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ORIGINAL ARTICLE

Tumor microenvironmental growth factors induce long-term estrogen deprivation resistance in breast cancer

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Abstract

Background Hormonal therapy is an effective treatment for luminal-like breast cancer. Aromatase inhibitor (AI) is widely used for estrogen receptor-positive, postmenopausal breast cancers. However, resistance is occurred and becomes a serious clinical concern. In general, progression of cancer strongly depends on tumor microenvironment, which may, therefore, also contribute to the development of AI resistance.

Methods We evaluated tumor microenvironment-derived factors with respect to AI resistance using typical estrogen receptor-positive breast cancer cell lines. We established tumor microenvironment-dependent AI-resistant models and elucidated the underlying mechanisms.

Results T-47D cells had a higher dependence on microenvironment-derived factors, such as estrogen or growth factors, for survival than MCF-7 cells. We, therefore, evaluated tumor microenvironment growth factors with respect to AI resistance using T-47D cells. We established three resistant cell lines (V1, V2, and V3) that survived estrogen deprivation and growth factor-supplemented conditions. These cell lines were deficient in estrogen receptor α expression and estrogen-dependent growth. Among six representative growth factors, epidermal growth factor was the most influential. In these models, HER2 protein was overexpressed without gene amplification and intracellular phosphorylation pathways were activated compared to parental cell lines. Molecular targeting inhibitors revealed that V1 and V2 primarily rely on the PI3 K pathway for survival, whereas V3 relies on the MAPK pathway.

Conclusions This study demonstrates the importance of tumor microenvironment-derived factors for the development of AI resistance. These resistant models did not utilize the same resistance mechanism, suggesting that flexible strategies are essential in conquering resistance.

Keywords Breast cancer · Aromatase inhibitor · Hormonal therapy resistance · Tumor microenvironment

Introduction

Approximately 70% of breast cancers express estrogen receptor α (ERα), which plays a pivotal role in breast cancer development and prognosis. Most ERα-positive breast cancers respond to hormone therapies such as anti-estrogens and aromatase inhibitors (AIs) [1]. Anti-estrogens work as competitive inhibitors of estrogen and block ERα function. The selective modulators of estrogen receptor block estrogen binding sterically [2], [3], whereas selective downregulators of estrogen receptor degrade ERα through the ubiquitin proteasome pathway [4], [5]. AIs affect the enzyme aromatase and block estrogen synthesis from androgens [6]. AI is the first choice in adjuvant therapy for postmenopausal ERα-positive breast cancer and is aimed at reducing regional estrogen production. It is very effective and has less adverse

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effects than chemotherapy, although drug resistance develops and is becoming a serious concern.

Most cancer cells interact with other cells, including non-cancerous ones, through secreted proteins such as growth factors, and form tumor microenvironment [7], [8]. These interactions affect the regulation of cell behavior, and some of them are suggested to contribute to the development of treatment resistance [9–12]. We have previously shown that carcinoma-associated fibroblasts (CAFs) educated by cancer cells to produce a favorable environment for each other encourage the development and progression of ERα-positive breast cancer [13], [14]. Aromatase is a member of cytochrome P450 family and synthesizes estrogen from androgens [15]. It is overexpressed in CAFs surrounding breast cancer cells, causing regional estrogen synthesis that supports estrogen-dependent breast cancer growth in postmenopausal ERα-positive breast cancer [16]. Therefore, aromatase is considered to be an important target for treatment and its inhibitors are frequently used for the treatment of postmenopausal ERα-positive breast cancers. Moreover, we have demonstrated that CAFs derived from breast cancer specimens secrete not only estrogen but also growth factors, and they appear to support breast cancer growth [13], [17], [18].

Our laboratory aims to identify distinct resistant mechanisms and, to this effect, we have established several AI-resistant breast cancer cell lines by estrogen deprivation [19–21]. However, the effect of microenvironmental factors on hormonal therapy response remains unclear. Therefore, we aimed to elucidate the effect of growth factors present in the breast cancer microenvironment on AI resistance. We attempted to establish growth factor-dependent AI-resistant cell models from typical breast cancer cell lines and to analyze the contribution of growth factors to AI resistance. Characterizing these resistance mechanisms might lead to new rationales for therapeutic strategies to address AI resistance.

Materials and methods

Reagents

Estradiol (E2) and fulvestrant were purchased from Sigma-Aldrich (St. Louis, MO, USA), p110α specific-PI3 K inhibitor BYL719, also known as alpelisib, and pan-PI3 K inhibitor BKM120, also as buparlisib, were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). MEK inhibitor U0126 was purchased from Cell Signaling Technology (Danvers, MA, USA). The antibodies used for western blotting were as follows: total HER2 (D8F12), total ERα (D8H8), phospho-p44/42 MAPK (Erk1/2, Thr202/Tyr204; D13.14.4E), total MAPK (Erk1/2; 137F5), phospho-Akt (Thr308; C31E5E), phospho-Akt (Ser473; D9E), total Akt (C67E7), and β-tubulin (Cell Signaling Technology). Secondary antibodies conjugated to horseradish peroxidase or alkaline phosphatase were purchased from Cell Signaling Technology and Bio-Rad Laboratories Inc. (Hercules, CA, USA), respectively.

