Am J Cancer Res 2022;12(6):2798-2816
www.ajcr.us /ISSN:2156-6976/ajcr0143036

Original Article

Upregulation of CYP1B1 by hypoxia is mediated by ERα activation in breast cancer cells

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Received March 22, 2022; Accepted May 24, 2022; Epub June 15, 2022; Published June 30, 2022

Abstract: 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-1α) and CYP1B1 with tumorigenesis is well known, the association between HIF-1α 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. 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; these cell lines were then subjected to immunoblotting, transient transfection, luciferase assays, gene silencing using small interfering RNA, polymerase chain reaction analysis, chromatin immunoprecipitation, co-immunoprecipitation, and mammalian two-hybrid assays. Furthermore, immunofluorescence analysis of the tumor microarrays was performed, and the pub2015 and the Cancer Genome Atlas patient datasets were analyzed. HIF-1α expression in response to hypoxia occurred in both normal and breast cancer cells, whereas CYP1B1 was induced only in estrogen receptor α (ERα)-positive breast cancer cells under hypoxia. HIF-1α activated ERα through direct binding and in a ligand-independent manner to promote CYP1B1 expression. Therefore, we suggested the mechanism by which hypoxia and ER-positivity orchestrate breast cancer relapse.

Keywords: CYP1B1, hypoxia, hypoxia-inducible factor-1-alpha, estrogen receptor, breast cancer, tumorigenesis, tumor relapse, tumor microarray

Introduction

Breast cancer is one of the most prevalent cancers in women, and malignant breast cancer is known to be the leading cause of cancer-related deaths in women worldwide [1]. Breast cancer is classified depending on the expression of estrogen receptor alpha (ERα), epidermal growth factor receptor 2, and progesterone receptor; the lack of expression of all three receptors implies 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 ERα positive and are usually treated with anti-hormonal therapy [5]. Several molecules have been developed for hormone neutralization in women with ERα-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 resistance to these therapeutics are not well understood, and there is a lack of therapeutic targets to overcome tumor relapse.

Hypoxia-induced hypoxia-inducible factor-1α (HIF-1α) 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-1α, which regulates the expression of several essential genes [9]. Under normoxic conditions, HIF-1α protein is ubiquitinated and degraded; however, under hypoxia, it is stabilized and translocated to the nucleus by HIF-1 beta (β) dimerization [10-12]. Several studies have reported that the overexpression of HIF-1α is associated with the initiation, progression, and recurrence of breast cancer [8, 13-15].

Several studies have shown that metabolites of 17β-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 Erα (estrogen receptor α (ERα)-positive breast cancer cells) and the complex translocates to the nucleus, where it binds the estrogen response element (ERE) site, and induces Cytochrome P450 1B1 (CYP1B1) transcription [18].

CYP1B1, a monooxygenase expressed in the 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 as it induces the metabolites of intracellular E2 along with environmental carcinogens [16, 21-23]. It metabolizes E2 to 4-hydroxy-E2 to generate free radicals forming 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-1α and CYP1B1 has not yet been studied. In this study, we assessed the role of hypoxia-induced HIF-1α in regulating CYP1B1 in breast cancer and the mechanism underlying this interaction. Our findings suggest that HIF-1α is involved in the regulation of CYP1B1 in breast cancer cells and that suppressing HIF-1α-induced CYP1B1 expression in patients with recurrent cancer can be utilized as a novel therapeutic strategy for breast cancer.

Materials and methods

YC-1, PD98059, LY294002, 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-β-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-ERα, and horseradish peroxidase-linked anti-mouse and rabbit IgG were obtained from Cell Signaling Technologies (Danvers, MA, USA). Antibodies against β-actin and CYP1B1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The HIF-1α antibody was obtained from Abnova (Taipei City, Taiwan), and the ERα antibody was obtained from Abcam (Cambridge, UK). The antibody against p-ERα (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% carbon dioxide 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% oxygen 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 dimethyl sulfoxide (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 the proteins were quantified using the Bradford protein assay (Bio-Rad, Irvine, CA, USA). The lysates were electroblotted on polyvinylidene difluoride membranes and were reacted with the primary 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-β-gal using the Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, MA, USA). The CYP1B1 promoter-Luc FL vector (-1635 to +1588) was provided to us 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 the 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-1α and ERα were knocked down using small interfering (si) RNA, and the results were analyzed using real-time quantitative polymerase chain reaction (qPCR), chromatin immunoprecipitation, and immunoblot analysis. siRNAs targeting *HIF-1A* and *ER* messenger (m) RNA and non-targeting siRNAs were purchased from OriGene (OriGene Inc., Rockville, MD, USA). Cells were transiently transfected with HIF-1α-siRNA, ERα-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 β-actin mRNA in MCF7 cells were compared with those in the 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 ERα or non-specific mouse IgG using an EZ CHIP kit (Upstate, Lake Placid, NY, USA). Input experiments were performed using approximately 5% of the sample. 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 HIF-1α antibodies. Antibody-bound beads were washed with PBS, electrophoresed on SDS-PAGE, and then immunoblotted with ERα.

Mammalian two-hybrid (M2H) assay

Vectors (pBIND-ERα 1-180 [N-terminal domain], 180-302 [DNA binding domain, DBD], 302-595 [ligand-binding domain, LBD], pACT-HIF-1α 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 lysates were added to the luminescent white plates. Luciferase activity was measured using a luminometer (SpectraMax M5). Relative reporter assay activity was measured using the pG5-Luc control vector.

