SRD5A1 are involved in the maintenance of ex vivo and in vivo ER activities, while STS and HSD17B1 are only involved in the maintenance of ER activity in vivo.

Based on these results and previous reports, we hypothesized that ER activation by local production of estrogenic steroids is functional, especially in the postmenopausal state. We therefore analyzed ER activity and expression levels of these non-aromatase steroid-metabolizing enzymes, before and after menopause. The 45 ER-positive breast cancer cases were divided into a low-expression group and a high-expression group, based on below- or above-average expression levels of the steroid-metabolizing enzymes HSD3B1, STS, and SDR5A1,

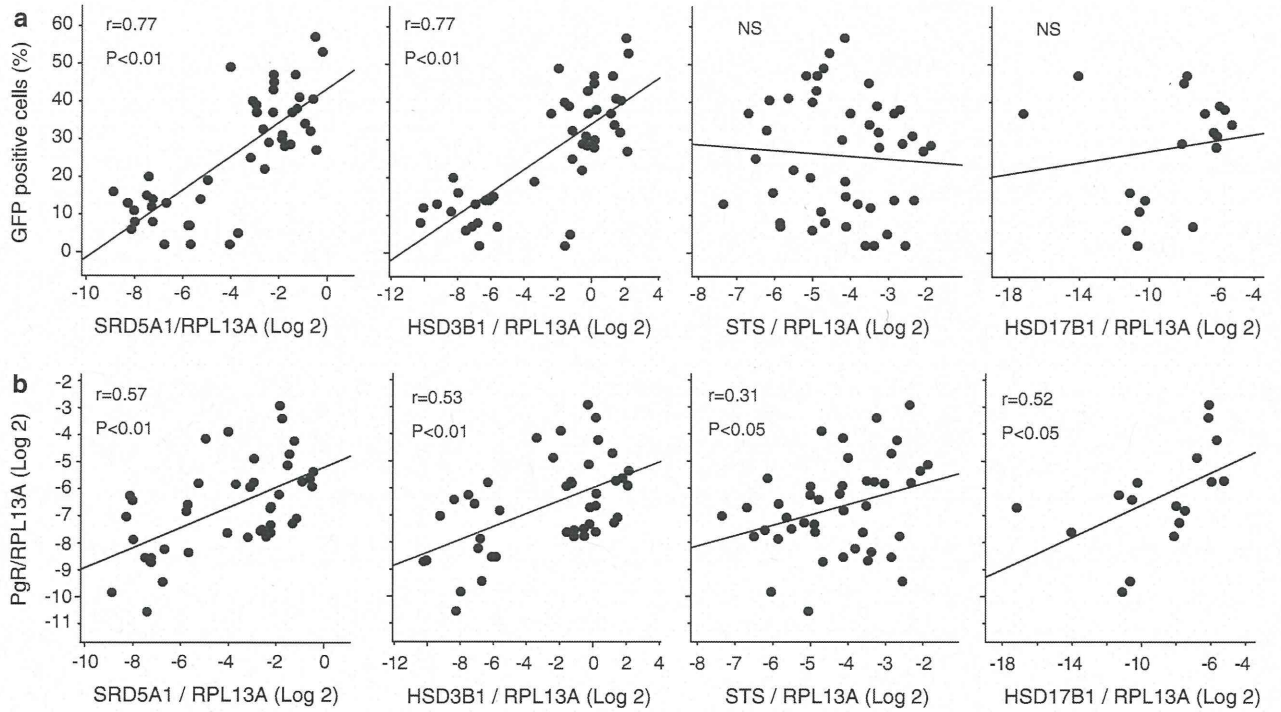

Fig. 2 Relationships between the expression of non-aromatase steroid-metabolizing enzymes and ER activity in breast cancer tissue. **a** and **b** Expression levels of SRD5A1, HSD3B1, STS, and HSD17B1 mRNA were assessed using real-time PCR. The relationships between these steroid-metabolizing enzymes, and ER activities ex vivo (**a**) and in vivo (**b**) determined by ERE-GFP assay [GFP-positive cells (%)], and PgR mRNA expression levels [PgR/PRL13A(log2)], respectively, were analyzed by Pearson's correlation. Pearson's correlation coefficients (r), and p values are indicated