Cells and cell culture

T-47D and MCF-7 breast cancer cell lines were stably transfected with estrogen receptor element-green fluorescent protein (ERE-GFP) reporter plasmids as previously reported [14] and named T-47D-TE8 and MCF-7-E10. For brevity, T-47D-TE8 and MCF-7-E10 were named T-47D and MCF-7, respectively, in this report. GFP fluorescence was monitored by fluorescence microscopy (Leica DM, Leica Microsystems GmbH, Wetzlar, Germany) and visualized using a BZ-9000 (KEYENCE, Tokyo, Japan). T-47D and SK-BR-3 cell lines were maintained in RPMI 1640 medium (Sigma-Aldrich) supplemented with 5% fetal calf serum (FCS; Gibco BRL, Grand Island, NY, USA) and penicillin (100 U/ml)/streptomycin (100 µg/ml) (Gibco). T-47D derived cell lines which exhibit estrogen-independent growth (estrogen deprivation-resistant cells; EDR cells) were generated by long-term culture under estrogen-deprived conditions. These cells represent a model of breast cancer treated with hormonal therapy. Establishment of EDR cells was performed in phenol red-free RPMI 1640 supplemented with 10% dextran-coated charcoal-treated FCS (DCC-FCS) as the estrogen-deprived medium, or supplemented with DCC-FCS and growth factor mixture [CXCL12/SDF-1α (20 ng/m), CXCL12/SDF-1β (20 ng/ml), EGF (20 ng/ml), HGF (20 ng/ml), IGF-I (20 ng/ml), and TGF-α (20 ng/ml) (R&D systems, Minneapolis, MN, USA)] as growth factor supplied medium. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Cloning of estrogen-deprived resistant T-47D cells

T-47D cells were cultured for > 3 months in estrogen-deprived and growth factor-supplemented medium. Some colonies expressing GFP were selected and separately seeded. When each colony had grown, further screening was performed by assessing ER activities through the GFP fluorescence, and the scale of the culture was gradually expanded. Ultimately, three cell lines named V1, V2, and V3 were obtained.

Cell growth assay

In growth factor sensitivity assays, cells were plated in 24-well plates at a density of 10,000 cells/well in phenol red-free RPMI 1640 supplemented with 10% DCC-FCS. They

were treated with each growth factor at 100 ng/ml concentration and cultured for 10 days. Then, cells were harvested and counted using a Sysmex CDA-500 automated cell counter (Sysmex Corporation, Kobe, Japan). Cell images (Fig. 3a) for the growth factor sensitivity assay were obtained using crystal violet staining and photographed using a BZ-9000 microscope.

Cell viability assay

In inhibitor and estrogen sensitivity assays, all cell lines were plated in 96-well plates at a density of 10000 cells/well in growth factor medium. They were precultured for 24 h and then treated with drugs and cultured for 72 h. Cell viability was calculated with a WST assay using a cell counting kit-8 (Dojindo, Kumamoto, Japan), according to the manufacturer's instructions. Viability of the vehicle control was set at 100%.

Real-time RT-PCR

Total RNA was extracted using IsoGen lysis buffer (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. Extracted RNA was converted to cDNA using a QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA). Transcripts were detected using a Step One™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, Inc., Santa Clara, CA, USA). Relative copy numbers were calculated from a standard curve and normalized to housekeeping genes. The sequences of primers are provided in Supplementary Table 1.

Western blot analysis

Cell lysates were prepared using Lysis-M Reagent (Roche Diagnostics GmbH, Mannheim, Germany) supplemented with Phos STOP phosphatase inhibitor cocktail (Roche Diagnostics) according to the manufacturer's instructions. Extracted proteins were separated on SDS-PAGE and proteins were transferred to PVDF membrane. Protein expression was determined by western blotting with specific antibodies listed in the Reagents section, and expression signals were detected on an ImageQuant™ LAS 4000 image analyzer (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

HER2 copy-number assay

Copy numbers were determined using TaqMan® Copy Number Assay (Applied Biosystems) according to the manufacturer's protocol. Briefly, genomic DNA was extracted using a DNA Mini Kit (Qiagen) and a solution of 5 ng/µl concentration was prepared. Each 20 µl reaction mixture contained 20 ng DNA and primer–probe mix for HER2 (Hs00817646_cn) as the target, a mix for RNase P as a reference (Applied Biosystems), and TaqPath™ ProAmp master mix (Applied Biosystems). Copy-number changes were measured using the following cycle conditions: denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. We determined relative quantification using a Step One Plus™ Real-Time PCR System (Applied Biosystems). The experiments were conducted in triplicates. Experimental results were imported into CopyCaller Software v2.1 (Applied Biosystems) for analysis.