Immunofluorescence analysis of tumor microarrays (TMAs)

A human breast cancer tissue microarray (TMA, #BC081120e) containing 110 cases

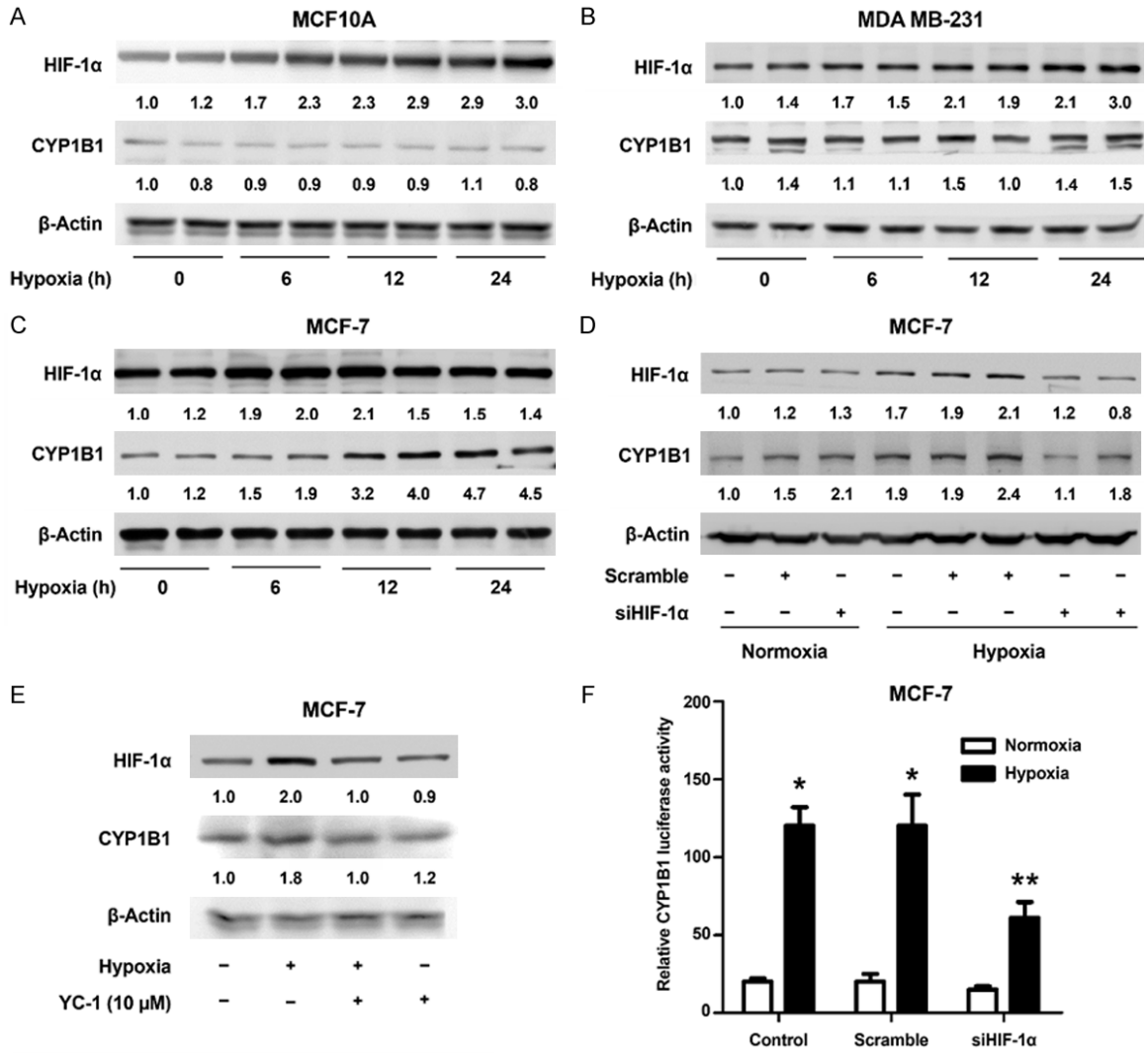


Figure 1. Effects of hypoxia on cytochrome P450 1B1 (CYP1B1) and hypoxia-inducible factor-1-alpha (HIF-1α) expression, were determined by immunoblot analysis in lysates of MCF10A (A), MDA-MB-231 (B), and MCF-7 (C) cells cultured for 0, 6, 12, and 24 h under hypoxia. (D) Validation of hypoxia-induced HIF-1α-mediated CYP1B1 expression in MCF-7 cells. HIF-1α and CYP1B1 levels in whole-cell lysates after 24 h incubation under normoxia or hypoxia in cells transiently transfected with scrambled or HIF-1α small interfering (si) RNA were detected by immunoblotting. (E) Further validation of HIF-1α-mediated induction of CYP1B1 expression in MCF-7 cells exposed to YC-1 (10 μM) 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-1α 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.

was purchased from Biomax (Rockville, MD). The immunofluorescent image was quantified using a quantitative analysis system (Thunder, Leica). Image analysis was performed using LAS-X software (Leica) by acquiring fluorescence images of DAPI, CYP1B1, and HIF-1α 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-1α in ERα-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 its retrospective nature; we used only