respectively. Of note, one case with undetectable HSD3B1 expression was classified in the low-expression group. HSD17B1 mRNA expression levels were divided into a negative-expression group (undetectable) and a positive-expression group (detectable). The ER activities in the low- and high-expression groups were also compared according to their menopausal status (pre or post).

High HSD3B1 and SRD5A1 expression levels were associated with greater ex vivo ER activity compared with the low-expression groups, regardless of menopausal status (Fig. 3a, b). However, for STS and HSD17B1, there were no significant differences in ex vivo ER activity between the high- and low-expression groups (Fig. 3c, d).

There were no significant differences in in vivo ER activity between the low- and high-expression groups with respect to any of the steroid-metabolizing enzymes in premenopausal cases. In contrast, high-expression levels tended to be associated with higher in vivo ER activity with respect to all the enzymes studied in postmenopausal cases, though the differences were not significant for STS and HSD17B1 (Fig. 4a, d).

Although the results of ex vivo and in vivo assays were inconsistent, they still suggest that non-aromatase steroid-metabolizing enzymes are involved in the activation of the ER in vivo, especially in the postmenopausal state.

Steroid-metabolizing enzymes could function in pathways

We analyzed the relationships among the expression levels of non-aromatase steroid-metabolizing enzymes using Pearson's correlation (Fig. 5). Expression levels of SRD5A1 and HSD3B1, and of STS and HSD17B1, were positively correlated. Conversely, no correlations were observed for other combinations. These results suggest that these non-aromatase steroid-metabolizing enzymes could function in pathways producing estrogenic steroids in an aromatase-independent manner (Fig. 6).

Discussion

The results of this study suggest that steroid-metabolizing enzymes, such as 5α-reductase type 1, 3β-HSD type 1, STS, and 17β-HSD type 1, could contribute to ER activation, especially in the postmenopausal state. Furthermore, the tendencies of SRD5A1 and HSD3B1, and STS and HSD17B1 to be co-expressed with each other suggest that these enzymes might function together in pathways responsible for producing estrogenic steroids in an aromatase-independent manner.