Statistical analyses

Statistical analysis was performed using JMP® 13 software (SAS Institute Inc., Cary, NC, USA). One-way ANOVA with a post hoc Tukey honestly significant difference test was used to assess the significance of differences among groups. Data were expressed as mean ± SD and $P < 0.05$ was considered significant and all experiments were conducted in triplicates.

Results

Establishment of tumor microenvironment-dependent AI-resistant models

We previously established ERE-GFP reporter-expressing stably transfected MCF-7 and T-47D cells, (named MCF-7-E10 and T-47D-TE8, respectively) to monitor ERα activity using GFP fluorescence [22], [14]. Several AI-resistant models have been previously established from these cell lines by long-term estrogen deprivation [19–21]. The MCF-7-derived resistant models were obtained by simple estrogen deprivation; however, we faced difficulty in establishing a resistant model by simple estrogen deprivation in T-47D cells and all the cells died under these conditions. In other words, they entirely depended on estrogen and may have required alternative exogenous factors derived from the tumor microenvironment for survival. In this report, we selected 6 typical protein or peptide factors (CXCL12/SDF-1α, CXCL12/SDF-1β, EGF, HGF, IGF-I, and TGF-α) from numerous factors that exist in tumor microenvironment, as described in our previous reports [13], [17], [18]. We established estrogen deprivation-resistant (EDR) cell models from T-47D cells using a culture medium containing these six factors (Fig. 1a). To obtain resistant cells with ERα activity, colonies expressing strong GFP fluorescence were selected and separately cultured for another 7–18 weeks. These cell lines expressed strong GFP fluorescence at 2 weeks, but they lost almost all that fluorescence within 8 weeks (Fig. 1b). The