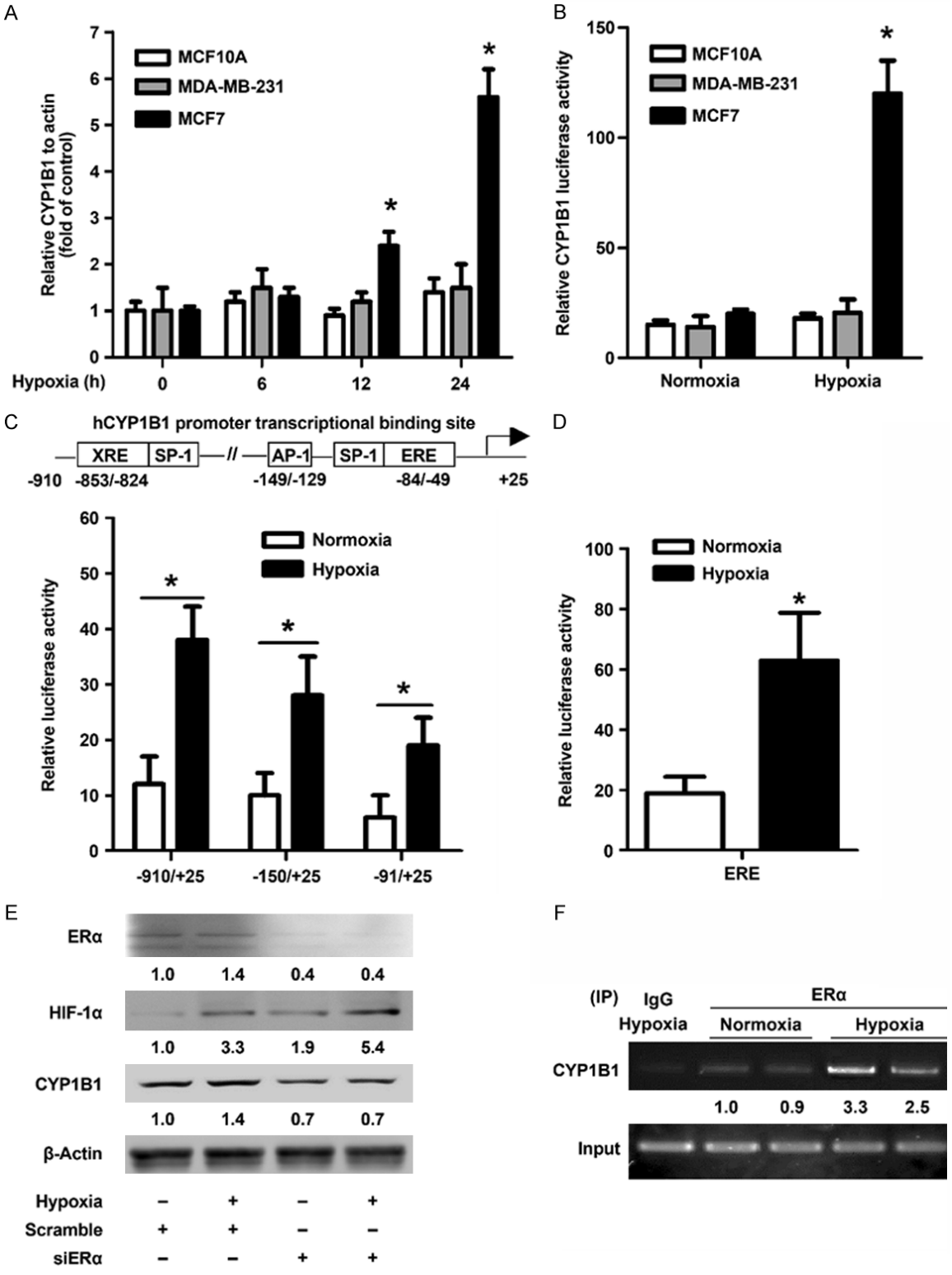


Figure 2. Identification of promoter binding sites involved in hypoxia-mediated CYP1B1 expression. A. Comparison of CYP1B1 messenger (m) RNA expression in different breast cells under hypoxic and normoxic conditions. *, P<0.01 versus normoxic control cells, determined by Newman-Keuls test. B. Comparison of CYP1B1 promoter activity in cells cultured under normoxia or hypoxia for 24 h after transformation with CYP1B1-Luc and pCMV-β-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-me-

diated CYP1B1 expression in MCF-7 cells (below) transfected with CYP1B1 promoter deletion constructs (-910/+25, -150/+25, and -91/+25) and pCMV-β-gal, and incubated under normoxia or hypoxia for 24 h. *, P<0.01 versus normoxic control, Newman-Keuls test. D. Estrogen response element (ERE) activity in MCF-7 cells transfected with ERE-Luc and pCMV-β-gal vectors, incubated under normoxic or hypoxic conditions for 24 h. *, P<0.01, by Newman-Keuls test. E. Immunoblot analysis for measuring estrogen receptor-α (Erα), HIF-1α, and CYP1B1 expression for the validation of the role of ERα on hypoxia-induced CYP1B1 expression in MCF-7 cells transfected with scrambled- or ERα-siRNA, cultured under normoxic or hypoxic conditions for 24 h. F. Chromatin immunoprecipitation (ChIP) assay was performed using the ERα antibody to verify the role of ERα on hypoxia-induced CYP1B1 expression in cells cultured under normoxic or hypoxic conditions and the ERE region of the CYP1B1 promoter was amplified by polymerase chain reaction (PCR).

