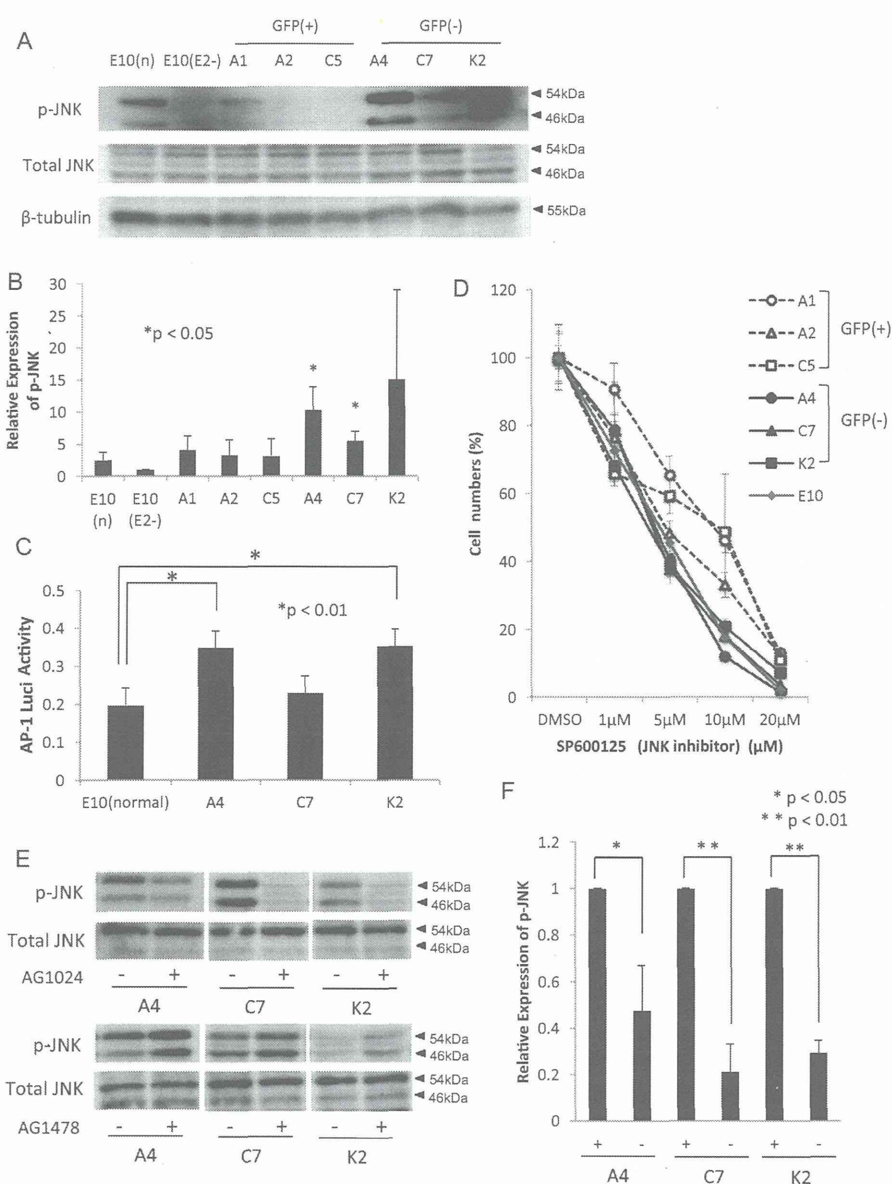

Fig. 5. The IGF1R-JNK pathway may contribute to the proliferation of GFP-negative EDR cells. (A) Western blot analysis using whole-cell lysates from MCF-7-E10 and EDR cells was performed using antibodies against JNK, p-JNK and β-tubulin as described in Section 2. (B) Densitometry results on p-JNK blots. Densitometry was performed on three blots and the results were indicated as values relative to the intensity with E10 (E2−). Statistical analysis of the indicated averages was performed using the Student's *t*-test, $p < 0.05$. (C) The AP-1 luciferase assay was carried out in MCF-7-E10 cells and GFP-negative EDR cells (A4, C7, K2) showing the phosphorylation of high levels of JNK as described in Section 2. Statistical analysis of the indicated averages was performed using the Student's *t*-test, $p < 0.01$. (D) The growth of GFP-negative cells was inhibited by the JNK inhibitor. MCF-7-E10 cells were plated at 5.0×10^3 cells/well into a 24-well plate and were cultured in RPMI1640 with 10% FCS. EDR cells (A1, A2, C5, A4, C7, K2) were also plated at 5.0×10^3 cells/well into a 24-well plate and were cultured in phenol red-free RPMI1640 with 10% DCC-FCS. Cells were treated with the JNK inhibitor, SP600125 (DMSO, 1 μM, 5 μM, 10 μM, 20 μM) for 5 days. The number of cells was counted using a Coulter Counter. (E) Phosphorylated JNK were suppressed by the IGF-1R inhibitor. GFP-negative EDR cells (A4, C7, K2) were plated at 1.0×10^5 cells/6-cm dish and were cultured in phenol red-free RPMI1640 with 10% DCC-FCS. After 3 days, the cells were incubated with DMSO as a control and with 10 μM AG1024 and AG1478 for 1 h. Western blot analysis using these whole-cell lysates from GFP-negative EDR cells were performed using the antibody against p-JNK. (F) Densitometry results on p-JNK blots inhibited by the IGF-1R inhibitor, AG1024. Densitometry was performed on the three blots and the results were indicated as values relative to the intensity with the control. Statistical analysis of the indicated averages was performed using the Student's *t*-test, $p < 0.01$.

such as A1, A2, and C5 strongly expressed *pS2*, *PgR*, *EGR3* [17] and *Bcl-2*, while the ERE-GFP-negative cells, A4, C7 and K2, did not show any significant mRNA expression of these ER-target genes. Since ER-expressing breast cancer was previously suggested to be less sensitive to chemotherapy [18], the strong expression of *Bcl-2* observed in ERE-GFP-positive EDR cells may be a useful biomarker for