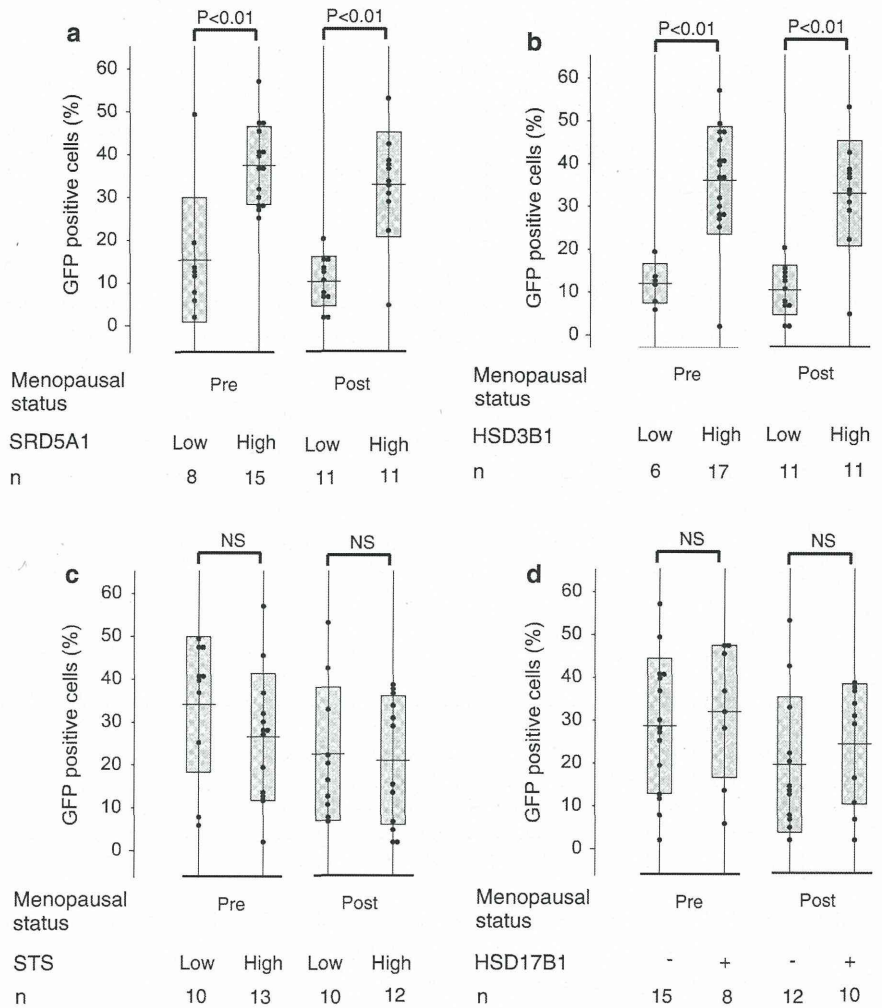

Fig. 3 Relationships between non-aromatase steroid-metabolizing enzymes and ex vivo ER activity before and after menopause. (**a**–**d**) mRNA expression levels of the non-aromatase steroid-metabolizing enzymes, SRD5A1 (**a**), HDS3B1 (**b**), STS (**c**) and HSD17B1 (**d**), were analyzed by real-time PCR in 45 breast cancer cases from either pre (Pre)- or post (Post)-menopausal patients. Samples were further divided into two groups of below (Low)- and above (High)-average expression levels of the steroid-metabolizing enzymes SDR5A1 (**a**), HSD3B1 (**b**) and STS (**c**). For HSD17B1 (**d**), mRNA expression levels were divided into two groups with undetectable (−) and detectable (+) expression levels. Ex vivo ER activities, measured by ERE-GFP assay [GFP-positive cells (%)], in low/− and high/+ expression groups were compared. Unpaired two-tailed *t* tests were performed to assess differences between two groups. *p* Values and sample numbers (*n*) are indicated; not significant (NS)

Despite a prominent reduction in serum E2 concentrations associated with the menopause, concentrations of estrogens in breast tissues of pre and postmenopausal women are known to be comparable [37]. This reflects the local biosynthesis of estrogens, mainly by aromatase [37], and is consistent with our observation that in vivo ER activity in breast cancer tissue was not solely dependent on the concentrations of circulating E2. The ER-activating abilities of non-estradiol steroids have been reported [17, 18], and these steroids and E2 were shown to be generated by multiple aromatase-independent pathways [1, 14, 15, 19, 21, 24]. We therefore focused on the ER activity and aromatase-independent metabolic pathways that produce estrogenic steroids, including E2.

In the adenovirus ERE-GFP assay system, GFP expression is fully dependent on ER activity because of the consensus ERE used in the reporter sequence [29], suggesting that GFP expression reflects highly specific ER activity; however, this does not represent normal physiological conditions. In contrast, the expression levels of PgR, which is a well-known ER target gene [32], are considered to reflect ER activity in a more physiological environment; however, the PgR gene has other regulatory regions, in addition to the half ERE in its promoter region [32]. Because these two methods have different advantages and disadvantages, we used both methods to assess ER activity. In contrast with the in vivo results, expression levels of STS and HSD17B1 were not correlated with ex vivo ER activity. This discrepancy between the ex vivo and in vivo assays can be explained as follows. Steroid sulfate conjugate might not be contained in the ex vivo assay system. Because steroid-depleted medium was used in these assays, the steroid included in the ex vivo system was considered to be derived from the cancer cells or stromal cells. Steroid sulfate conjugates, which are metabolized by the STS-HSD17B1 pathway, might rarely be included in these cells. There were no significant differences in in vivo ER activity between the low- and high-expression groups in premenopausal cases, in respect of any of the steroid-metabolizing enzymes. ER activity in