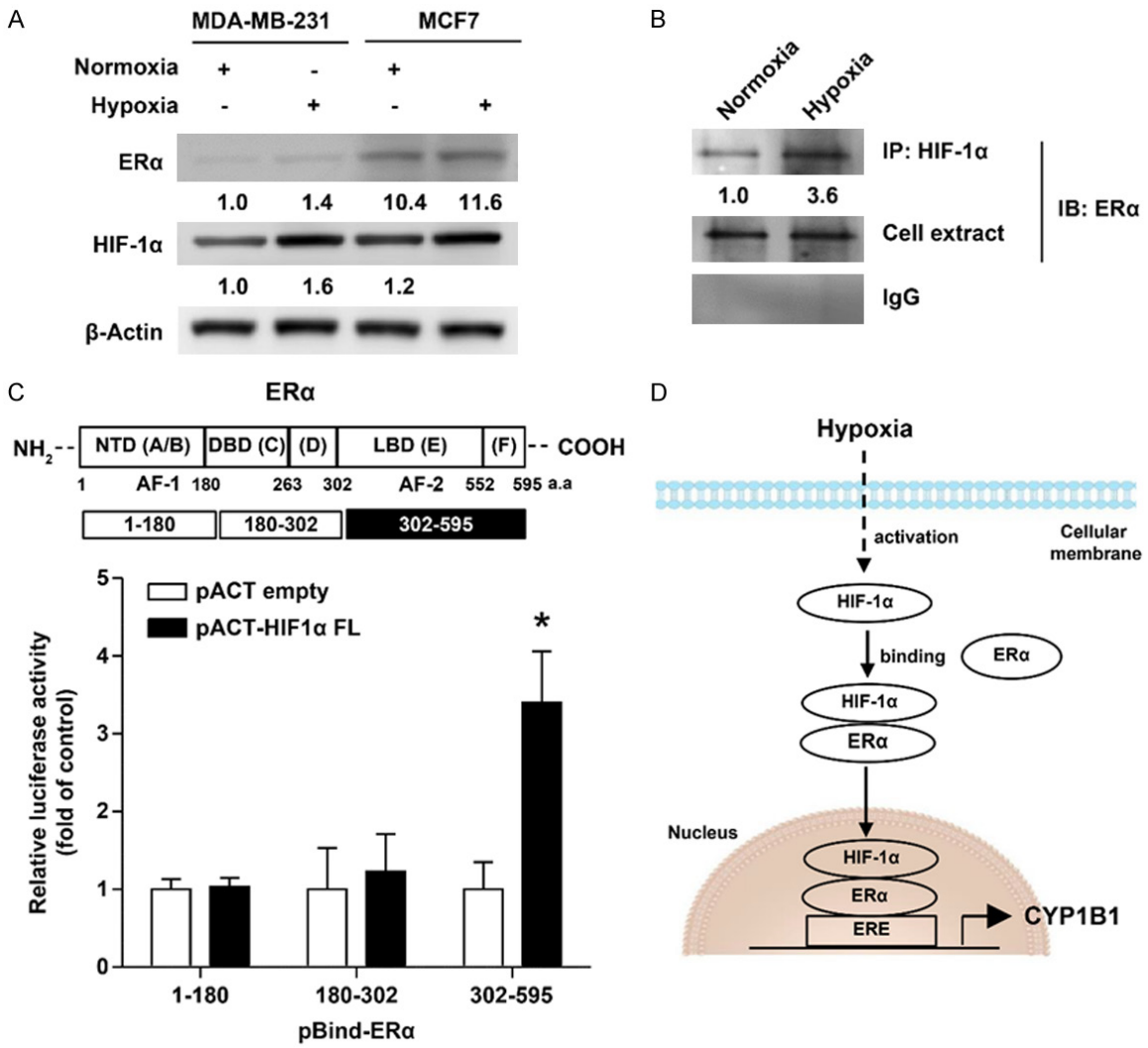


Figure 3. Validation of the interaction between HIF-1α and ERα. A. Expression of HIF-1α and ERα confirmed by immunoblot in cell lysates of MDA-MB-231 and MCF-7 cells cultured under hypoxic or normoxic conditions for 24 h. B. Validation of HIF-1α and ERα binding in whole-cell lysates of MCF-7 cells cultured under hypoxic or normoxic conditions and immunoprecipitated with HIF-1α antibody, followed by co-IP analysis by immunoblotting with ERα antibody. C. Schematic representation of the functional domains of ERα and deletion regions (top). Analysis of the ERα domain that binds HIF-1α by mammalian two-hybrid (M2H) assay (below) in MCF-7 cells transfected with pBIND-ERα constructs (1-180, 180-302 or 302-595), empty pACT vector, or pACT-HIF-1α, and incubated under hypoxia for 24 h. *, P<0.01 vs. empty vector control determined by Newman-Keuls test. D. Schematic diagram of HIF-1α and ERα-mediated signaling regulating hypoxia-induced CYP1B1 expression.

publicly available data. Transcriptome analysis data and the pathological data of the 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