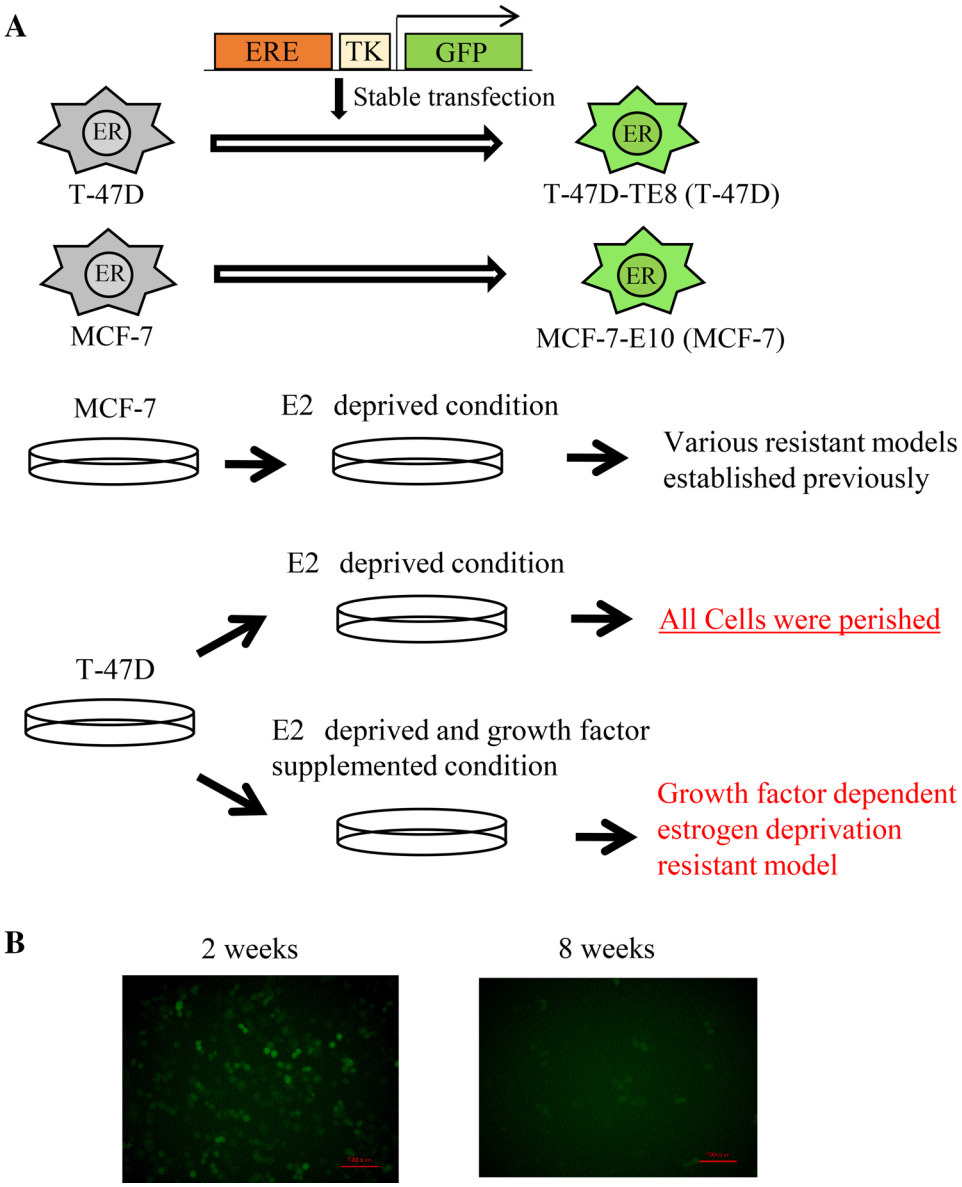

Fig. 1 Establishment of tumor microenvironment-dependent AI-resistant models. **a** Schematic figure describing cell lines and conditions. MCF-7 and T-47D cells were transfected with estrogen receptor element (ERE)–green fluorescent protein (GFP) reporter plasmids. The T-47D-resistant cell models were obtained after long-term estrogen (E2) deprivation and growth factor supplementation. **b** Picture of GFP fluorescence monitoring of ERE activity at the indicated point

dynamics of GFP fluorescence reflecting ERE activity suggest that, in 2 weeks, ERα protein was not silenced yet and seemed to be activated by growth factor induced phosphorylation signals to sustain ERE activity in these cells. Then, by 8 weeks, they adapt to estrogen deprivation and lost ERα activity. We ultimately established three cell lines (V1, V2, and V3) as microenvironment factor-dependent estrogen deprivation-resistant cell models.

Resistant cell models lost ERα expression, target gene expression, and ERα-dependent growth

Because EDR cells seemed to lose ERα activity, we examined the expression of ERα and ERα target genes. V1, V2, and V3 lost both ERα mRNA and protein expression. As representative ERα target genes, we selected progesterone receptor (PgR) and pS2 (also known as TFF-1). Both PgR and pS2 were downregulated in EDR cells, which was associated with the loss of ERα expression (Fig. 2a). Moreover, we analyzed estrogen and anti-estrogen sensitivity and found that EDR cells lost the sensitivity to estrogen nor anti-estrogen (Fig. 2b). These results suggest that established EDR cells grow independently of the estrogen/ERα axis.

HER family ligands result in robust growth and upregulation of HER2 expression

Because we used a growth factor cocktail to establish resistant cell models, we then elucidated the effect of the individual factors on growth stimulation under conditions of estrogen deprivation. These six factors were tested individually using EDR cells and evaluated by cell number and cell