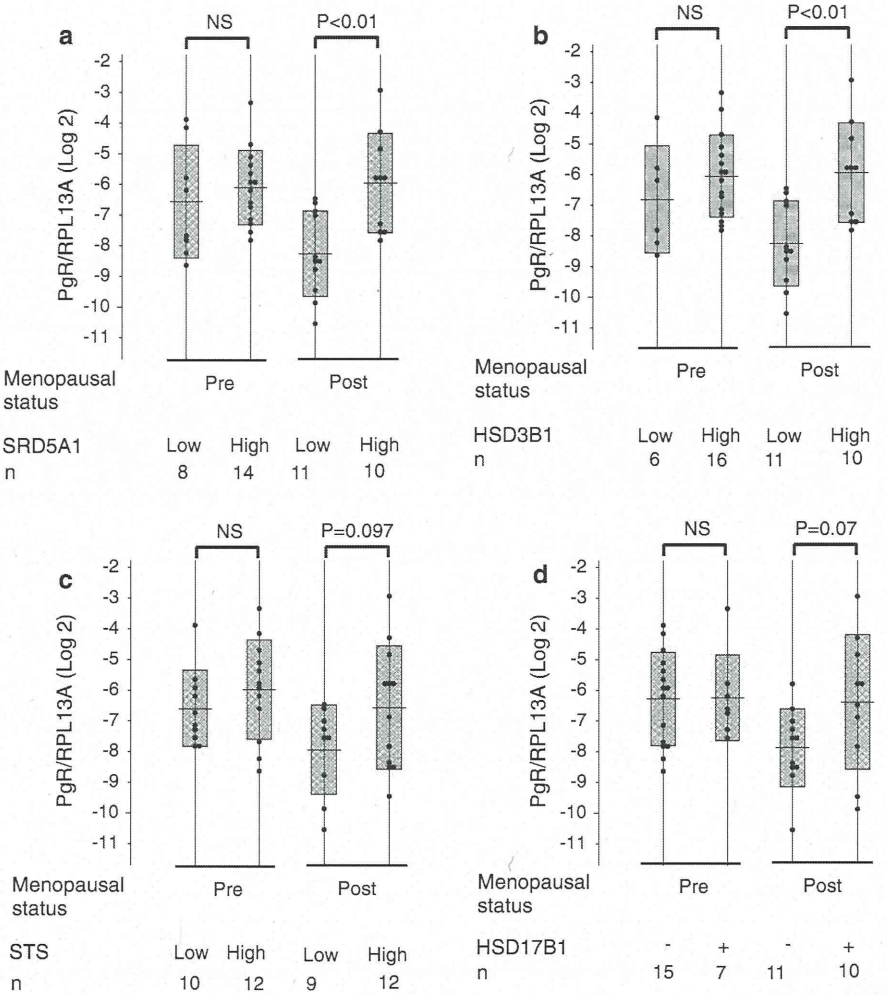

Fig. 4 Relationships between non-aromatase steroid-metabolizing enzymes and in vivo ER activity before and after menopause. (**a**–**d**) mRNA expression levels of the non-aromatase steroid-metabolizing enzymes, SRD5A1 (**a**), HDS3B1 (**b**), STS (**c**), and HSD17B1 (**d**), were analyzed by real-time PCR in 45 breast cancer cases from either pre (Pre)- or post (Post)-menopausal patients. Samples were further divided into two groups of below (Low) and above (High) average expression levels for the steroid-metabolizing enzymes SRD5A1 (**a**), HSD3B1 (**b**), and STS (**c**). For HSD17B1 (**d**), mRNA expression levels were divided into two groups with undetectable (−) and detectable (+) expression levels. In vivo ER activities determined by PgR mRNA expression, in low/− and high/+ expression groups were compared. Unpaired two-tailed *t* tests were performed to assess differences between two groups. p Values and sample sizes (*n*) are indicated; not significant (NS)