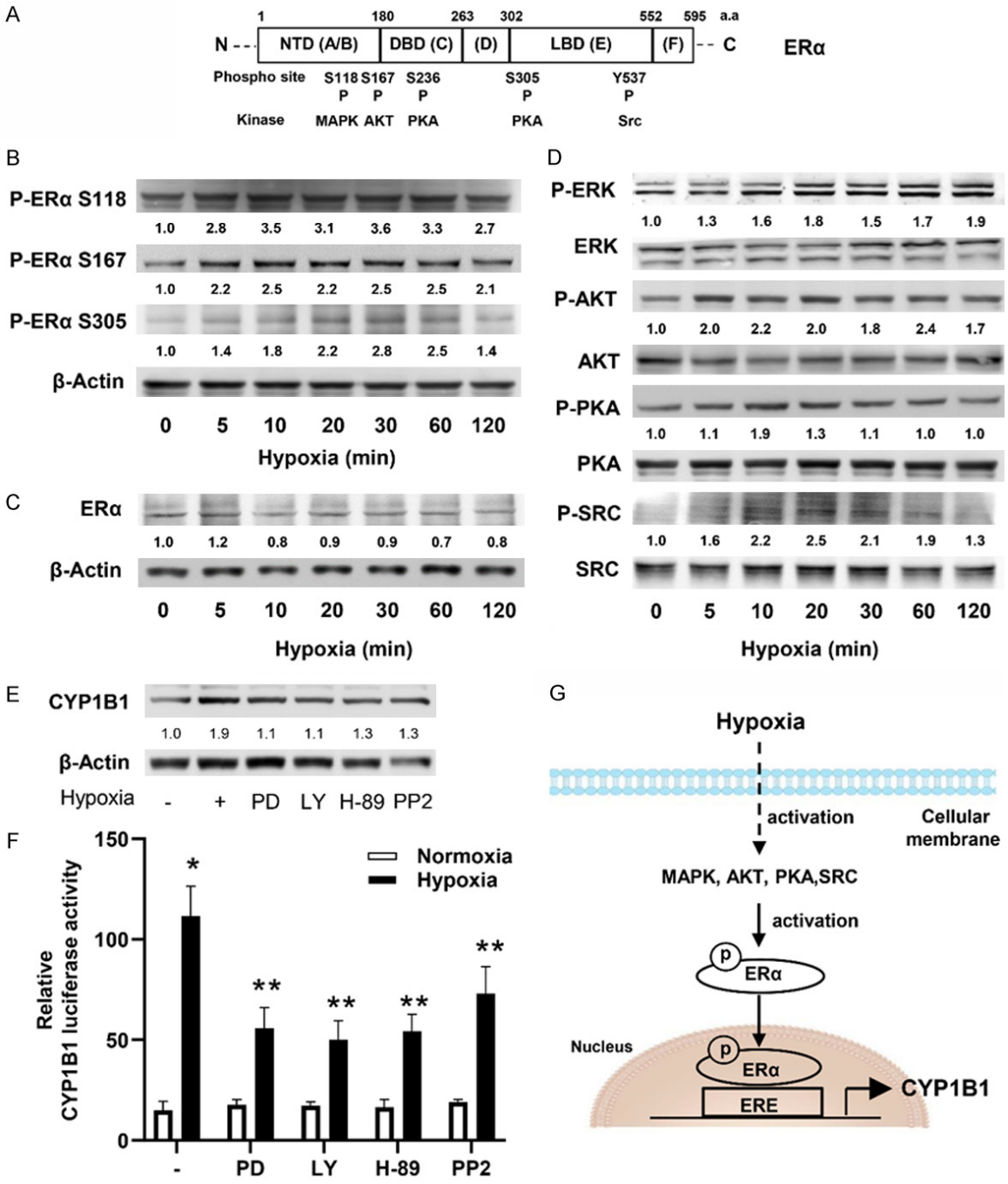
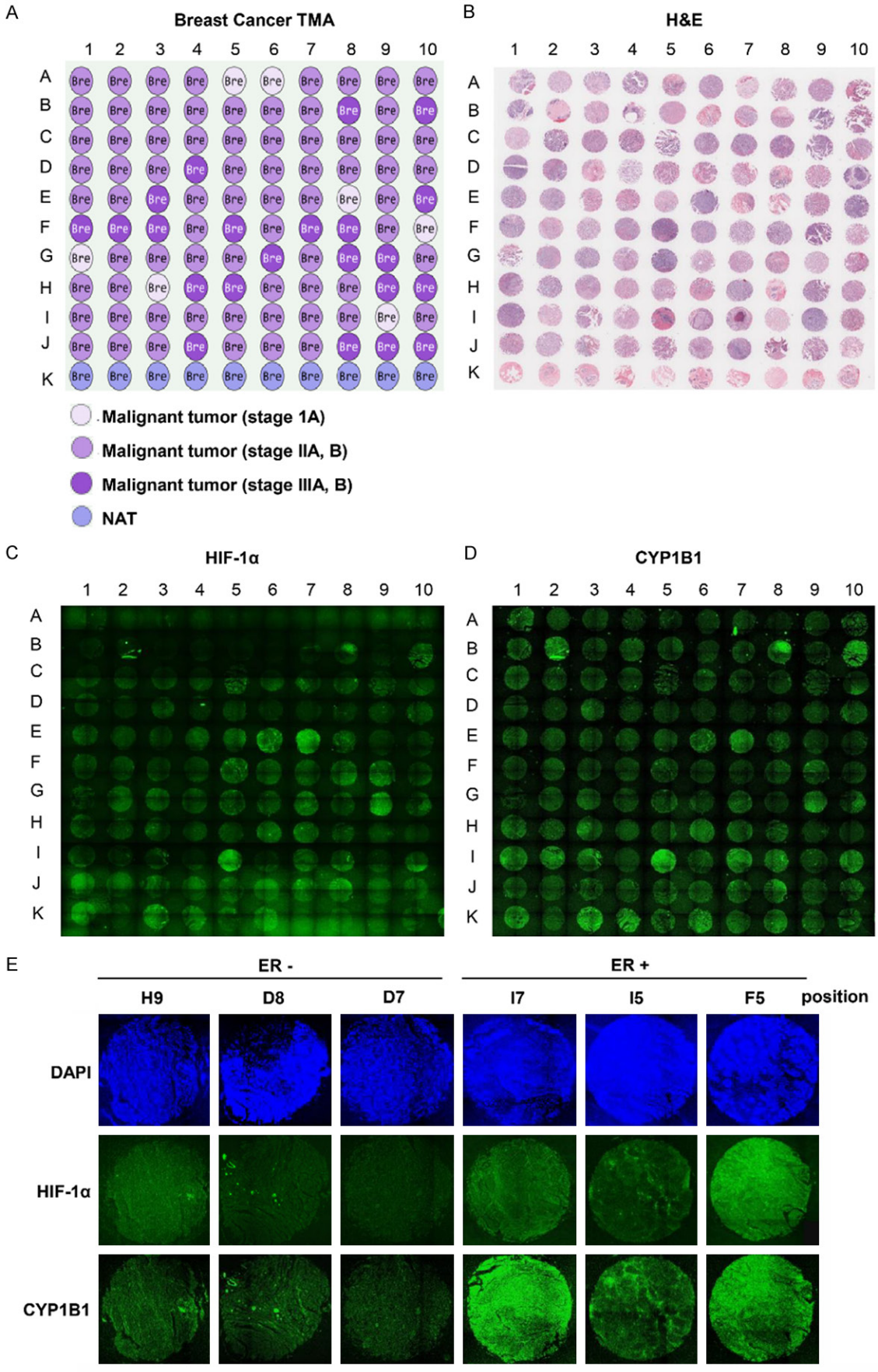