the treatment of recurrent breast cancer following hormonal therapy. Furthermore, the expression of *CyclinD1* in all ERE-GFP-positive and -negative cells was equivalent to that of the parental cells. These results indicate that ER transcription in ERE-GFP-expressing cells was constitutively activated in an estrogen-independent manner, while ERE-GFP-negative cells mostly lost ER transcriptional activity.

Taking these findings together with other results, ERE-GFP-positive EDR cells were shown to be dependent on the constitutive activation of ERα in terms of their growth, while the proliferation of ERE-GFP-negative cells could be supported by another signaling pathway.

3.5. Phosphorylation of ERα on Ser167 by the PI3K-Akt pathway may contribute to estrogen-independent proliferation in the ERE-GFP-positive EDR cells

ERα was previously shown to be activated by protein phosphorylation [19–22]. We analyzed intracellular signaling factors, such as Erk and Akt, which had been reported to be related to estrogen signaling, and investigated the phosphorylation status of ERα in ERE-GFP-positive and -negative EDR cells. The status of Akt on Thr308 appeared to be increased in A1, A2, and C5 cells, while the expression of total Akt did not change (Fig. 4A and B). However, we could not find a marked change in the total and phosphorylation status of p44/42 MAPK (Erk1/2) in these cells (Fig. 4A). On the other hand, among the ERE-GFP-negative cells, K2 cells had a high p44/42 MAPK (Erk1/2) phosphorylation status (Supplemental Data 2A). ERE-GFP-positive cells showed higher phosphorylation levels of ERα on Ser167, but not on Ser118 (Fig. 4A and B). These results suggested that activation of the PI3K-Akt signal phosphorylated ERα on Ser167 in breast cancer cells. Furthermore, growth was significantly inhibited by LY294002, a PI3K inhibitor, in ERE-GFP-positive cells (Fig. 4C), which indicated that the PI3K-Akt pathway may be involved in their growth and ER constitutive activation. On the other hand, the proliferation of ERE-GFP-negative cells was not significantly diminished by LY294002 (Supplemental Data 2B). We then examined whether PI3K-Akt pathway signaling activated ERα in ERE-GFP-positive EDR cells using an ERE-GFP assay. Fig. 4D shows that the activity of ERα in these cells was inhibited by LY294002. These results suggest that the phosphorylation of ERα on Ser167 by the PI3K-Akt pathway may contribute to the estrogen-independent and constitutive activation of ERα in ERE-GFP-positive EDR cells.