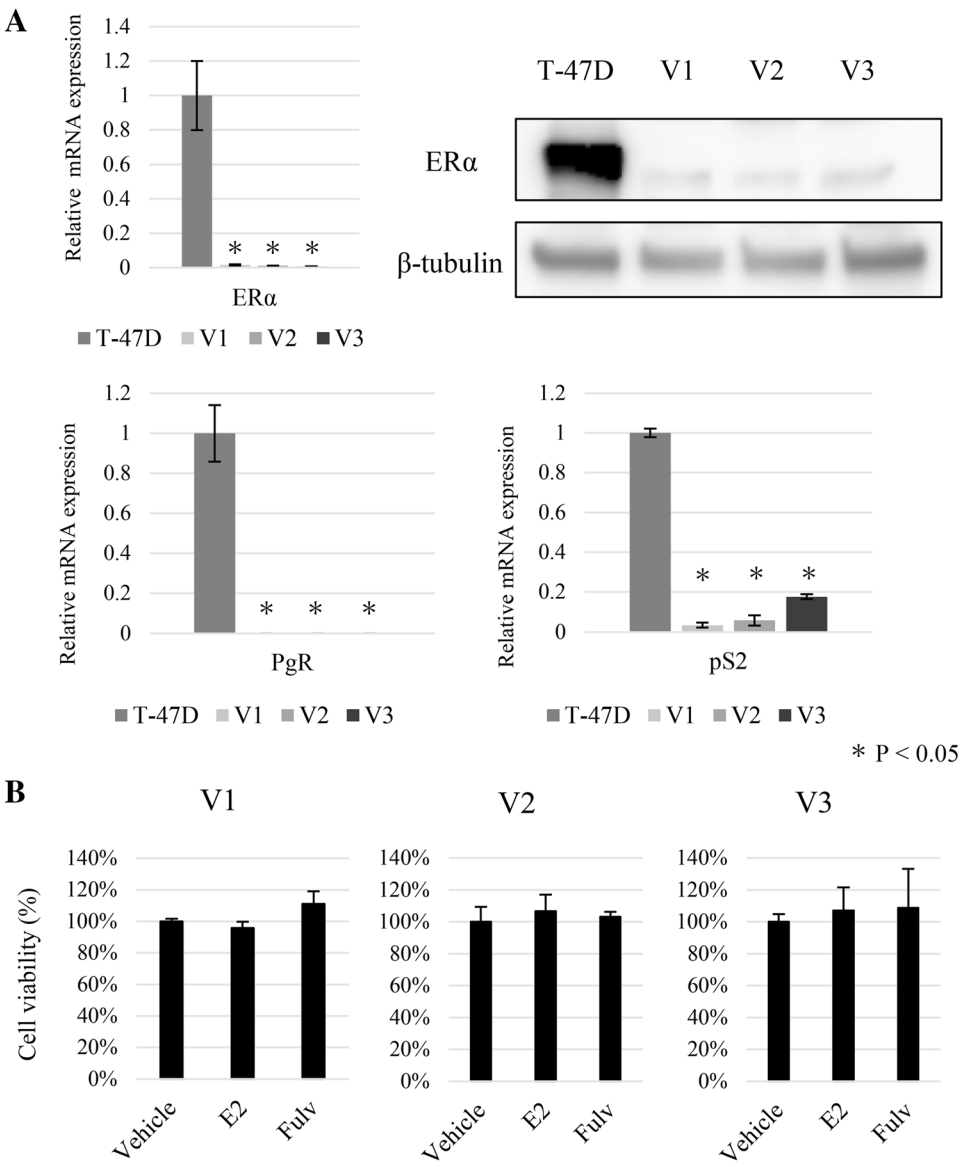

Fig. 2 The status of ERα expression, target gene expression, and ERα-dependent growth. **a** Total ERα and target gene expression in EDR cells and the parental cell line as determined by real-time RT-PCR and western blot analysis. Data were normalized to an internal control (RPL13A) in real-time RT-PCR, and the relative expression level is the ratio compared to the parental cells. Data are presented as mean ± SD ($n = 3$), *$P < 0.05$. In western blot analysis, β-Tubulin is shown as an internal control. T-47D cell lines were analyzed after growth in normal medium and EDR cell lines in growth factor-containing medium. **b** Estrogen (E2) and anti-estrogen sensitivity were analyzed using WST assay. T-47D cells were plated under estrogen deprivation and EDR cells were plated in medium supplemented with growth factors and conditions of estrogen deprivation. Cells were treated with vehicle, 1 nM of estrogen (E2) and 100 nM of the anti-estrogen fulvestrant (Fulv) for 72 h. The values are presented relative to the vehicle control and as mean ± SD ($n = 3$)

density. Figure 3a shows the results from V3 cells, all three variant cell lines produced similar results. EGF and TGF-α dramatically stimulated cell growth, whereas others did so to a lesser extent (Fig. 3a). Because EGF and TGF-α are typical HER family stimulating growth factors, we investigated their relationship with HER family receptors. Especially, HER2 expression was upregulated at the mRNA (Fig. 3b) and protein (Fig. 3c) levels in EDR cells. To elucidate the mechanism underlying HER2 overexpression, we performed a copy-number assay for the HER2 gene. The EDR cells exhibited no changes in copy number compared to the parent cells, suggesting that the HER2 upregulation in EDR cells was due to transcriptional upregulation (Fig. 3d). These results suggest that these EDR cells lost ERα-dependent growth and switched their proliferation to an HER2 signal-dependent mode.

Activation of intracellular phosphorylation signaling and growth-dependent pathways

Because the EDR cells exhibited HER2 ligand-dependent growth and HER2 overexpression, we addressed the activation of intracellular phosphorylation signaling. Here, we focused on two major intracellular signaling pathways' downstream of HER2 signal: PI3 K/Akt and MAPK. PI3 K and MAPK pathways were considerably activated in all EDR cells compared to that in the parent cells; particularly, in V3 cells, phosphorylation was remarkably observed in both pathways (Fig. 4a). We next assessed the efficacy of selective inhibitors of MAPK and PI3 K pathways. V1 and V2 cells exhibited relatively higher growth inhibition by both specific and pan-PI3 K inhibitors rather than by the MAPK inhibitor, whereas V3 cells were more sensitive to the MAPK inhibitor (Fig. 4b). These results