Figure 4. ERα and CYP1B1 transcriptional activity by hypoxia-mediated kinase phosphorylation. A. Schematic representation of the activation sites of ERα. B. Analysis of ERα phosphorylation in cells under hypoxia by immunoblotting using the phospho-ERα antibody. C. ERα expression under hypoxia in MCF-7 cells incubated for 0-60 min under hypoxia. D. Immunoblot analysis showing activation of kinases extracellular signal-regulated kinase (ERK), protein kinase B (Akt), protein kinase A (PKA), and proto-oncogene tyrosine-protein kinase SRC by phosphorylation in cells incubated for 0-60 min under hypoxia in a time-dependent manner. E. Decreased hypoxia-mediated CYP1B1 expression under the influence of ERK, Akt, PKA, and SRC kinase inhibitors (ERK [PD; 10 μM], Akt [LY; 5 μM], PKA [H-89, 10 μM], or SRC [PP2, 20 μM]) in cells under hypoxia for 24 h. F. Hypoxia-mediated CYP1B1 promoter luciferase activity under the influence of ERK, Akt, PKA, and SRC kinase inhibitors (ERK [PD; 10 μM], Akt [LY; 5 μM], PKA [H-89, 10 μM], or SRC [PP2, 20 μM]) in cells transfected with CYP1B1-Luc and subjected to hypoxia for 24 h. G. Schematic diagram of CYP1B1 regulation by hypoxia-induced kinase activation-mediated ERα signaling. *, P<0.01 versus normoxic control cells and **, P<0.01 versus control treated cells under hypoxia, Newman-Keuls test.


A
Breast Cancer TMA
1 2 3 4 5 6 7 8 9 10
A B C D E F G H I J K
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Malignant tumor (stage 1A)
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Malignant tumor (stage IIIA, B)
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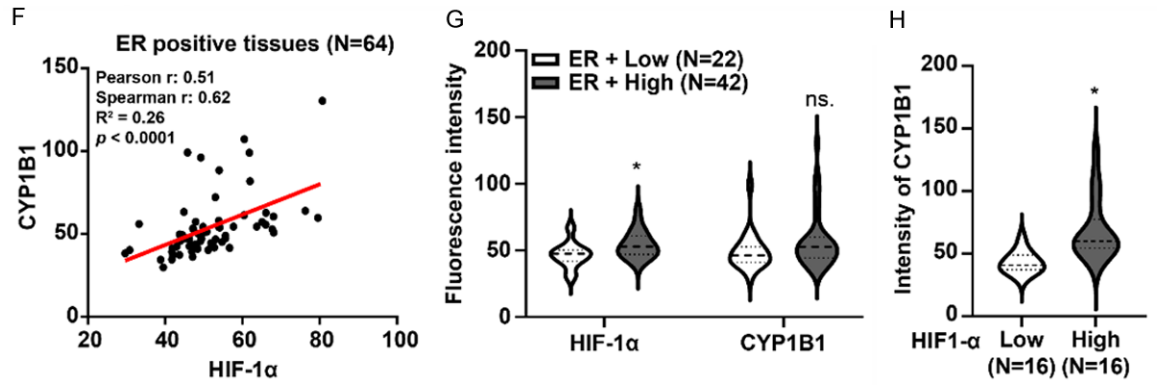