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ERα has two transactivation domains, A/B and E, which mediate ligand-independent and ligand-dependent transactivation, respectively. Phosphorylation site Ser167 of ERα is included in the A/B domain. We investigated whether ERE-GFP-positive cells showed constitutive activation of the A/B domain by PI3K-Akt pathway relative to the parental cells. To analyze transcriptional activities of the A/B and E domains in ERE-GFP-positive cells, we performed a luciferase assay using expression vectors containing either an A/B domain or E domain fused to a GAL4-DNA-binding domain, together with the reporter plasmid with the GAL4-DBD response element [11]. As expected, activation of the E domain was induced by E_2 in MCF-E10 cells, while that of the A/B domain was not (data not shown). Under estrogen-deprivation conditions, the A/B domain-dependent activity of ERE-GFP-positive cells was higher than that of parental cells (Fig. 4E). In addition, A/B domain-dependent activity was inhibited by LY29402 in ERE-GFP-positive EDR cells (Fig. 4E). These results suggested that the PI3K-Akt pathway activated the ligand-independent transactivation domain of ERα in ERE-GFP-positive EDR cells.

3.6. Constitutive JNK activation of GFP-negative EDR cells

We further analyzed the representative proteins involved in the intracellular phosphorylation signaling pathway that had been reported to be related to estrogen signaling, and found that only c-Jun N-terminal kinase (JNK) was constitutively activated in ERE-GFP-negative EDR cells (Fig. 5A and B). Activator protein 1 (AP-1) luciferase activity of A4 and K2 cells was significantly higher than that of E10 cells (Fig. 5C). Furthermore, as shown in Fig. 5D, the JNK inhibitor, SP600125, more effectively inhibited the growth of ERE-GFP-negative cells than that of ERE-GFP-positive cells. These results suggest that the JNK-related signaling pathway could be important for the survival of ERE-GFP-negative and ER-independent MCF-7 cells.

3.7. The IGF1R-JNK pathway may be important for the proliferation of ERE-GFP-negative cells

To explore the upstream signals of the JNK pathway in these cells, we examined whether EGFR and insulin-like growth factor receptor (IGF1R) were involved in the phosphorylation of JNK in ERE-GFP-negative EDR cells. Fig. 5E shows that p-JNK were significantly down-regulated after treatment with the IGF1R inhibitor, AG1024, in these three EDR cells (Fig. 5E and F). On the other hand, the expression of p-JNK was not altered or slightly increased by treatment with the EGFR inhibitor, AG1478. Considering that JNK may be an important factor for the growth of ERE-GFP-negative EDR cells (Fig. 5D), these results suggest that the IGF-1R-JNK signal pathway may be a significant mechanism for the proliferation of these cells; however, further studies will be needed to elucidate these details.

4. Discussion

The majority of women suffering from breast cancer have ER-positive tumors. Thus, hormonal therapy targeting ERα or its signaling pathway could be an efficacious therapeutic strategy for breast cancer. However, approximately one-third of ER-positive patients do not respond to hormonal therapy in an adjuvant setting. They show resistance to antiestrogens or aromatase inhibitors. In the last three decades, tamoxifen has been used as a standard antiestrogen drug in hormonal therapy for breast cancer [23]. Thus, numerous studies on tamoxifen resistance have been conducted and several mechanisms have been postulated [24,25]. However, to date, none of these has practically contributed to a clinical benefit for breast cancer patients. In recent years, the superior efficacy of third generation aromatase inhibitors has been indicated in several randomized clinical trials [3]. Thus, this type of drug is now widely used globally for the treatment of ER-positive breast cancer. The strategy of this type of drug is the blockade of estrogen production in cancerous regions to inhibit the action of estrogen in cancer cells. However, AI-resistant patients have often been reported, even in ER-positive cases. The acquisition of resistance to estrogen deprivation could be a major mechanism in these cases. Accordingly, studies on estrogen-deprivation resistance have also been performed by several laboratories; however, no consensus regarding the mechanisms involved has been obtained. The reason for this may be that multiple mechanisms may have all played a role in previous studies using estrogen-deprivation-resistant cells. In this study, we used EDR cells, which were transfected with the ERE-GFP reporter gene, and demonstrated for the first time that more than two clearly distinct mechanisms exist in estrogen-deprivation resistance. Furthermore, these results suggest that two mechanisms may simultaneously occur, which means that breast cancers with different AI-resistance mechanisms can concomitantly occur and mingle in an individual breast cancer case. This phenomenon may be involved in the acquisition of further resistance for a second or third therapy. Therefore, it is important to elucidate the detailed mechanisms for developing an appropriate therapy.

