

# Upregulation of CYP1B1 by hypoxia is mediated by ER $\alpha$ activation in breast cancer cells

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## Research Article

**Keywords:** CYP1B1, hypoxia, HIF-1 $\alpha$ , estrogen receptor (ER $\alpha$ ), breast cancer

**Posted Date:** February 4th, 2022

**DOI:** <https://doi.org/10.21203/rs.3.rs-1319305/v1>

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# Abstract

**Background:** Endocrine therapy for breast cancer often leads to drug resistance and tumor recurrence; tumor hypoxia is also associated with mortality and tumor relapse. Cytochrome P450 1B1 (CYP1B1) regulates estrogen metabolism in breast cells and is known to be overexpressed in breast cancer tissue. Although the individual association of hypoxia-induced hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) and CYP1B1 with tumorigenesis is well known, the association between HIF-1 $\alpha$  and CYP1B1 leading to tumorigenesis has not been investigated. Here, we investigated the correlation between hypoxia and CYP1B1 expression in breast cancer cells for tumorigenesis-related mechanisms.

**Methods:** Hypoxia was induced in the human breast cancer cell lines MCF-7 (Er-positive) and MDA-MB-231 (triple-negative) and the normal breast epithelial cell line MCF10A, and then subjected to immunoblotting, transient transfection and luciferase assays, gene silencing using small interfering RNA, PCR analysis, and chromatin immunoprecipitation, co-immunoprecipitation, and mammalian two-hybrid assays. Furthermore, immunofluorescence analysis of tumor microarrays was performed and the pub2015 and The cancer genome atlas patient datasets were analyzed.

**Results:** HIF-1 $\alpha$  expression in response to hypoxia occurred in both normal and breast cancer cells, whereas CYP1B1 was induced only in estrogen receptor  $\alpha$  (ER $\alpha$ )-positive breast cancer cells under hypoxia. HIF-1 $\alpha$  activated ER $\alpha$  by direct binding as well as in a ligand-independent manner to promote CYP1B1 expression.

**Conclusions:** Therefore, we established the mechanism by which hypoxia and ER-positivity orchestrate breast cancer relapse.

## Background

Breast cancer is one of the most prevalent cancers in women, and malignant breast cancer is known to be the leading cancer-related deaths in women worldwide [1]. Breast cancer is classified depending on the expression of estrogen receptor  $\alpha$  (ER $\alpha$ ), epidermal growth factor receptor 2, and progesterone receptor; the lack of expression of all three receptors is called triple-negative breast cancer [2]. These classifications drive the therapeutic approach for breast cancer patients in clinical practice [3, 4]. Approximately 70% of breast cancers are ER $\alpha$  positive and are usually treated with anti-hormonal therapy [5]. Several molecules have been developed for hormone neutralization in women with ER $\alpha$ -positive cancers, including ER modulators such as tamoxifen, ER degraders, and aromatase inhibitors [6, 7]. However, many patients develop drug resistance to anti-hormonal therapy, and approximately 50% of patients with malignant breast cancer do not respond to ER modulators in the initial setting [7]. Moreover, the underlying mechanisms of acquiring resistances to these therapeutics are not well understood and there is a lack of therapeutic targets to overcome tumor relapse.

Hypoxia-induced hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) is one of the factors determining resistance to anti-hormone therapy and is a therapeutic target to overcome tumor relapse in breast cancer [8]. Intracellular

signaling in hypoxia is mediated by the HIF-1 $\alpha$ , which regulates the expression of several essential genes [9]. Under normoxic conditions, HIF-1 $\alpha$  protein is ubiquitinated and degraded, but under hypoxia, it is stabilized and translocated to the nucleus by HIF-1 $\beta$  dimerization [10-12]. Several studies have reported that the overexpression of HIF-1 $\alpha$  is associated with the initiation, progression, and recurrence of breast cancer [8, 13-15].

Several studies have shown that metabolites of 17 $\beta$ -estradiol (E2) induce breast tumor development, therefore, studies have been conducted to better understand the mechanisms involved in estrogen metabolism and estrogen metabolites [16, 17]. In breast cancer cells, E2 binds to ER $\alpha$  and the complex translocates to the nucleus, where it binds the estrogen response element (ERE) site and induces CYP1B1 transcription [18].

CYP1B1, a monooxygenase expressed in endocrine regulatory tissues such as the breast, uterus, and ovary, is a major enzyme involved in E2 metabolism [19, 20]. CYP1B1 is also known to be involved in hormone-dependent carcinogenesis by induction of metabolites of intracellular E2 and environmental carcinogens [16, 21-23]. It metabolizes E2 to 4-hydroxy-E2 to generate free radicals and form DNA adducts, which cause intracellular DNA damage and carcinogenesis in tissues, including breast tissue [17, 18, 24-26].

Although the association of hypoxia with CYP1B1 has been reported, that between HIF-1 $\alpha$  and CYP1B1 has not yet been studied. In this study, we assessed the role of hypoxia-induced HIF-1 $\alpha$  in regulating CYP1B1 in breast cancer as well as the mechanism underlying this interaction. Our findings suggest that HIF-1 $\alpha$  is involved in the regulation of CYP1B1 in breast cancer cells and that suppressing HIF-1 $\alpha$ -induced CYP1B1 expression in patients with recurrent cancer can be utilized as a novel therapeutic strategy for breast cancer.

## Methods

### *Materials*

YC-1, PD98059, LY 294002, and H-89, and PP2 were purchased from Sigma Aldrich Chemical Co. (St. Louis, Mo, USA). Penicillin–streptomycin solution, fetal bovine serum (FBS), trypsin, TurboFect, and Lipofectamine 2000 were obtained from Thermo Fisher Scientific (Waltham, MA, USA). The pCMV-b-gal plasmid was obtained from Clontech (Palo Alto, CA, USA). The Dual-Glo luciferase assay kit, pERE-Luc plasmid, and the mammalian-two-hybrid system were obtained from Promega (Madison, WI, USA). Antibodies against PKA/p-PKA, Akt/p-Akt, ERK/p-ERK, SRC/p-SRC, p-ER $\alpha$ , and horseradish peroxidase-linked anti-mouse and rabbit IgG were obtained from Cell Signaling Technologies (Danvers, MA, USA). Antibodies against  $\beta$ -actin and CYP1B1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The HIF-1 $\alpha$  antibody was obtained from Abnova (Taipei City, Taiwan), and the ER $\alpha$  antibody was obtained from Abcam (Cambridge, UK). The antibody against p-ER $\alpha$  (Ser305) was obtained from Bethyl Laboratories (Montgomery, Texas, USA). PE-conjugated goat anti-mouse IgG and Alexa Fluor 488-conjugated goat anti-rabbit IgG were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Oligonucleotide primers for polymerase chain reaction (PCR) were custom synthesized by Bioneer (South Korea). All chemicals used were of the highest commercially available grade.

### *Cell culture and treatment*

The human breast cancer cell lines MCF-7 (Er-positive) and MDA-MB-231 (triple-negative) were obtained from the Korea Cell Line Bank (KCLB, Seoul, South Korea) and the normal breast epithelial cell line MCF10A was obtained from the Laboratory Animal Resource Center (Korea Research Institute of Bioscience and Biotechnology, South Korea). MCF-7 and MDA-MB-231 cells were cultured in DMEM from HyClone (Thermo Fisher Scientific) supplemented with 10% FBS in a humidified 5% CO<sub>2</sub> incubator at 37 °C. MCF10A cells were cultured in Human Mammary Epithelial Cell Systems (HMEM) BulletKit medium from Lonza (Basel, Switzerland) and only cells up to passage 15 were used in the experiments. To induce hypoxia, these cells were cultured in a hypoxia chamber (Billups-Rothenberg, San Diego, CA, USA) containing a 1% O<sub>2</sub> gas mixture. Cell lines were DNA fingerprinted by the Korea Cell Line Bank (KCLB, Seoul, South Korea). The stock solution of the inhibitor was diluted with dimethylsulfoxide (DMSO) and added directly to the culture medium. Control cells were treated with DMSO only, and the final DMSO concentration was always <0.2%.