Figure 5. Analysis of HIF-1α and CYP1B1 expression in tissue microarray (TMA) samples from breast cancer patients. (A) Schematic diagram of the tumor stages of each breast tissue core. (B) Hematoxylin and eosin (H&E) staining images provided by the manufacturer. Immunofluorescence images of tumor microarrays (TMA) stained with anti-hypoxia-inducible factor-1 alpha (HIF-1α) (C) and anti-cytochrome P450 1B1 (CYP1B1) (D) antibodies. Images were acquired at 20X magnification using a Leica Thunder microscope. Whole tissue slide images were mosaic merged via LAS-X software. (E) 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. (F) Scatter plots showing fluorescence intensity correlation analysis of HIF-1α and CYP1B1 in 64 ER-positive TMAs. (G) Violin plots of fluorescence intensities of *HIF-1α* and *CYP1B1*. Samples were classified into low (immunohistochemical [IHC] score: 1+, 2+) and high (IHC score: 3+) groups according to the estrogen receptor (ER) expression status of the TMA specification sheet, and the correlation between *HIF-1α* and *CYP1B1* expression was analyzed. (H) Analysis of fluorescence intensity of CYP1B1 against HIF1α expression in ER-positive TMA. The fluorescence intensity of CYP1B1 was analyzed by classifying the fluorescence intensity of HIF1-α based on the top and bottom 25% ER expression. *, P<0.0001 versus ER-positive tumor tissue with low HIF1-α fluorescence intensity by Mann-Whitney test.

(1,100 cases), and BRCA-METABRIC (1,904 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 the cBioPortal's online instructions, and analyzed using 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 ERα-positive high profile/HIF1α-CYP1B1 high group were filtered (q-value <0.01, $|Log_2(FC)| > 0.58$). These were then subjected to gene enrichment analysis using the Metascape platform (http://metascape.org) for gene annotation verification. The filtered data were subjected to hierarchical clustering and visualized as a heatmap.

Statistical analysis

Data are presented as the ± standard error of the mean (SEM). All statistical analyses were performed using the Mann-Whitney U-test when data were not normally distributed. Statistical significance was set at P<0.05.

Results

HIF-1α is associated with CYP1B1 expression in breast cancer cells

To investigate the effect of hypoxia on CYP1B1 expression in breast cell lines, HIF-1α and CYP1B1 expression were analyzed by immunoblotting. HIF-1α protein level was induced by hypoxia at 6, 12, and 24 h; however, CYP1B1 expression was not altered in MCF10A normal breast epithelial cells (**Figure 1A**), or in ERα-negative MDA-MB 231 cells (**Figure 1B**). In contrast, both HIF-1α and CYP1B1 expressions increased due to hypoxia in MCF-7 cells, which were ERα-positive (**Figure 1C**). To verify that hypoxia-induced CYP1B1 expression is associated with HIF-1α, ERα-positive MCF-7 cells were cultured under hypoxic conditions after transient transfection with HIF-1α-siRNA. The increase in CYP1B1 expression due to hypoxia was suppressed by HIF-1α knockdown (by HIF-1α-siRNA) at 24 h, suggesting that CYP1B1 expression under hypoxia is induced by HIF-1α (**Figure 1D**). As a negative control, the use of

Table 1. Clinical characteristics of the breast tissue cores in the TMA

Feature	Subgroup	Counts of sample	
		ER-negative (non-measurable*)	ER-positive
Type	NAT	4 (4*)	6
	Malignant	36 (2*)	64
TNM	-	4	6
	T1N0M0	2	5
	T1N1M0	1	1
	T2N0M0	15	27
	T2N1M0	8	11
	T2N2M0	1	3
	T3N0M0	2	6
	T3N1M0	3	3
	T3N2M0	0	2
	T4N0M0	1	3
	T4N1M0	1	1
	T4N2M0	2	2
Grade	-	3	6
	*	2	1
	1~2	0	1
	2	18	47
	2~3	1	1
	3	16	14
Stage	-	4	6
	IA	2	5
	IIA	16	28
	IIB	10	17
	IIIA	4	8
	IIIB	4	6

*Measurement not possible. ER, estrogen receptor; NAT, Histologically normal tissue adjacent to the tumor; TMA, tumor microarrays.

scrambled siRNA instead of HIF-1α-siRNA did not reduce CYP1B1 expression under hypoxic conditions (**Figure 1D**). To confirm the role of HIF-1α and hypoxia on CYP1B1 expression, cells were treated with YC-1(3-(5'-hydroxymethyl-2'-furyl)-1-benzyl indazole), a HIF-1α inhibitor developed for the treatment of circulatory disorders, and immunoblotting was performed. YC-1 inhibited CYP1B1 expression under hypoxic conditions (**Figure 1E**). Furthermore, to determine whether CYP1B1 activity is regulated by hypoxia, MCF-7 cells were transiently transfected with CYP1B1-Luc and HIF-1α-siRNA under hypoxic conditions. Hypoxia-induced CYP1B1 luciferase activity was reduced by HIF-1α knockdown with HIF-1α-siRNA (**Figure 1F**). These results suggest that CYP1B1 expression under hypoxia is mediated by HIF1-α.

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 ERα-positive MCF-7 cells by hypoxia, but not in ERα-negative MCF10A or MDA-MB-231 cells, suggesting that CYP1B1 regulation by hypoxia is ERα-dependent (**Figure 2A**, **2B**). Based on these findings, we used the CYP1B1 promoter vector to verify whether ERα is involved in regulating CYP1B1 expression in hypoxia. The human CYP1B1 promoter FL (-910/+25 bp; **Figure 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) that is important for regulating CYP1B1 transcription [18, 26, 28]. We observed that CYP1B1 promoter activity was increased even in the construct where XRE and AP-1 were deleted. Additionally, 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 (**Figure 2C**). Moreover, it was established that hypoxia directly regulates CYP1B1 through luciferase activity of the ERE-Luc vector in MCF-7 cells (**Figure 2D**). ERα-mediated hypoxia-induced CYP1B1 expression was further verified by immunoblot analysis in MCF-7 cells treated with ERα-siRNA. ERα-siRNA treatment suppressed CYP1B1 expression in MCF-7 cells under hypoxia (**Figure 2E**). Additionally, ChIP analysis confirmed the binding of ERα to ERE in the CYP1B1 promoter region under hypoxia (**Figure 2F**). These results suggest that hypoxia-activated ERα can induce CYP1B1 expression by binding the CYP1B1 promoter in ERα-positive MCF-7 cells.