We subsequently characterized our EDR cells, which appeared to have distinct mechanisms of resistance. ERE-GFP-positive EDR cells showed the overexpression of ERα and maintained high ER activity in estrogen-deprivation conditions. The up-regulation of ER-target genes confirmed that these cells showed ER-dependent proliferation. Furthermore, significant induction of the MAP kinase pathway was not observed in our ERE-GFP-positive EDR cell lines. On the other hand, Campbell et al. reported that activation of

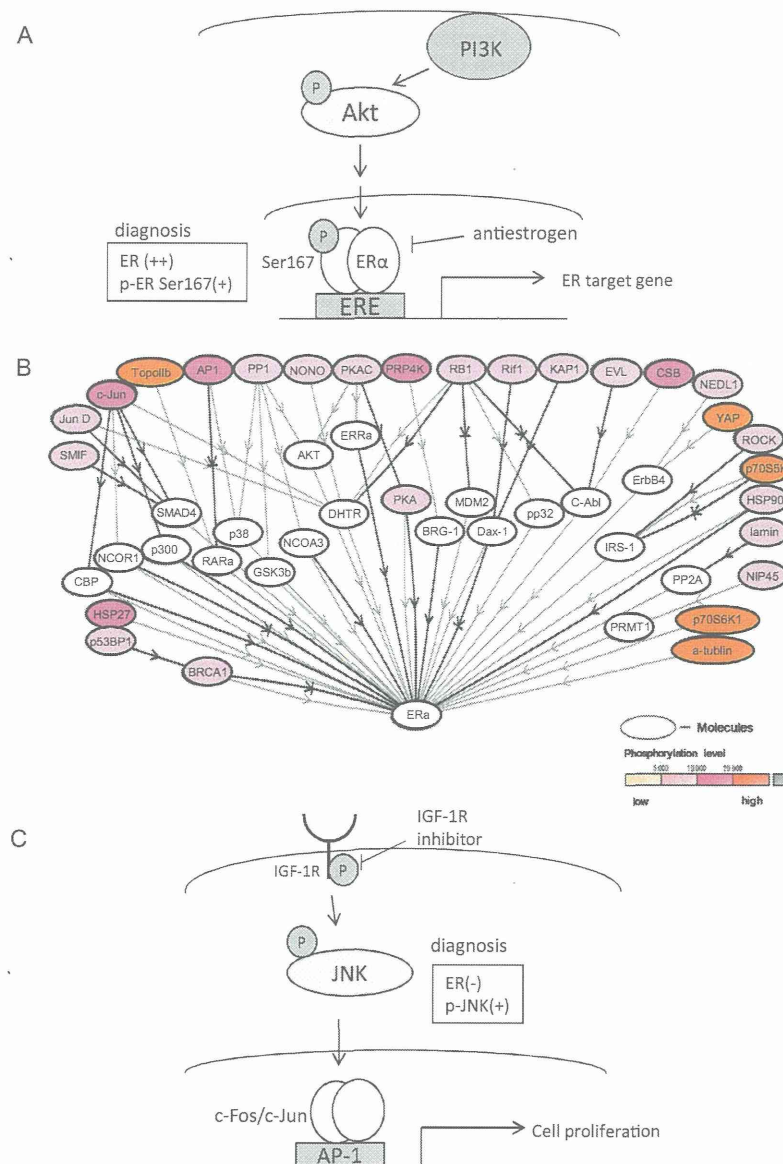

Fig. 6. Models of the mechanisms for estrogen-independent growth in GFP-positive and -negative EDR cells. (A) In GFP-positive EDR cells, the overexpression of ERα and activation of Akt phosphorylated ERα on Ser167, inducing constitutive ERα activation and ERα-mediated transcription. (B) The possible signal pathway connected to ERα involving molecules that were phosphorylated more in GFP-positive cells (A1) than in parental MCF-7-E10 cells were investigated by KeyMolnet analysis in Section 2. These pathways indicated several possibilities for the mechanisms of ER activity in ERE-GFP-positive EDR cells. The red circle shows molecules with higher phosphorylation levels in A1 than in MCF-7-E10; the lines show that the relationship was confirmed by a review (black lines) or a single study (gray lines). (C) In GFP-negative EDR cells, the IGF-1R pathway induced the activation of JNK and significant activation of AP-1 transcription activity. This signal pathway may be important for the survival of ER-independent MCF-7 cells.