premenopausal patients was considered to be independent of steroid-metabolizing enzymes in cancer tissues because of their high circulating E2 concentrations. In contrast, patients with high HSD3B1 or SRD5A1 expression levels showed higher ex vivo ER activity compared with the low-expression groups, regardless of menopausal status. SRD5A1-HSD3B1-pathway-dependent ER activity might have been unmasked in the ex vivo system compared with the in vivo system because of the lower levels of E2. Nevertheless, we consider that the results of the ex vivo assay are supplementary of those of the in vivo assay.

DHT is synthesized from TS in an irreversible reaction catalyzed by 5α-reductase, and is a highly potent androgen with inhibitory effects in hormone-responsive breast cancer cells [38–40]. Thus, high expression of 5α-reductase type 1 alone is considered to have an adverse effect on the survival of breast cancer cells. However, DHT can be further metabolized by 3β-HSDs to 3β-diol, which has substantial estrogenic activity [17, 18, 24, 25]. Although evidence for the function of HSD3B1 in human breast cancer is limited, we recently found that ectopic expression of HSD3B1 induced DHT-dependent ER activation and cell proliferation in E10 cell lines derived from MCF-7 breast cancer cells [28]. Together, these data suggest that breast cancer cell survival might rely on 3β-HSD type 1 as a means of reducing the inhibitory effect of DHT. In support of this hypothesis, we demonstrated co-expression of SRD5A1 and HSD3B1, which was also in agreement with the results of a previous report [23].

In the present study, we demonstrated that ER activity in clinical breast cancers was positively correlated with the expression levels of steroid-metabolizing enzymes involved in the production of 3β-diol. This finding is consistent with other studies that demonstrated the estrogenic activity of 3β-diol in in vitro models [14]. Intratumoral DHT concentrations were also shown to be significantly higher in breast cancer tissues following AI treatment [22], suggesting that these alternative metabolic pathways which produce estrogenic androgen from androgens can potentially function as escape routes from