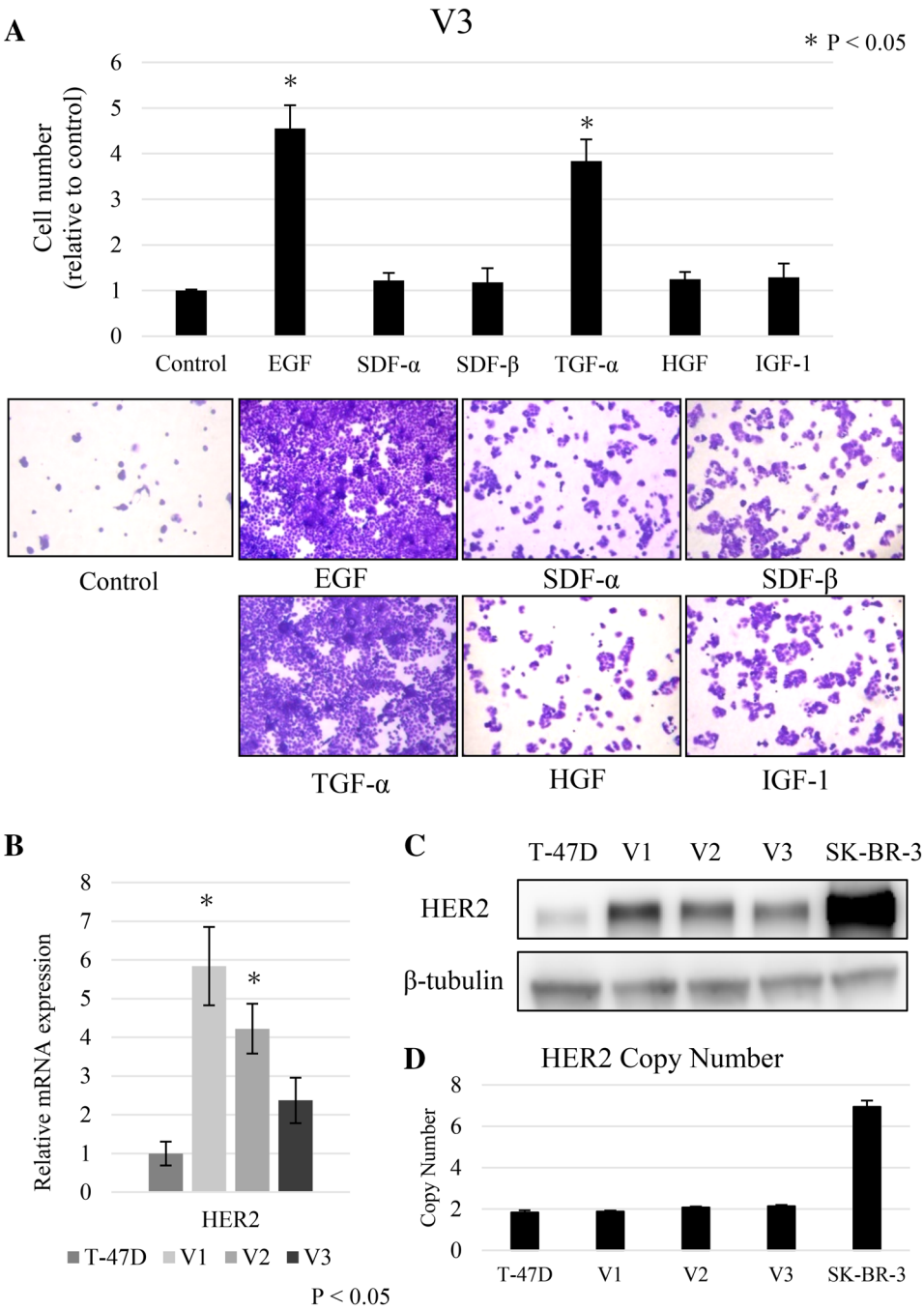

Fig. 3 The effect of individual growth factors. **a** The results of cell growth assay on V3 cells. Cells were plated in estrogen-deprived medium with 100 ng/ml of the individual growth factors and cultured for 10 days. They were stained with crystal violet and imaged using BZ9000; then, cell numbers were counted using CDA-500. The values are shown relative to the control, and data are presented as mean ± SD ($n = 3$), *$P < 0.05$. **b** HER2 expression was analyzed using real-time RT-PCR. The data were normalized by an internal control (RPL13A) and the relative expression level is the ratio compared to that of parental cells. Data are presented as mean ± SD ($n = 3$), *$P < 0.05$. **c** HER2 protein expression was determined using western blotting. β-Tubulin was used as internal control. **d** The copy number of the HER2 gene in the indicated cells. The values are presented relative to reference (RNase P) and presented as mean ± SD ($n = 3$)

suggest that at least two distinct resistant mechanisms, PI3 K dominant or MAPK dominant, were utilized during EDR evolution from the same parental cells.

Discussion

Hormonal therapy resistance is a serious concern in the treatment of ERα-dependent breast cancers and to overcome the resistance is urgent problem. T-47D and MCF-7 are the most frequently used and well-studied models of ERα-positive luminal-like breast cancer [23], [24]. We have used these cell lines to elucidate the mechanisms of AI resistance in breast cancer.