the PI3K-Akt signal that phosphorylates ERα on Ser167 in breast cancer cells is implicated in tamoxifen resistance [20]. However, ERE-GFP-positive EDR cells that showed a marked change in the phosphorylation status of ERα on Ser167 and Akt on Thr308 were responsive to antiestrogen drugs, such as tamoxifen. A possible reason for some discrepancies between our results and previous studies is that the estrogen-deprivation-resistant cells in previous studies may have exhibited multiple mechanisms. The new strategy, using ERE-GFP-introduced EDR cells, established by cloning from a single colony, enabled us to avoid this complication. Another report in which the phosphorylation of ERα on Ser167 induced binding on ERE and transcriptional activation [26] also explains the high ER activity in our ERE-GFP-positive EDR cells. Additionally, the results of immunostaining using specimens from breast cancer patients showed that patients with the phosphorylation of high levels of ERα on Ser167 were responsive to antiestrogen drugs for hormonal therapy [27], which is consistent with the effect of antiestrogens on the cell growth of ERE-GFP-positive EDR cells. The reduction in ER activity and growth in ERE-GFP-positive EDR cells by inhibiting the PI3K-Akt signal pathway also suggests that the phosphorylation of ERα on Ser167 by the PI3K-Akt pathway may contribute to the constitutive activation of ERα and proliferation in ERE-GFP-positive EDR cells (Fig. 6A). Taken together, these results suggest that the overexpression and phosphorylation of ERα on Ser167 may be a useful diagnostic marker for cases that are ER-dependent.

We also carried out comprehensive phosphoproteomic analysis [12,13] as described in Section 2 to explore the mechanism

of estrogen-independent and constitutive activation of ERα in ERE-GFP-positive EDR cell lines by another approach. Comprehensive phosphoproteomic and pathway analyses of these cells supported our hypothesis that the PI3K-Akt pathway phosphorylates ERα (Fig. 6B) and suggested that other signaling pathways may be involved in the activation of ERα. Previous studies have indicated that the PKA signal pathway was important for tamoxifen resistance in breast cancer cells [28–30]. Methods such as comprehensive phosphoproteomic analysis may contribute to identifying the particular molecules related to these mechanisms in detail for the next step.

From a clinical perspective, these mechanisms of estrogen-deprivation resistance in ERE-GFP-positive EDR cells suggest that SERM and SERD may be more effective for AI-resistant breast cancer showing ERα overexpression and the phosphorylation of ERα on Ser167.

On the other hand, the three ERE-GFP-negative cell lines could have acquired estrogen-independent and ER-independent growth mechanisms. The expression of ER-target genes was downregulated and antiestrogens were often less effective in ERE-GFP-negative EDR cells than in ERE-GFP-positive cells. Tamoxifen inhibits the binding of estrogen to ER, while fulvestrant has a similar effect to tamoxifen and also degrades the ER receptor. In Fig. 2B, A4 and K2 were significantly inhibited by fulvestrant, which suggests that it is more effective in these cells than tamoxifen considering the mechanisms of this drug. Interestingly, the growth of all ERE-GFP negative cells was diminished by toremifene, which had the same effect as tamoxifen. Two reasons have been suggested for this phenomenon. Firstly, the concentration of toremifene used in this experiment was high, and was previously shown to be effective in some postmenopausal breast cancer patients [31]. Secondly, several reports have suggested that toremifene inhibited the growth of hormone-dependent MCF-7 tumors and anaplastic thyroid carcinoma cell lines via IGFR [32,33]. In addition, IGF1R was suggested to be related to the growth of ERE-GFP-negative cells in Fig. 5. Considering these results together with previous reports, the inhibition of growth in ERE-GFP-negative cells by toremifene could be via IGF1R.