### *Immunoblot*

Protein lysates were prepared from cells cultured under hypoxia or normoxic conditions, and proteins were quantified using the Bradford protein assay (Bio-Rad, Irvine, CA, USA). The lysates were electroblotted on polyvinylidene difluoride membranes and reacted with the primary antibodies and secondary antibodies. The membranes were visualized with enhanced chemiluminescence (ECL) solution and analyzed using a ChemiDoc Imager (Bio-Rad).

### *Transient transfection and luciferase assays*

Cells were transiently transfected with the human CYP1B1 promoter-luciferase (Luc) full length (FL), pERE-Luc, and pCMV-b-gal using the Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, MA, USA). The CYP1B1 promoter-Luc FL vector (-1635 to +1588) was gifted by Dr. Robert Barouki [27]. The CYP1B1-Luc FL and deletion plasmids (-910 to +25 and -91 to +25) were used to investigate the hypoxia-mediated CYP1B1 promoter binding sites. After transfection, cells were incubated for 24 h under normoxic or hypoxic conditions, and cell lysates for luciferase reporter assay were prepared. Cell lysates were mixed with an equal volume of luciferase assay substrate reagent and analyzed using a luminometer (SpectraMax M5; Molecular Devices).

### *Gene silencing using small interfering RNA (siRNA)*

The expressions of HIF-1 $\alpha$  and ER $\alpha$  were knocked down using siRNA, and the results were analyzed by real-time qPCR, chromatin immunoprecipitation, and immunoblot analysis. siRNAs targeting *HIF-1A* and *ESR1* mRNA and non-targeting siRNAs were purchased from OriGene (OriGene Inc, Rockville, MD, USA).

Cells were transiently transfected with HIF-1 $\alpha$ -siRNA, ER $\alpha$ -siRNA, or scrambled control siRNA using the Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, MA, USA).

#### *RNA extraction and digital PCR analysis*

Under hypoxia or normoxia, total cellular RNA was isolated using the RNeasy Mini Kit (Qiagen, Germany) and reverse-transcribed using RNA-to-cDNA EcoDry reagent (TaKaRa, Japan). PCR products were directly monitored during PCR assays using QuantStudio 3 real-time PCR system (Applied Biosystems) and detected via SYBR Gene reporter dye enhancement. The levels of CYP1B1 and  $\beta$ -actin mRNA in MCF7 cells were compared with those in control cells using the comparative cycle threshold (Ct) method.

#### *Chromatin immunoprecipitation (ChIP) assay*

Cells cultured under normoxic or hypoxic conditions were treated with formaldehyde to cross-link protein and DNA. The cells were then sonicated and chromatin-DNA complexes were precipitated using an antibody against ER $\alpha$  or non-specific mouse IgG using an EZ CHIP kit (Upstate, Lake Placid, NY, USA). PCR was performed using purified DNA, oligonucleotide PCR primers, and Taq DNA polymerase (TaKaRa). PCR products were analyzed on an agarose gel using the SYBR Safe DNA Gel Stain kit.

#### *Co-immunoprecipitation (Co-IP) assay*

Cells were cultured under hypoxic or normoxic conditions and lysed with IP buffer (Roche). Protein lysates were pre-removed by incubation with Protein A beads (GE Healthcare) and then incubated with beads and ER $\alpha$  antibodies. Antibody-bound beads were washed with PBS, electrophoresed on SDS-PAGE, and then immunoblotted with HIF-1 $\alpha$ .

#### *Mammalian two-hybrid (M2H) assay*

Vectors [pBIND-ER $\alpha$  1-180 (N-terminal domain, NTD), 180-302 (DNA binding domain, DBD), 302-595 (ligand-binding domain, LBD), pACT-HIF-1 $\alpha$  FL, and pG5-Luc] were used for the M2H assay. Transfection was performed using the TurboFect transfection reagent (Thermo Fisher Scientific). The cells were lysed using lysis buffer (Promega) and the lysates were added to luminescent white plates. Luciferase activity was measured using a luminometer (SpectraMax M5). Relative reporter assay activity was calculated using the pG5-Luc control vector.

#### *Immunofluorescence analysis of tumor microarrays (TMAs)*

A human breast cancer tissue microarray (TMA, #BC081120e) containing 110 cases was purchased from Biomax (Rockville, MD). The immunofluorescent image was quantified using a quantitative analysis system (Thunder, Leica). Image analysis was performed using the LAS-X software (Leica) by acquiring fluorescence images of DAPI, CYP1B1, and HIF-1 $\alpha$  for each TMA spot and scoring the sum of the target pixel intensities. Tumors were divided into high and low groups according to the ER expression score

(according to the patient information sheet). The correlation of CYP1B1 and HIF-1 $\alpha$  in ER $\alpha$ -positive breast tumors was assessed using linear (Pearson) and nonparametric (Spearman) correlation coefficients.

### *The cancer genome atlas (TCGA) data and cBioPortal*

Ethical approval for the study was not required due to the retrospective nature of this study using only publicly available data. Transcriptome analysis data and pathological data of human breast cancer biopsy samples were obtained from the following dataset in cBioPortal platform: BRCA-TCGA-pub2015 [817 cases with mRNA data (RNA Seq V2 RSEM)], BRCA-TCGA [1100 cases], BRCA-METABRIC [1904 cases with mRNA data (microarray)]. Gene expression levels were normalized (Z-score) and scaled (log-transformed). Co-expression, overall survival, and volcano plots were calculated according to cBioPortal's online instructions and analyzed using the GraphPad Prism 9.00 software. The BRCA-METABRIC dataset was analyzed using the cBioPortal online tool for exploration and comparison between the two patient groups, and 304 genes differentially expressed in the ER $\alpha$ -positive high profile/HIF1 $\alpha$ -CYP1B1 high group was filtered ( $q$ -value < 0.01,  $|\text{Log}_2(\text{FC})| > 0.58$ ) and subjected to gene enrichment analysis using the Metascape platform (<http://metascape.org>) for gene annotation. The filtered data were subjected to hierarchical clustering and visualized as a heatmap.

### *Statistical analysis*

Data are presented as  $\pm$  standard error of the mean (SEM). All statistical analyses were performed using Student's  $t$ -test or the Mann–Whitney U-test when data were not normally distributed. Statistical significance was set at  $P < 0.05$ .

## **Results**

### *HIF-1 $\alpha$ is associated with CYP1B1 expression in breast cancer cells*

To investigate the effect of hypoxia on CYP1B1 expression in breast cell lines, HIF-1 $\alpha$  and CYP1B1 expression were analyzed by immunoblotting. HIF-1 $\alpha$  protein level was induced by hypoxia at 6, 12, and 24 h but CYP1B1 expression was not altered in MCF10A normal breast epithelial cells (Fig. 1a) or ER $\alpha$ -negative MDA-MB-321 cells (Fig. 1b). In contrast, both HIF-1 $\alpha$  and CYP1B1 expression increased due to hypoxia in MCF-7 cells, which are ER $\alpha$ -positive (Fig. 1c). To verify that hypoxia-induced CYP1B1 expression is associated with HIF-1 $\alpha$ , ER $\alpha$ -positive MCF-7 cells were cultured under hypoxic conditions after transient transfection with HIF-1 $\alpha$ -siRNA. The increase in CYP1B1 expression due to hypoxia was suppressed by HIF-1 $\alpha$  knockdown (by HIF-1 $\alpha$ -siRNA) at 24 h, suggesting that CYP1B1 expression under hypoxia is induced by HIF-1 $\alpha$  (Fig. 1d). As a negative control, the use of scrambled siRNA instead of HIF-1 $\alpha$ -siRNA lead to no decrease in CYP1B1 expression under hypoxic conditions (Fig. 1d). To confirm the role of HIF-1 $\alpha$  and hypoxia on CYP1B1 expression, cells were treated with a YC-1, an HIF-1 $\alpha$  inhibitor developed for the treatment of circulatory disorders, and immunoblot was performed. YC-1 inhibited CYP1B1 expression under hypoxic conditions (Fig. 1e). Next, to determine whether CYP1B1 activity is regulated by hypoxia, MCF-7 cells were transiently transfected with CYP1B1-Luc and HIF-1 $\alpha$ -siRNA under

hypoxic conditions. Hypoxia-induced CYP1B1 luciferase activity, which was reduced by HIF-1 $\alpha$  knockdown with HIF-1 $\alpha$  siRNA (Fig. 1f). These results suggest that CYP1B1 expression under hypoxia is mediated by HIF1- $\alpha$ .