First, we observed a difference in the response to estrogen deprivation between MCF-7 and T-47D cells. We were able to establish estrogen deprivation-resistant cell lines from MCF-7 by simple estrogen deprivation [19–21]; however, we could not from T-47D cells. Therefore, we speculated that these cells require additional factors to survive estrogen

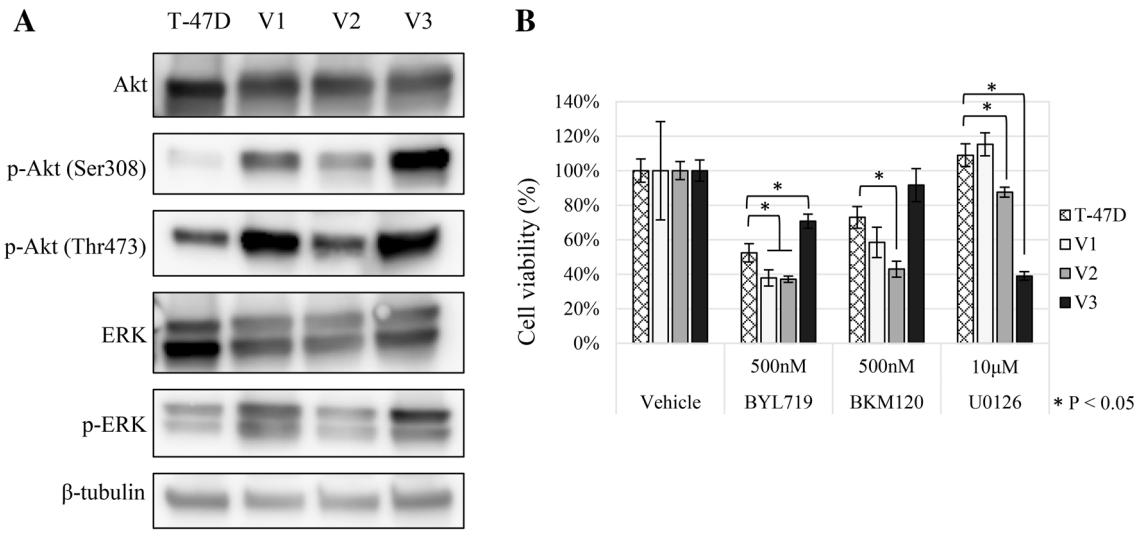

Fig. 4 Activation of intracellular phosphorylation signaling and growth-dependent pathways. **a** Western blot analysis for comparing T-47D and EDR cells. T-47D cells were cultured under conditions of estrogen deprivation and EDR cells under conditions of estrogen deprivation and growth factor supplementation. Cells were treated with the indicated drugs, p110α specific-PI3 K inhibitor BYL719, pan-PI3 K inhibitor BKM120, and MEK inhibitor U0126, at the indicated concentration for 72 h. Whole lysates were subjected to western blot analysis to detect the indicated proteins using specific antibodies. β-Tubulin was used as an internal control. **b** The sensitivity to the indicated drugs was analyzed using WST assay. The values are presented relative to the vehicle control and as mean ± SD ($n = 3$), $^*P < 0.05$

deprivation. The previous reports analyzing breast cancer specimens have described the presence of growth factors that might stimulate cancer development and cell growth [13], [18]. Although numerous such factors exist in the tumor microenvironment, we selected six proteins or peptide factors (CXCL12/SDF-1α, CXCL12/SDF-1β, EGF, HGF, IGF-I, and TGF-α) as representative factors and used them to establish a tumor microenvironment-dependent AI-resistant cell model from T-47D cells.

T-47D cells seemed retain estrogen receptor activity; however, in the long term, they exhibited estrogen receptor-independent and HER family receptor-dependent growth. Regarding intracellular phosphorylation signaling, V1 and V2 required PI3 K signaling, whereas V3 required MAPK signals. We have schematically described these results in Fig. 5. Our previous reports on AI-resistant models have suggested that MCF-7 easily switches their survival approach from ERα to other intracellular signals [19]. In contrast, T-47D cells depend on microenvironmental-derived exogenous factors such as steroids or growth factors [22]. These models suggest that there are diverse properties of breast cancer and some are more dependent on tumor microenvironmental factors for the development of resistance.

AIs are the first choice of treatment for estrogen receptor-positive postmenopausal breast cancers. Breast cancer cells are surrounded by stromal cells, some of which express aromatase to enable regional estrogen synthesis [14–16]. The AIs block estrogen production and, therefore, do not target cancer cells directly, rather the tumor microenvironment. To determine the mechanism underlying the development of AI resistance, we have established several types of AI-resistant models and classified the following three types of AI-resistant mechanisms from the viewpoint of ERα dependence: (1) alternative ERα ligand-dependent mechanisms; (2) ligand-independent ERα-dependent mechanisms; and (3) ERα-independent mechanisms [25]. In this report, we established a tumor microenvironment-dependent AI-resistant cell model and classified this type of resistant mechanism as an ERα-independent mechanism. These cells lost ERα expression and estrogen-dependent growth, and exhibited HER family ligand-dependent growth. Notably, T-47D, an ERα-positive luminal-like breast cancer cell line, acquired an HER2-like character by HER2 protein overexpression rather than by HER2 gene amplification after estrogen deprivation and growth factor supplementation. In ERα-positive luminal-like breast cancers, ERα and HER2 competitively repress each other [26], [27]. Our results suggest that some luminal-like cancer cells can easily switch to an HER2-type-like character, and thus, there could be some extent of specimens to obtain HER2-type-like character after AI resistance.