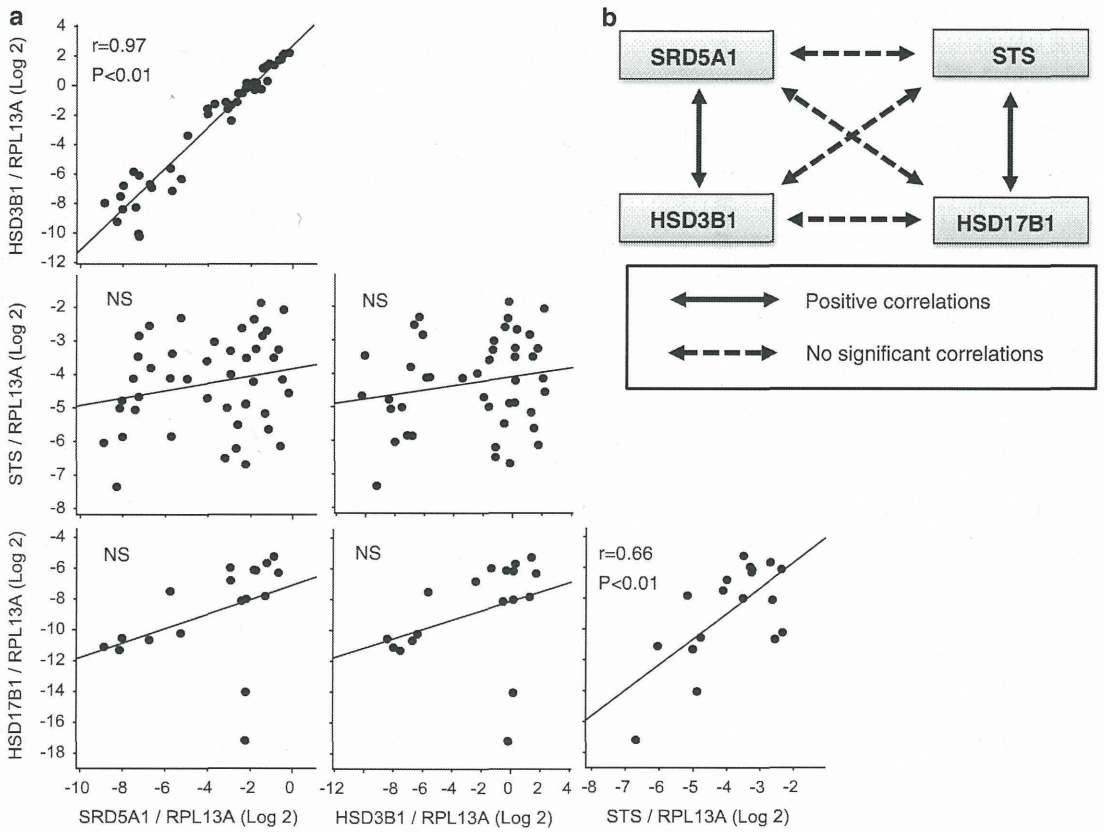

Fig. 5 Relationships between expression levels of non-aromatase steroid-metabolizing enzymes. **a** Scatterplots of relationships between mRNA expression levels of non-aromatase steroid-metabolizing enzymes, SRD5A1, HSD3B1, STS, and HSD17B1. The mRNA expression level of each enzyme is presented as a ratio of the control gene RPL13A (log2). Relationships between the different non-aromatase steroid-metabolizing enzymes were examined using Pearson's correlation. Pearson's correlation coefficients (r) and p values are indicated; not significant (NS). **b** Schematic representation of the relationships between expression levels of non-aromatase steroid-metabolizing enzymes. *Solid arrows* represent positive correlations; *dashed arrows* represent non-significant correlations

AI treatment. It is therefore interesting to speculate that 3β-HSD type 1 could represent a novel therapeutic target. Trilostane has been reported to act as a specific inhibitor of 3β-HSD type 1 [41]. A previous cross-over study of aminoglutethimide and trilostane in advanced postmenopausal breast cancer [42] found no differences in response rates of either drug, or in the average time of disease progression. Our results suggest that inhibition of 3β-HSD type 1 might contribute to reduced 3β-diol-mediated ER activation in breast cancer tissue, suggesting that the clinical efficacy of trilostane should be reconsidered.

Steroid sulfates such as E1S and DHEAS are metabolized to E1 and DHEA, respectively, by STS. E1 and DHEA are further metabolized to E2 and A-diol, respectively [2, 21, 27]. A-diol has been shown to have potent estrogenic activity and to stimulate the proliferation of breast cancer cells in vitro [17–20]. Although MCF-7 breast cancer cells are insensitive to sulfated estrogen because of their low endogenous STS level [43], overexpression of STS in MCF-7 cells resulted in increased cell proliferation in response to E1S or DHEAS [44], suggesting that the STS pathway could function as an estrogenic steroid-producing pathway in vitro.