#### *The estrogen response element (ERE) of the CYP1B1 promoter is sufficient for hypoxia-induced upregulation of CYP1B1*

CYP1B1 expression and luciferase activity were induced in ER $\alpha$ -positive MCF-7 cells by hypoxia but not in ER $\alpha$ -negative MCF10A or MDA-MB-231 cells, suggesting that CYP1B1 regulation by hypoxia is ER $\alpha$ -dependent (Fig. 2a and 2b). Based on these findings, we used the CYP1B1 promoter vector to verify whether ER $\alpha$  is involved in regulating CYP1B1 expression in hypoxia. The human CYP1B1 promoter full length (-910/+25 bp; Fig. 2c) contains a xenobiotic response element (XRE; -853/-824 bp), an active protein 1 binding site (AP-1; -149/-129 bp), and the E2 response element (ERE; -84/-49 bp), which is important for regulating CYP1B1 transcription [18, 26, 28]. We found that CYP1B1 promoter activity was increased even in the construct in which XRE and AP-1 were deleted. In addition, hypoxia-induced CYP1B1 activity was increased even in -91/+25 containing only the ERE site, confirming that the ERE site is essential for hypoxia-mediated CYP1B1 regulation (Fig. 2c). Moreover, it was established that hypoxia directly regulates CYP1B1 through the luciferase activity of the ERE-Luc vector in MCF-7 cells (Fig. 2d). ER $\alpha$ -mediated hypoxia-induced CYP1B1 expression was further verified by immunoblot analysis in MCF-7 cells treated with ER $\alpha$ -siRNA. ER $\alpha$ -siRNA treatment suppressed CYP1B1 expression in MCF-7 cells under hypoxia (Fig. 2e). Next, ChIP analysis confirmed the binding of ER $\alpha$  to ERE in the CYP1B1 promoter region under hypoxia (Fig. 2f). These results suggest that hypoxia-activated ER $\alpha$  can induce CYP1B1 expression by binding the CYP1B1 promoter in ER $\alpha$ -positive MCF-7 cells.

#### *HIF-1 $\alpha$ interacts with ER $\alpha$ in MCF-7 cells*

To further confirm that ER $\alpha$  regulates CYP1B1 expression by hypoxia, we evaluated the effect of hypoxia in two types of breast cancer cells, namely, MDA-MB-231 and MCF-7 with different ER $\alpha$  expression. HIF-1 $\alpha$  expression in MDA-MB-231 cells was induced by hypoxia but not in MCF-7 cells with high ER $\alpha$  expression (Fig. 3a). Also, there was no change in ER $\alpha$  expression when MCF-7 cells were incubated for 0–60 min under hypoxia (Fig. 3b). Therefore, it was established that the ER $\alpha$  expression is not regulated by hypoxia.

As ER $\alpha$  expression is not regulated by HIF-1 $\alpha$ , we investigated the association between HIF-1 $\alpha$  and ER $\alpha$  by a co-immunoprecipitation assay using cell lysates of MCF-7 cells cultured under hypoxic conditions. Cell lysates under normoxic and hypoxic conditions were immunoprecipitated using ER $\alpha$  antibodies, and the immunoprecipitates were subjected to immunoblot analysis using HIF-1 $\alpha$  antibodies. HIF-1 $\alpha$  and ER $\alpha$  were bound in MCF-7 cells under hypoxia (Fig. 3c). To identify the binding domain of ER $\alpha$  required for interaction with HIF-1 $\alpha$ , M2H assay was performed using ER $\alpha$  truncated mutants. ER $\alpha$  has three functional domains—activation domain 1 (AF1; 1-180 aa), DNA binding domain (DBD; 180-302 aa), and activation domain 2 (AF2; 302-595 aa) (Fig. 3d). To determine the sites required for binding between ER $\alpha$  and HIF-1 $\alpha$ , a deletion construct of these regions (pBind-ER $\alpha$  deletion construct) was designed. Luciferase

activity by the interaction of HIF-1 $\alpha$  with ER $\alpha$  AF2 (LBD) was approximately 2.5-fold higher than that using vectors containing AF1 or DBD (Fig. 3d). LBD is a region with hormone binding sites, homo- and hetero-dimerization interfaces, and ligand-dependent co-regulator binding [29]. These findings suggest that HIF-1 $\alpha$  and ER $\alpha$  physically interact with each other under hypoxia in MCF-7 cells via the LBD of ER $\alpha$  (Fig. 3e).

#### *HIF-1 $\alpha$ induces ER $\alpha$ activation in a ligand-independent manner*

Phosphorylation of phosphatidylinositol-3-kinase (PI3K) [29, 30] and extracellular signal-regulated kinase (ERK) [31, 32] are known to modulate the HIF-1 $\alpha$  signaling pathway. A recent study showed that the cAMP-dependent protein kinase A (PKA) phosphorylates HIF-1 $\alpha$  and increases its activity in cancer cells [33]. Although ER $\alpha$  can be activated by genomic pathways [34], non-genomic pathways mediated by phosphorylation by various kinases are also important in the regulation of ER $\alpha$  activity [35]. Figure 4a shows the phosphorylation sites of ER $\alpha$  protein. Serine 118, 167, and 305 are activated by the kinases ERK, Akt, and PKA, respectively [36-38], and tyrosine 537 is activated by Src [39]. Each of these phosphorylation sites is responsible for E2-independent activation and non-genomic pathways [40]. To investigate whether ER $\alpha$  is activated by hypoxia-induced HIF-1 $\alpha$  protein signaling cascade, we analyzed the degree of ER $\alpha$  phosphorylation in lysates of cells cultured under hypoxia by immunoblotting. When MCF-7 cells were incubated under hypoxia lead to phosphorylation of ER $\alpha$  at S118, 167, and 305 in a time-dependent manner (Fig. 4b). These kinases showed increased activation in a time-dependent manner in MCF-7 cells under hypoxia for up to 60 min (Fig. 4c). To further validation of the upstream signaling pathways involved in hypoxia, we evaluated the effects of several kinase inhibitors on CYP1B1 protein expression. PD, LY, and H-89, inhibitors of ERK, Akt, and PKA, respectively, decreased hypoxia-induced CYP1B1 expression (Fig. 4d). Moreover, these inhibitors reduced hypoxia-mediated CYP1B1 mRNA expression and CYP1B1 promoter luciferase activity in breast cancer cells under hypoxia. Thus, hypoxia induces ligand-independent ER $\alpha$  activation as a non-genomic mechanism, as well as the binding of ER $\alpha$  and HIF-1 $\alpha$  (Fig. 4f). PI3K and ERK are known to be hypoxia-induced modulators of HIF-1 $\alpha$  signaling pathways.

#### *HIF-1 $\alpha$ positively correlates with CYP1B1 expression in breast cancer patients*

To clinically evaluate the correlation between HIF-1 $\alpha$  and CYP1B1, we performed immunofluorescence analysis using a breast cancer patient TMA. In the TMA specification sheet, 10 normal breast tissues and 100 breast cancer tissues were classified according to the immunohistochemical (IHC) notation of the ER (“-” indicates ER-negative and “+” indicates ER-positive; Supple Fig. 1a, b and Supple Table 1). Fluorescence intensity results of CYP1B1 and HIF-1 $\alpha$  in 100 breast cancer tissue samples from TMA (Supple Fig. 1c, d, Supple Tables 2, 3) were scored using the ImageJ software (National Institutes of Health, Bethesda, MD, USA). The fluorescence intensity of CYP1B1 and HIF-1 $\alpha$  in ER-positive breast cancer tissue was higher than that in ER-negative breast cancer tissue (Fig. 5a). Pearson's correlation analysis was performed on ER-positive TMA samples (N = 64), and there was a significant positive correlation between CYP1B1 and HIF-1 $\alpha$  levels ( $p < 0.0001$ ,  $r^2 = 0.26$ ) (Fig. 5b). When ER-positive breast

cancer tissues were divided into low (IHC score: 1+, 2+) and high (IHC score: 3+) expression groups according to ER expression status (TMA specification sheet), the expression of HIF-1 $\alpha$  was higher in benign tissues (Supple Fig. 1e). Next, after separating groups using the 25th or 75th percentile of HIF-1 $\alpha$  as a cutoff, the expression of CYP1B1 was established to be significantly increased in ER-positive tissues with high HIF-1 $\alpha$  expression (Fig. 5c).