These cell lines showed the robust induction of phosphorylated JNK and significant activation of AP-1 transcription activity. Since the JNK pathway has been shown to play a crucial role in the growth and development of breast cancer [34], the estrogen-deprivation resistance and ER signal-independence of these cells may have been acquired by the constitutive activation of this signaling pathway. Primary breast cancer is known to overexpress IGF1R, which is phosphorylated in many breast cancer subtypes [35]. A previous study has shown that IGF1R signaling is involved in the antiestrogen resistance of breast cancer cells [36]. In this study, we showed that the inhibition of IGF1R activity by AG1024 decreased phosphorylated JNK (Fig. 5E and F) and the proliferation of these cells was inhibited by SP600125 (Fig. 5D), which suggested that the IGF1R-JNK signal pathway induced the proliferation and survival of ERE-GFP-negative cells (Fig. 6C). On the other hand, K2 caused a marked change in the phosphorylation status of p44/42 MAPK (Erk1/2) (Supplemental Data 2A), and this result indicated that MAPK signal pathways may be partly involved in the activation of these cells. The involvement of the IGF-1R-JNK-AP-1 pathway in ERE-GFP-negative cells, as indicated by an in vitro experiment, suggests the possibility of a new molecular target to overcome hormonal therapy resistance. The activation of JNK may be a diagnostic marker and therapeutic target for the recurrence of luminal-type breast cancer. Considering these results together with previous reports, the molecular mechanism for the acquisition of estrogen-deprivation resistance is not uniform, and various intercellular signaling pathways could impact on the survival, growth, and development of hormonal therapy-resistant breast cancer. The distinction of these mechanisms of estrogen-deprivation resistance may contribute to the individualization of breast cancer to determine eligibility criteria for recurrent patients and the provision of appropriate therapy.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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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 $\geq$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

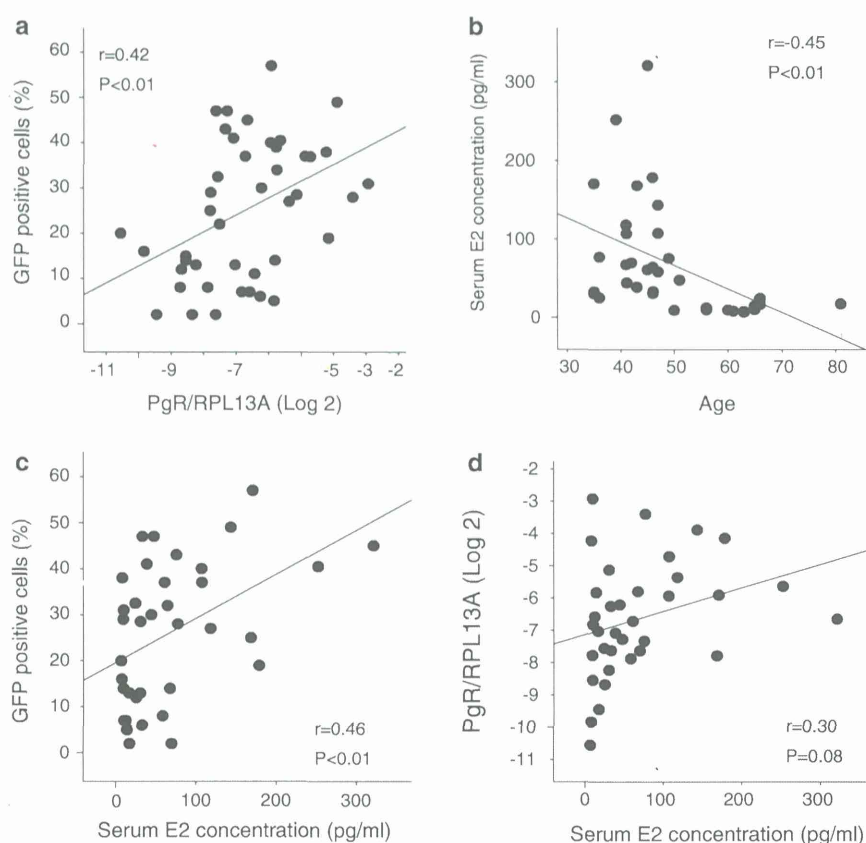

Fig. 1 Relationship between serum E2 concentration and ER activity in breast cancer tissue. **a** ER activity was assessed by adenovirus ERE-GFP assay [GFP-positive cells (%)] and real-time PCR for PgR expression levels [PgR/RPL 13A (log2)] in tumor cells isolated from ER-positive human breast cancers. 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,