Based on their high concentrations [45] and long half-lives [46] in blood, steroid sulfate conjugates such as DHEAS and E1S are thought to act as a central reservoir for the formation of biologically active estrogens, although they themselves are biologically inactive [21]. However, contrary to the results in vivo, expression levels of STS, or HSD17B1 were not correlated with ex vivo ER activity, suggesting that the continuous uptake of steroid sulfates from the reservoir (i.e., blood vessels) might be important for the production of estrogenic steroids by 17β-HSD type 1 and STS. The STS pathway has been noted as a therapeutic target, and its clinical application is already underway [47, 48]. However, there is currently insufficient evidence to support a role for the STS pathway in the regulation of ER activity through the production of estrogenic steroids in clinical breast cancer. Our results indicating that expression levels of STS and HSD17B1 tended

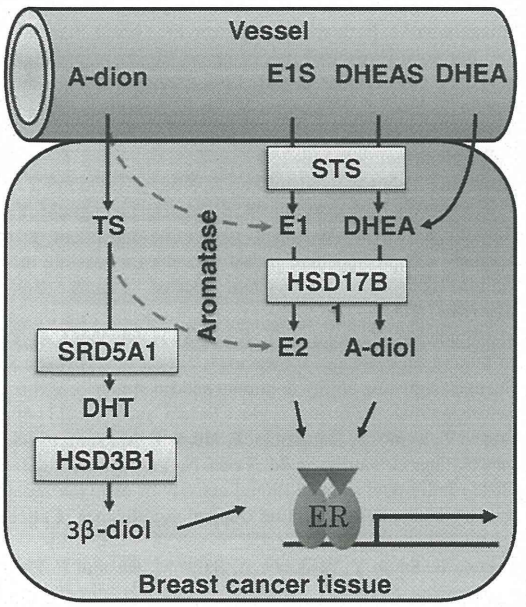

Fig. 6 Hypothesized mechanisms of local steroid metabolism to produce estrogenic steroids in an aromatase-independent manner in breast cancer. 5α-reductase type 1 (SRD5A1) and 3β-HSD type 1 (HSD3B1) produce 3β-diol from androgens. Steroid sulfatase (STS) and 17β-HSD type 1 (HSD17B1) produce E2 or A-diol from E1S, or dehydroepiandrosterone sulfate. These steroid-metabolizing enzymes might function together in pathways to produce estrogenic steroids such as 3β-diol, A-diol as well as E2 in an aromatase-independent manner

to correlate with in vivo ER activity are therefore important, eventhough the result was not statistically significant. We suggest that this finding should form the basis for future research in this field. STS and 17β-HSD type 1 expression levels were recently shown to increase following AI neoadjuvant therapy in postmenopausal ER-positive breast carcinoma patients [49]. All these data indicate that the STS pathway might contribute to the escape of breast cancer from AI therapy.

Our results concerning the correlations between enzyme expression levels and in vivo ER activities in postmenopausal patients suggested that the contribution of the 3β-diol-producing pathway to ER activity was greater than that of the STS pathway, which produces E2. This finding was inconsistent with the previous studies that found lower binding affinity of 3β-diol compared with E2 [17, 18]. Although mRNA expression levels are considered to reflect protein expression levels, they do not necessarily reflect actual protein expression levels exactly. We should therefore avoid making simple assumptions about the contributions of each pathway to ER activities. In this regard, further IHC evaluations of the enzymes are needed to validate the results.

In conclusion, the aim of present study was to verify the functions of non-aromatase steroid-metabolizing enzymes such asSRD5A1, HSD3B1, HSD17B1, and STS in untreated breast cancer. This was the first study to suggest that these enzymes function in together and contribute to ER activation especially in postmenopausal women. This was a small observational study utilizing clinical samples and further investigation is needed to provide the mechanistic insight about role of the SRD5A1-HSD3B1 and STS-HSD17B pathways, and to verify whether these pathways are actively involved in the AI-resistance mechanisms in the future. However, our study provides novel findings into the possible role of the SRD5A1-HSD3B1 and STS-HSD17B pathways as an alternative estrogenic steroid-producing, aromatase-independent pathways.

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Ethical standards All experiments complied with the current laws of Japan.

Conflict of interest The authors declare that they have no conflict of interest.

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