To validate the clinical significance of HIF-1 $\alpha$  and CYP1B1 expression in ER-positive breast cancer patients, further analysis was performed using the breast cancer patient transcriptional public data set (cBioPortal study IDs: brca\_metabric, pub2015 and TCGA). After classifying ER-positive patients in all three data sets, a positive correlation between HIF1 $\alpha$  and CYP1B1 expression was confirmed (Supple. Fig. 2a, B, C). Additional analysis was performed based on the brca\_metabric dataset with the largest number of patient data. Analysis of 1459 ER-positive patients from the brca\_metabric dataset showed a statistically significant positive correlation between the expression of CYP1B1 and HIF-1 $\alpha$  (Fig. 6a). In this dataset (metabric), patients with high CYP1B1 and HIF-1 $\alpha$  expression were classified as ER high (N = 26, top 25%) and ER low (N = 33, bottom 25%) based on ER expression. Of the 18 042 genes expressed in this patient group, 304 genes (DEG; q-value < 0.01, 0.58-|Log<sub>2</sub>(FC)|) that changed in the ER low versus ER high group were identified (red spots, increase = 136 and blue spots, decrease = 168) (Fig. 6b). Gene ontology (GO) and enrichment analysis (metascape) were performed based on the differentially expressed genes (DEG) of the ER/HIF1 $\alpha$ /CYP1B1 triple high group. Genes associated with tumor recurrence were identified at the top and visualized as a percentage (% of number) and -Log<sub>10</sub> (q-value) of shared genes (Fig. 6c, Supple Table 4 and Supple Fig. 3a); 43 such genes, including cell cycle-related genes such as aurora kinase, associated with tumor recurrence were analyzed by hierarchical clustering and visualized as heat maps (Supple. Fig. 3b, Supple. Table 5). We also found that the survival rate is significantly lower in patients with high ER/HIF1 $\alpha$ /CYP1B1 breast cancer (Fig. 6d). The pub2015 and TCGA patient datasets, as well as the metabric dataset, showed a remarkable correlation between high ER/HIF1 $\alpha$ /CYP1B1 and lower survival rates and cancer recurrence in breast cancer patients (Supple. Fig. 4a and b). Taken together, our results suggest that the overexpression of HIF-1 $\alpha$  and CYP1B1 in ER-positive breast cancer patients is associated with negative clinical outcomes.

## Discussion

Approximately 70% of breast cancers are ER-positive [41] and treated with anti-hormonal therapy; therefore, the recurrence of breast cancer is high and significantly negatively impacts patient survival. Although many studies have been conducted to find biomarkers for breast cancer recurrence [42, 43], recurrence and metastasis remain unresolved. Previous studies have shown that HIF-1 $\alpha$  is overexpressed in advanced breast cancer and that the hypoxic tumor microenvironment [44] is involved in tumorigenesis, refractory cancer, and recurrence [13-15, 45]. Therefore, several studies have developed drugs that target the HIF-1 $\alpha$  signaling pathway. Inhibition of HIF-1 $\alpha$  by YC-1 is known to decrease cell growth and metastasis in breast cancer [46]. In addition, antiangiogenic therapy that suppresses hypoxia

is an effective treatment approach because hypoxia can induce tumor progression and metastasis [47-49].

CYP1B1 is an E2 hydroxylase enzyme involved in the production of DNA damage inducers through estrogen biosynthesis and metabolism [22]. Several studies have reported that CYP1B1 is overexpressed in malignancies of various tissue origins, including the breast, colon, lung, brain, skin, prostate, ovarian, and liver cancers [50-54]. As CYP1B1 expression is increased in tumor tissues compared to normal tissues [53, 55, 56], CYP1B1 has garnered considerable research attention as a potential therapeutic target in tumors [57].

Although the individual association of HIF-1 $\alpha$  and CYP1B1 with tumorigenesis is well known, to the best of our knowledge, their correlation has not been studied in breast cancer. We investigated the relationship between CYP1B1 expression and HIF-1 $\alpha$  in human ER $\alpha$ -positive breast cancer. We found that hypoxia-induced CYP1B1 expression occurred only in ER $\alpha$ -positive breast cancer MCF-7 cells. HIF-1 $\alpha$  can bind ER $\alpha$  directly, and this complex translocates from the cytoplasm to the nucleus and binds the ERE site of the CYP1B1 promoter in MCF-7 cells. We also found that hypoxia activates ER $\alpha$  in a ligand-independent manner in the absence of E2 through the activation of signaling kinases (Fig. 7). Moreover, immunofluorescence analysis of tissue microarrays from ER-positive breast cancer patients confirmed a positive correlation between HIF-1 $\alpha$  and CYP1B1. A positive correlation between cancer recurrence and mortality and high ER $\alpha$ , HIF-1 $\alpha$ , and CYP1B1 expression was also confirmed in a dataset of breast cancer patients.

A limitation of this study is that we were unable to verify whether inhibition of HIF-1 $\alpha$  and CYP1B1 inhibited cancer recurrence in recurrent ER $\alpha$ -positive breast cancer patients. Our findings provide the foundation for further studies and promote the use of HIF-1 $\alpha$  and CYP1B1 as therapeutic targets in the treatment of recurrent tumors.

## Conclusions

We report the mechanism by which hypoxia-induced HIF-1 $\alpha$  regulates CYP1B1 through the activation of ER $\alpha$  in breast cancer cells and the role of hypoxia and ER $\alpha$  positivity in tumor relapse. Our findings confirm the role of hypoxia in the pathogenesis and progression of ER $\alpha$ -positive breast cancer and suggest that the regulation of CYP1B1 expression under hypoxic tumor conditions can be targeted to overcome cancer recurrence, especially as an alternative to conventional hormone therapy.

## Abbreviations

ChIP, chromatin immunoprecipitation; Co-IP, Co-immunoprecipitation; Ct, cycle threshold; DMSO, dimethylsulfoxide; DEG, differentially expressed gene; ECL, enhanced chemiluminescence; FBS, fetal bovine serum; GO, Gene ontology; IHC, immunohistochemical; LBD, ligand-binding domain; M2H,

mammalian two-hybrid; PCR, polymerase chain reaction; siRNA, small interfering RNA; SEM, standard error of the mean; TCGA, The cancer genome atlas; TMA, tissue microarray

## **Declarations**

### **Acknowledgements**

We would like to thank Editage ([www.editage.co.kr](http://www.editage.co.kr)) for English language editing.

### **Funding**

This work was supported by the National Research Foundation of Korea (NRF), through grants funded by the Korean Ministry of Science and Information Technology (MSIT) (No. NRF-2020R1A2C1007764, NRF-2020R1C1C1012542), the National Research Council of Science & Technology (NST) (No. CAP-18-02-KRIBB), Korea Food Research Institute (No. E0210203) and the Korea Basic Science Institute (No. K115000, C170100 and C170300).

### **Authors' contributions**

EHH and HGJ conceived and designed the studies. JYM, GHL, TK, SWJ, HGK, JYH, and EHH performed the biochemical and cell-based experiments. JYM and SYL analyzed the human patient data. JYM, GHL, TK, SWJ, HGK, JYH, YHC, SKH, EHH, and HGJ analyzed the data. EHH and HGJ drafted the manuscript, and all authors contributed to figure and data preparation.

### **Ethics approval and consent to participate**

Ethical approval for the study was not required due to the retrospective nature of this study using only publicly available data.

### **Consent for publication**

Not applicable

### **Competing interests**

The authors declare that they have no competing interests.