PI3 K pathways play an important role in the development of resistance to hormonal therapy [28], [29]. To overcome this resistance, molecular inhibitors targeting PI3 K pathway components have been developed. The BOLERO-2 study showed that everolimus, an mTOR inhibitor, is effective against ER-positive postmenopausal metastatic breast

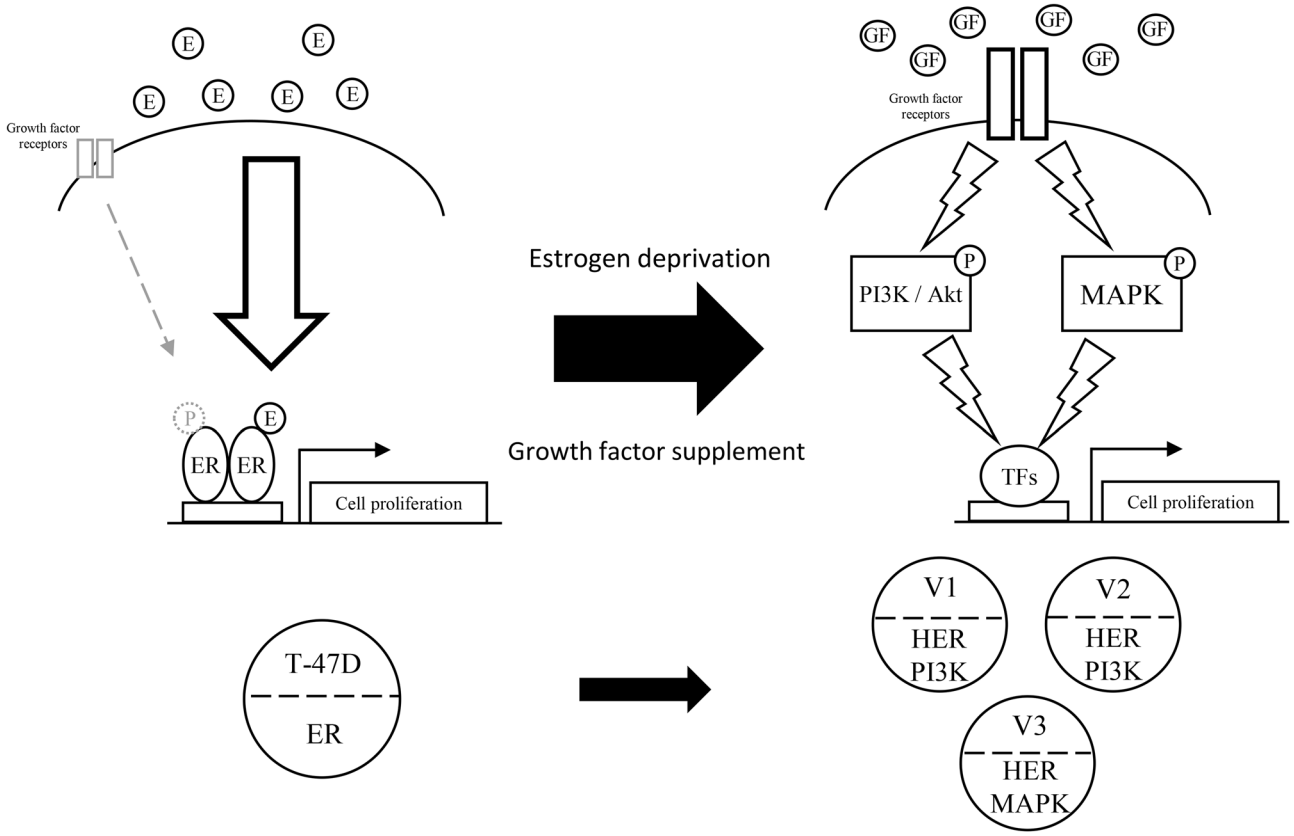

Fig. 5 Schematic illustration describing T-47D-derived growth factor-dependent resistance mechanisms

cancer and improves progression-free survival, but does not prolong overall survival [30], [31]. These results are consistent with the findings of our study and suggest that there are limitations to the efficacy of PI3 K pathway inhibitor in conquering hormonal therapy resistance; other survival pathways such as MAPK may also need to be inhibited using a combinatorial drug approach [32–35].

In conclusion, this study demonstrates the importance of tumor microenvironment-derived factors in the development of AI resistance. Analysis of intracellular phosphorylation signaling pathway showed that the same growth factor conditions did not always induce resistance in the cells by the same molecular mechanisms. There are multiple resistant mechanisms; hence, a diverse and flexible strategy is required to overcome resistance. Although further analysis of the key molecules involved in the different resistance mechanisms arising from the same conditions is required, we believe that our findings give some clues toward developing a strategy to overcome hormonal therapy resistance.

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Compliance with ethical standards

Conflict of interest Shin-ichi Hayashi received research grants from Novartis Pharma K.K, Astra Zeneca K.K, and Eisai K.K.

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