### **Availability of data and materials**

The METABRIC dataset analyzed principally in the current study is publicly available in the cBioPortal repository ([https://www.cbioportal.org/study/summary?id=brca\\_metabric](https://www.cbioportal.org/study/summary?id=brca_metabric)). RNA sequencing datasets further analyzed from Supple Fig. 2b, c and 3 are publicly available on the cBioPortal at the following link: pub2015; Cell 2015; [https://www.cbioportal.org/study/summary?id=brca\\_tcga\\_pub2015](https://www.cbioportal.org/study/summary?id=brca_tcga_pub2015) and TCGA;

Firehose Legacy; [https://www.cbioportal.org/study/summary?id=brca\\_tcga](https://www.cbioportal.org/study/summary?id=brca_tcga). The breast cancer TMA dataset analyzed in this study is available in the Supplementary information file. H&E image of Supple Fig. 1b is available in original version at <https://www.biomax.us/index.php?route=product/zoomify&park=59428>.

## Authors' information

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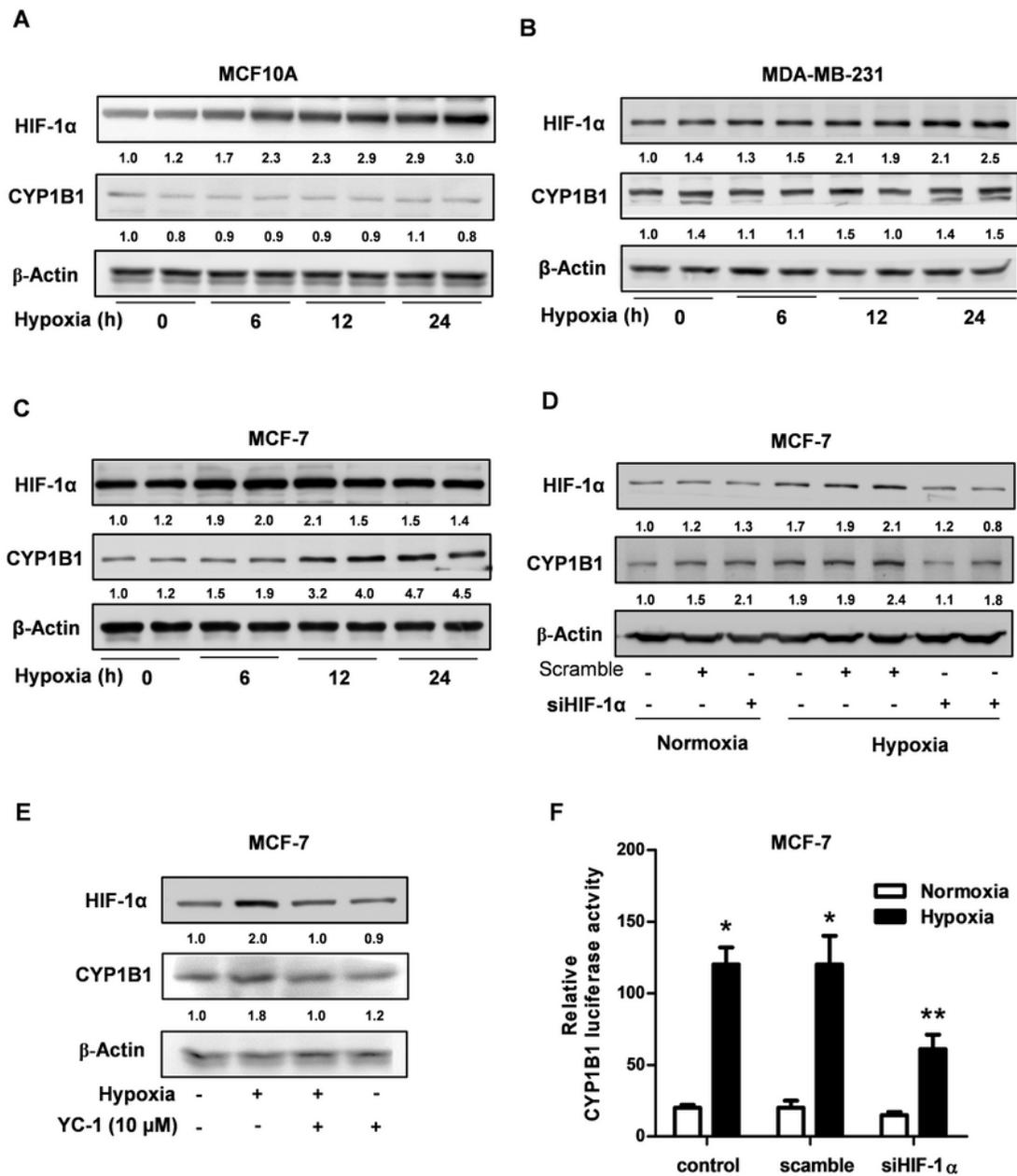
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## Figures

**Fig 1.**

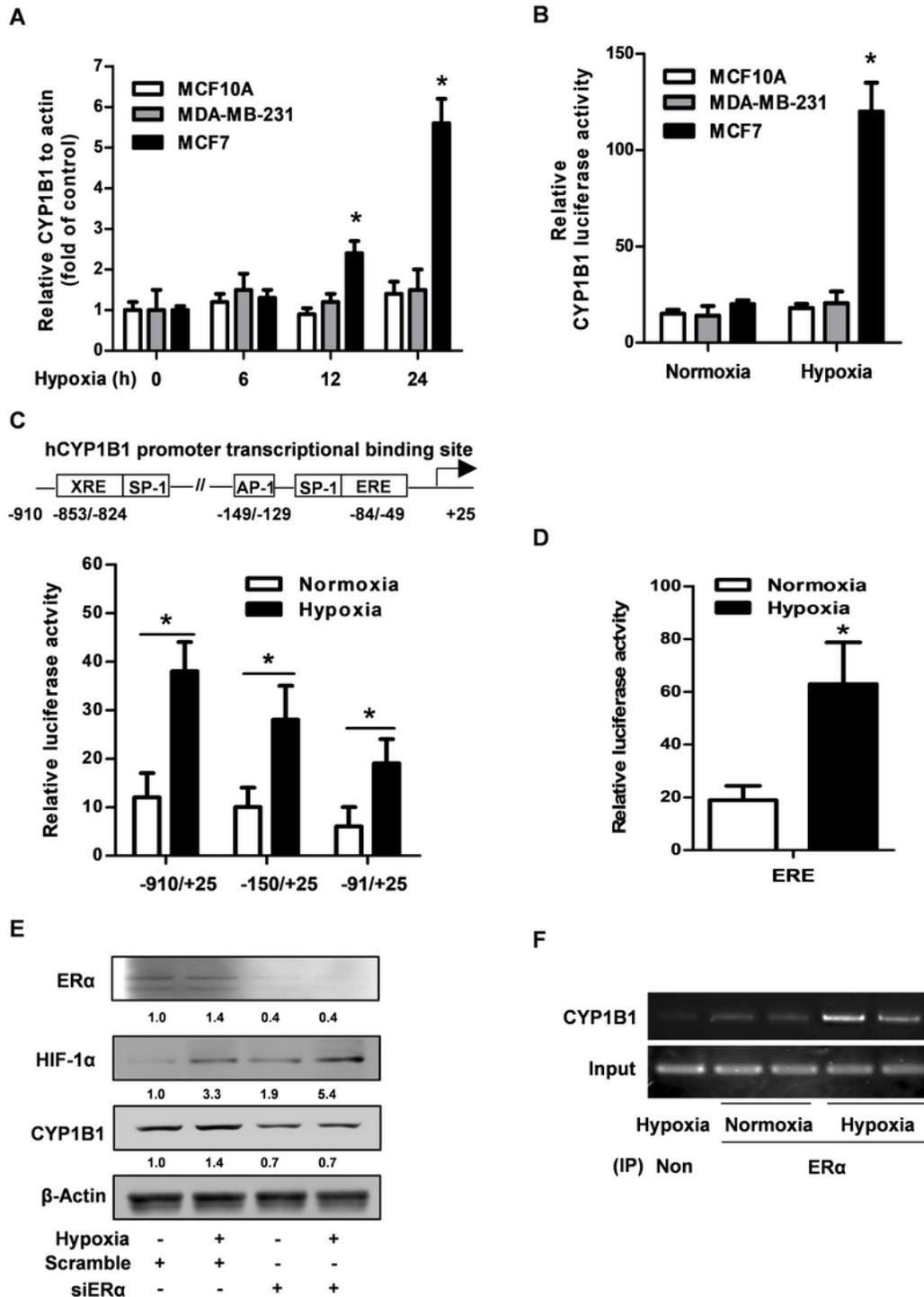


**Figure 1**

Effect of hypoxia on CYP1B1 and HIF-1α expression. CYP1B1 and HIF-1α expression were determined by immunoblot analysis in lysates of MCF10A (a), MDA-MB-231 (b), and MCF-7 (c) cells cultured for 0, 6, 12, and 24 h under hypoxia. (d) Validation of hypoxia-induced HIF-1α-mediated CYP1B1 expression in MCF-7 cells. HIF-1α and CYP1B1 levels in whole-cell lysates after 24 h incubation in normoxia or hypoxia in cells transiently transfected with scrambled or HIF-1α siRNA were detected by immunoblotting. (e) Further

validation of HIF-1  $\alpha$ -mediated induction of CYP1B1 expression in MCF-7 cells exposed to YC-1 (10  $\mu$ M) and incubated for 24 h under hypoxia by immunoblotting. (f) Effect of hypoxia on CYP1B1-promoter luciferase activity in MCF-7 cells transfected with CYP1B1-Luc and HIF-1 $\alpha$  or scrambled siRNA and incubated under hypoxic and normoxic conditions or for 24 h. \*,  $p < 0.01$  vs. normoxia and \*\*,  $p < 0.01$  vs. hypoxia control transfected cells, determined by Newman–Keuls test.

**Fig 2.**



**Figure 2**

Identification of promoter binding sites involved in hypoxia-mediated CYP1B1 expression. (a) Comparison of CYP1B1 mRNA expression in different breast cells under hypoxic and normoxic conditions. \*,  $p < 0.01$  versus normoxic control cells, determined by Newman–Keuls test. (b) Comparison of CYP1B1 promoter activity in cells cultured under normoxia or hypoxia for 24 h after transformation with CYP1B1-Luc and pCMV- $\beta$ -gal. \*,  $p < 0.01$  versus normoxic control cells, determined by Newman–Keuls test. (c) Schematic diagram of the transcription factor binding site in the human CYP1B1 promoter (above). Identification of promoter binding sites involved in hypoxia-mediated CYP1B1 expression in MCF-7 cells (below) transfected with CYP1B1 promoter deletion constructs (- 910/+25, - 150/+25 and - 91/+25) and pCMV- $\beta$ -gal, and incubated under normoxia or hypoxia for 24 h. \*,  $p < 0.01$  versus normoxic control, Newman–Keuls test. (d) ERE activity in MCF-7 cells transfected with ERE-Luc and pCMV- $\beta$ -gal vectors, incubated under normoxic or hypoxic conditions for 24 h. \*,  $p < 0.01$ , by Newman–Keuls test. (e) Immunoblot analysis for measuring ER $\alpha$ , HIF-1 $\alpha$ , and CYP1B1 expression for the validation of the role of ER $\alpha$  on hypoxia-induced CYP1B1 expression in MCF-7 cells transfected with scrambled- or ER $\alpha$ -siRNA, cultured under normoxic or hypoxic conditions for 24 h. (f) ChIP assay was performed using the ER $\alpha$  antibody to verify the role of ER $\alpha$  on hypoxia-induced CYP1B1 expression in cells cultured under normoxic or hypoxic conditions and the ERE region of the CYP1B1 promoter was amplified by PCR.

Fig 3.

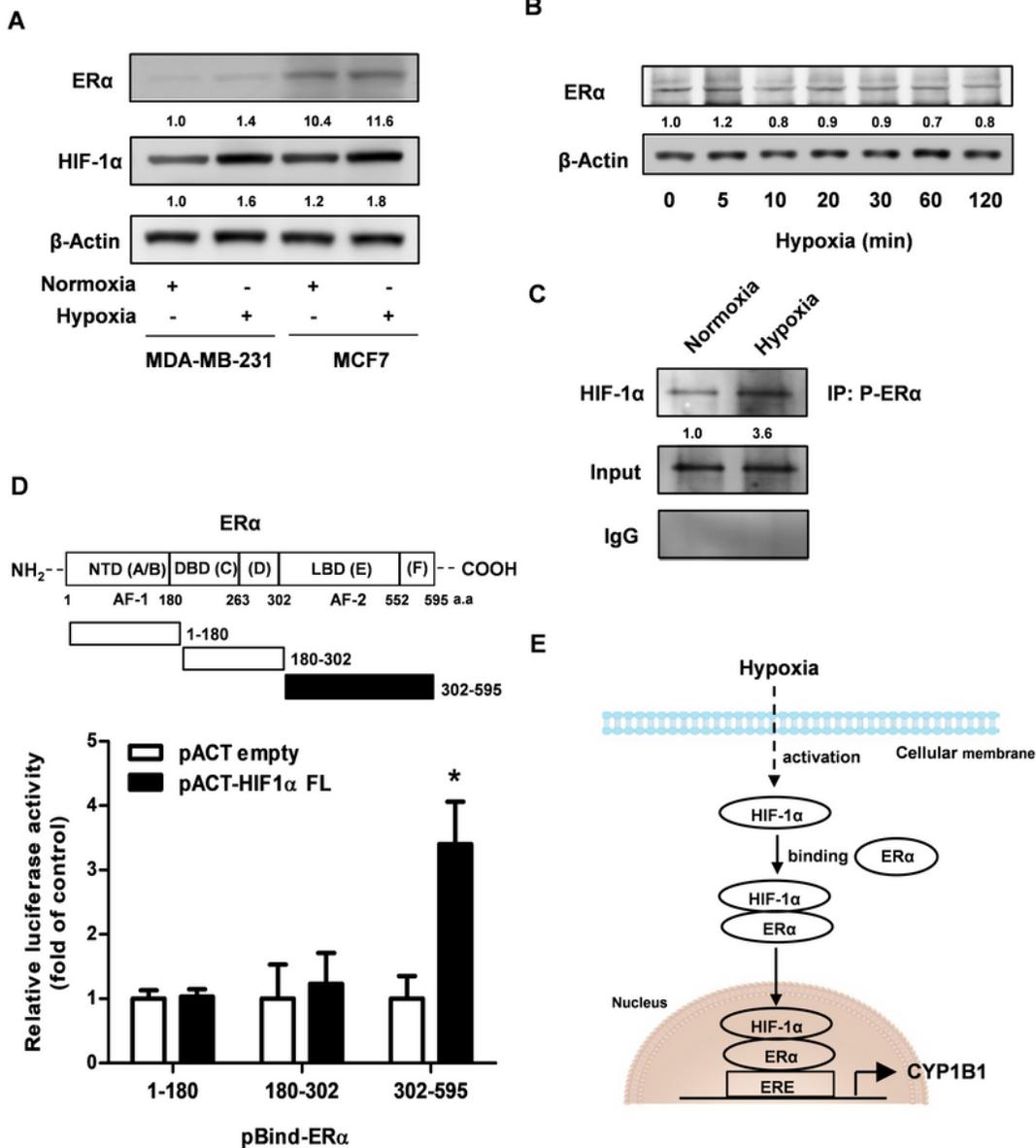
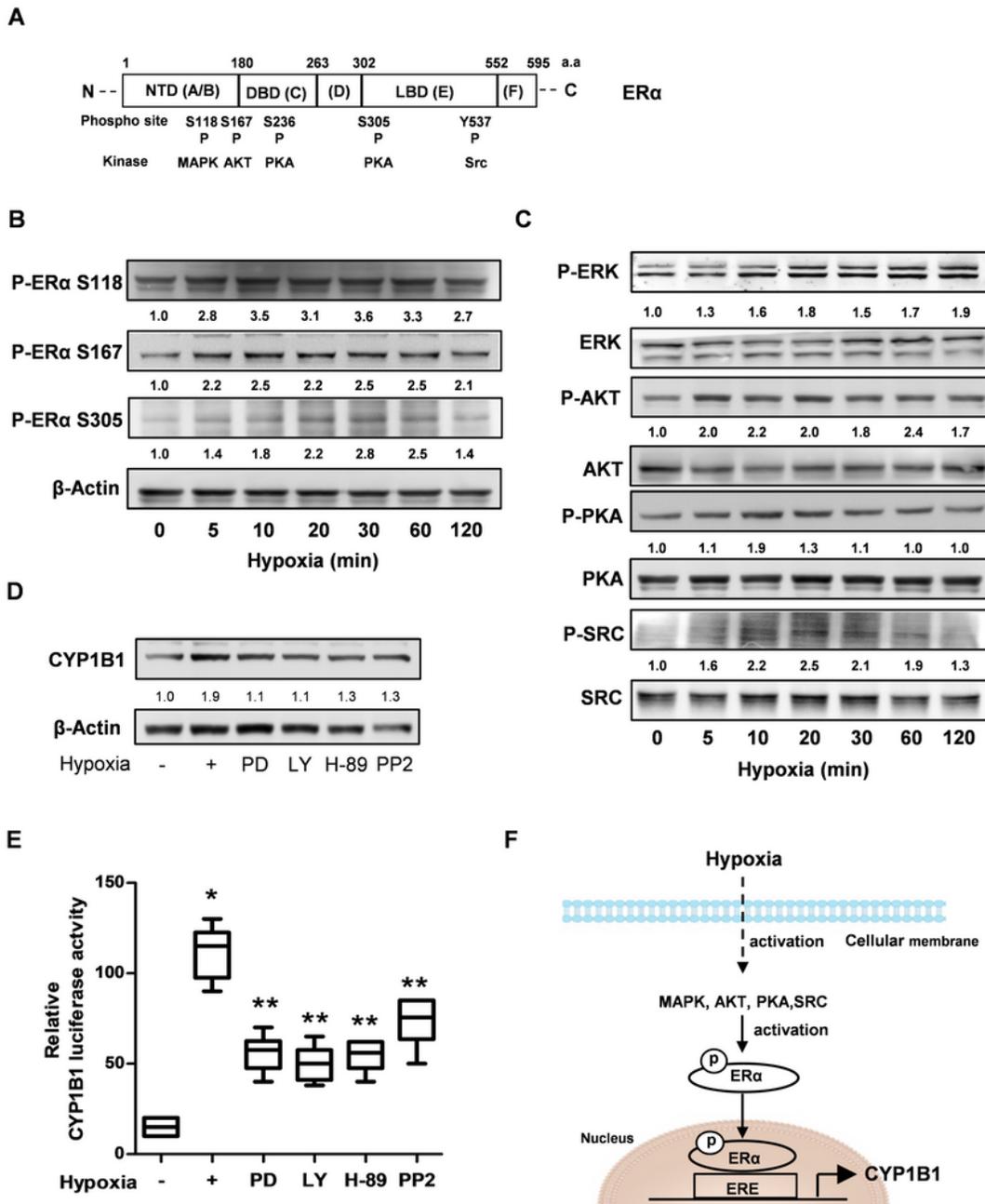


Figure 3

Validation of the interaction between HIF-1 $\alpha$  and ER $\alpha$ . (a) Expression of HIF-1 $\alpha$  and ER $\alpha$  confirmed by immunoblot in cell lysates of MDA-MB-231 and MCF-7 cells cultured under hypoxic or normoxic conditions for 24 h. (b) ER $\alpha$  expression under hypoxia in MCF-7 cells incubated for 0–60 min under hypoxia. (c) Validation of HIF-1 $\alpha$  and ER $\alpha$  binding in whole-cell lysates of MCF-7 cells cultured under hypoxic or normoxic conditions and immunoprecipitated with p-ER $\alpha$  antibody, followed by co-IP analysis

by immunoblotting with HIF-1 $\alpha$  antibody. (d) Schematic representation of the functional domains of ER $\alpha$  and deletion regions (top). Analysis of the ER $\alpha$  domain that binds HIF-1 $\alpha$  by mammalian two-hybrid (M2H) assay (below) In MCF-7 cells transfected with pBIND-ER $\alpha$  constructs (1-180, 180-302 or 302-595), empty pACT vector, or pACT-HIF-1 $\alpha$ , and incubated under hypoxia for 24 h. \*,  $p < 0.01$  vs. empty vector control determined by Newman-Keuls test. (e) Schematic diagram of HIF-1 $\alpha$  and ER $\alpha$ -mediated signaling regulating hypoxia-induced CYP1B1 expression.

**Fig 4.**



## Figure 4

ER $\alpha$  and CYP1B1 transcriptional activity by hypoxia-mediated kinase phosphorylation. (a) Schematic representation of the activation sites of ER $\alpha$ . (b) Analysis of ER $\alpha$  phosphorylation in cells under hypoxia by immunoblotting using the phospho-ER $\alpha$  antibody. (c) Immunoblot analysis showing activation of kinase ERK, Akt, PKA, and SRC by phosphorylation in cells incubated for 0-60 min under hypoxia in a time-dependent manner. (d) Decreased hypoxia-mediated CYP1B1 expression under the influence of ERK, Akt, PKA, and SRC kinase inhibitors (ERK [PD; 10  $\mu$ M], Akt [LY; 5  $\mu$ M], PKA [H-89, 10  $\mu$ M], or SRC [PP2, 20  $\mu$ M]) in cells under hypoxia for 24 h. (e) Hypoxia-mediated CYP1B1 promoter luciferase activity under the influence of ERK, Akt, PKA, and SRC kinase inhibitors (ERK [PD; 10  $\mu$ M], Akt [LY; 5  $\mu$ M], PKA [H-89, 10  $\mu$ M], or SRC [PP2, 20  $\mu$ M]) in cells transfected with CYP1B1-Luc and subjected to hypoxia for 24 h. \*,  $p < 0.01$  versus normoxic control cells and \*,  $p < 0.01$  versus control treated cells under hypoxia, Newman–Keuls test. (f) Schematic diagram of CYP1B1 regulation by hypoxia-induced kinase activation-mediated ER $\alpha$  signaling.

Fig 5.

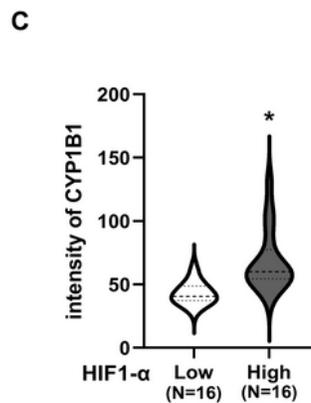
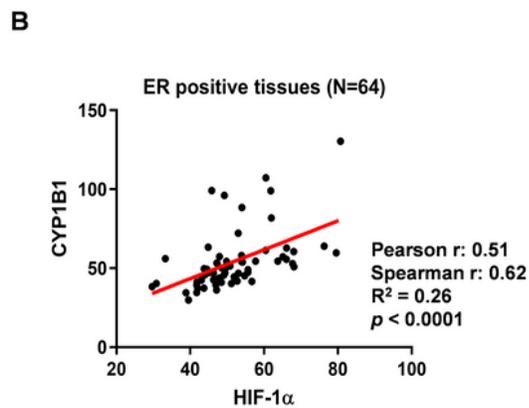
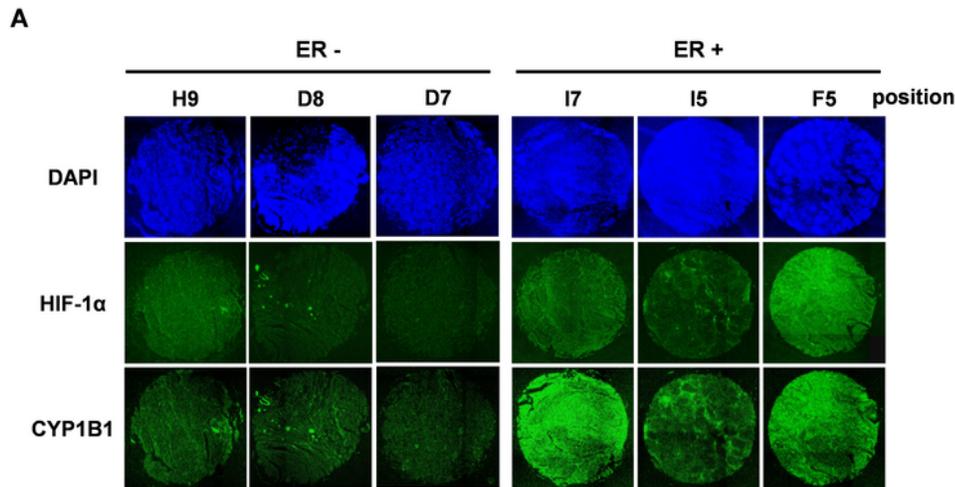
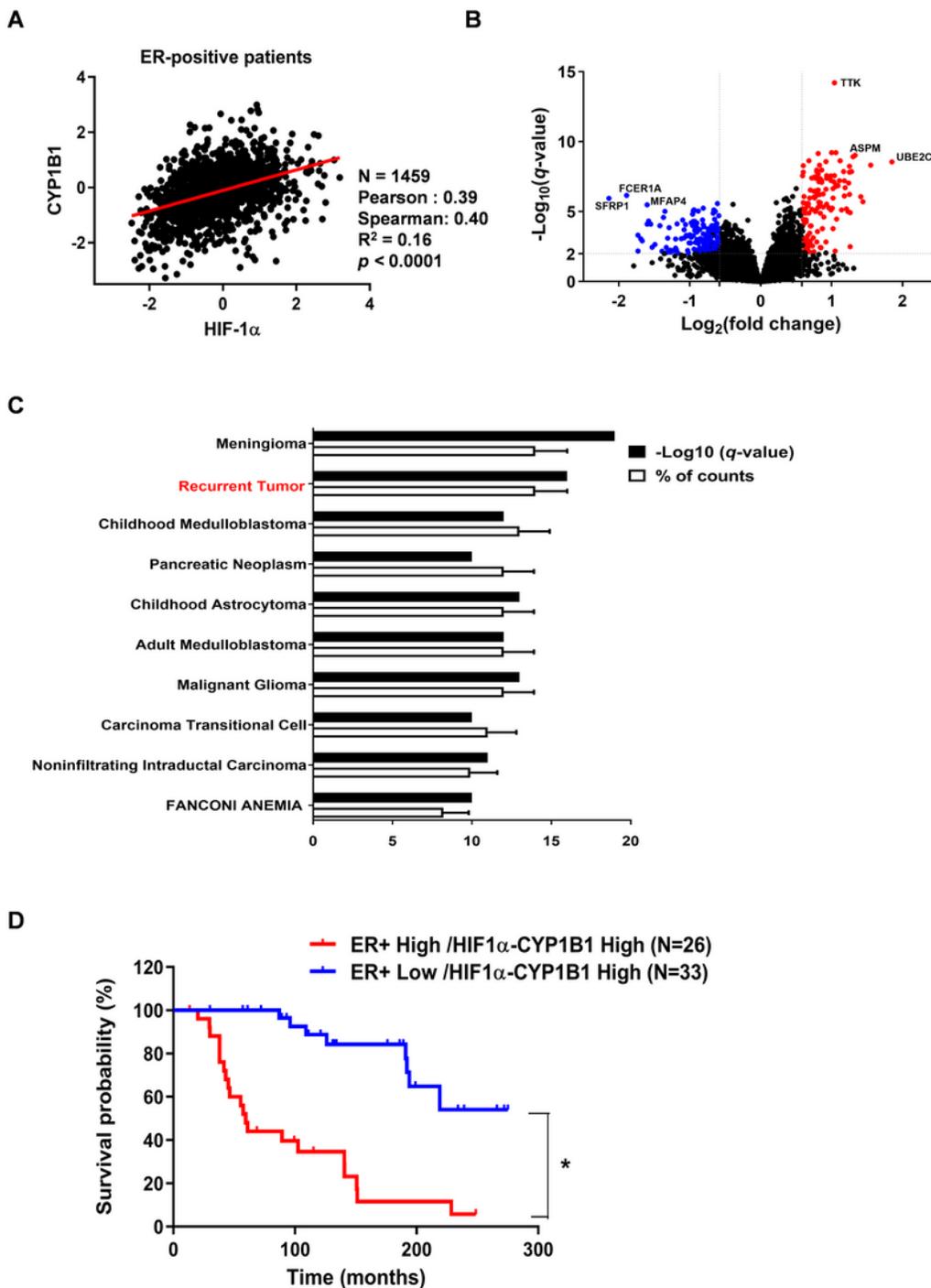


Figure 5

Analysis of HIF-1α and CYP1B1 expression in tissue microarray (TMA) samples from breast cancer patients. (a) Representative images of immunofluorescence analysis of HIF-1α (green) and CYP1B1 (green) in two slides of ER-negative and positive breast cancer TMA. Immunofluorescence images were acquired using a Leica Thunder microscope. (b) Scatter plots showing fluorescence intensity correlation analysis of HIF-1α and CYP1B1 in 64 ER-positive TMAs. (c) Analysis of fluorescence intensity of CYP1B1

against HIF1a expression in ER-positive TMA. The fluorescence intensity of CYP1B1 was analyzed by classifying the fluorescence intensity of HIF1- $\alpha$  based on the top and bottom 25% ER expression. \*,  $p < 0.0001$  versus ER-positive tumor tissue with low HIF1- $\alpha$  fluorescence intensity by Mann-Whitney test.

**Fig 6.**



**Figure 6**

Clinical significance analysis of CYP1B1 and HIF-1 $\alpha$  using the transcriptome dataset of breast cancer patients. (a) Scatter plots showing HIF-1 $\alpha$  and CYP1B1 mRNA expression in ER-positive breast cancer patients. Log<sub>2</sub>-transformed values of mRNA levels were used as z-scores for all samples. (b) Volcano plot of differentially expressed genes (DEGs) of the ER high HIF-1 $\alpha$ /CYP1B1 high breast cancer patient group and the ER low HIF-1 $\alpha$ /CYP1B1 high group. By significance (q-value < 0.01, |Log<sub>2</sub>(FC)| > 0.58), red spot indicates upregulated genes and blue spot indicates downregulated genes. (c) Gene ontology analysis of DEGs in the ER/HIF1 $\alpha$ -CYP1B1 high group. Bar charts visualizing the percentage of shared genes (% of number) and -Log<sub>10</sub> (q-value). (d) Kaplan–Meier survival rate analysis of patients with ER high HIF-1 $\alpha$ /CYP1B1 high breast cancer than the ER low HIF-1 $\alpha$ /CYP1B1 high group. \*,  $p < 0.05$  vs. ER-positive cancer patients with low ER expression.

Fig 7.

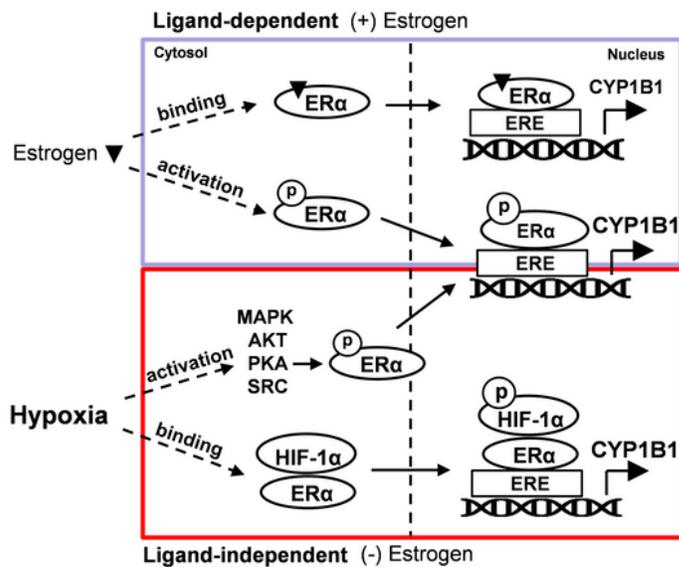


Figure 7

Mechanism of hypoxia-mediated CYP1B1 upregulation through ER activation. In the presence of estrogen as an ER ligand, estrogen directly binds ER or induces CYP1B1 expression through ER activation through a ligand-dependent pathway. Under hypoxia, even in the absence of estrogen, a ligand-independent pathway can be induced in ER-positive breast cancer cells by the activation of kinases that phosphorylate ER or by binding of increased HIF-1 $\alpha$  to ER to induce CYP1B1 expression.

## Supplementary Files

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