HIF-1α interacts with ERα in MCF-7 cells

To further confirm that ERα 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 ERα expressions.

In MDA-MB-231 and MCF-7 cells with different ERα expression, HIF-1α expression was indu-

Table 2. Immunofluorescence staining intensities in ER-positive breast cancer tissue cores in the tissue microarray

No.	Position	Tissue ID.	Fluorescence intensity		
			ER	HIF-1α	CYP1B1
1	A2	Fmg010622	++	42.003	37.252
2	A3	Fmg040237	+++	53.988	88.394
3	A5	Fmg100001	+++	38.83	34.435
4	A6	Fmg010733	+++	44.473	49.318
5	A7	Fmg120154	+	49.106	45.741
6	A9	Fmg020090	+++	48.433	41.056
7	B2	Fmg020179	+++	43.942	45.852
8	B3	Fmg030639	+++	47.931	43.768
9	B4	Fmg020081	+++	41.777	41.072
10	B5	Fmg020082	+++	63.692	54.442
11	B6	Fmg100066	+++	41.71	34.585
12	B8	Fmg020190	+++	54.115	53.868
13	B9	Fmg040118	+++	51.144	40.27
14	B10	Fmg140037	+++	60.467	107.167
15	C1	Fmg020770	+	44.861	63.264
16	C2	Fmg010624	+++	45.26	47.263
17	C3	Fmg040104	+++	57.682	54.486
18	C4	Fmg040001	+++	46.88	39.387
19	C5	Fmg110044	+++	54.698	45.156
20	C6	Fmg130073	+++	46.297	46.999
21	C7	Fmg030482	+++	55.627	47.424
22	C8	Fmg030496	+++	53.857	58.036
23	C9	Fmg030501	+	52.72	41.88
24	D1	Fmg130041	+++	55.627	49.057
25	D2	Fmg030645	+++	46.295	42.64
26	D3	Fmg100050	+++	65.07	57.144
27	D4	Fmg040661	+	43.738	49.644
28	D5	Fmg040074	+++	51.844	44.496
29	D6	Fmg010735	+++	67.698	52.79
30	D10	Fmg040048	+++	49.801	54.317
31	E1	Fmg010671	+++	68.05	60.504
32	E2	Fmg040768	+	50.125	51.819
33	E3	Fmg010531	++	56.683	41.655
34	E4	Fmg030523	+++	47.133	36.148
35	E8	Fmg010358	+	49.404	47.024
36	E9	Fmg050026	+++	52.998	72.101
37	F1	Fmg010536	+++	66.004	55.626
38	F2	Fmg010643	+	47.643	43.431
39	F3	Fmg030516	+++	60.422	61.298
40	F5	Fmg040082	+	42.98	42.46
41	F8	Fmg040675	+++	48.59	50.035
42	F9	Fmg040125	+++	47.259	52.995
43	G1	Fmg100010	++	30.781	40.244
44	G2	Fmg010492	++	47.593	43.128
45	G4	Fmg010540	+++	53.032	46.804

ced by hypoxia, but ERα expression was not altered by hypoxia (**Figure 3A**). As ERα expression is not regulated by HIF-1α, we investigated the association between HIF-1α and ERα 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 HIF-1α antibodies, and the immunoprecipitates were subjected to immunoblot analysis using ERα antibodies. HIF-1α and ERα were bound in MCF-7 cells under hypoxia (**Figure 3B**).

To identify the binding domain of ERα required an interaction with HIF-1α'; thus an M2H assay was performed using ERα truncated mutants. ERα has three functional domains: an activation domain 1 (AF1; 1-180 aa), DBD (180-302 aa), and AF2 (302-595 aa) (**Figure 3C**). To determine the sites required for binding between ERα and HIF-1α, a deletion construct of these regions (pBind-ERα deletion construct), was designed. Luciferase activity by the interaction of HIF-1α with ERα AF2 (LBD) was approximately 2.5-fold higher than that using vectors containing AF1 or DBD (**Figure 3C**). LBD is a region with hormone binding sites, homo- and hetero-dimerization interfaces, and ligand-dependent co-regulator binding [29]. Thus, HIF-1α and ERα physically interact with each other under hypoxia in MCF-7 cells via the LBD of ERα (**Figure 3D**).

HIF-1α induces ERα 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-1α signaling pathway. A recent study showed that the cAMP-dependent protein kinase A (PKA) phosphorylates HIF-1α and increases its activity in cancer cells [33]. Although ERα can be activated by genomic pathways [34], non-genomic pathways mediated by phosphorylation by various kinases are also important in the regulation of ERα activity [35]. **Figure 4A** shows the phosphorylation sites of ERα 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

46	G5	Fmg040132	+	47.925	57.278
47	G6	Fmg100103	++	68.085	50.821
48	G7	Fmg020026	+	41.747	39.227
49	G10	Fmg010729	+++	53.945	53.975
50	H1	Fmg010642	++	33.24	56.036
51	H2	Fmg010943	++	39.537	29.763
52	H4	Fmg080090	+++	61.95	81.873
53	H5	Fmg100104	+	49.621	50.133
54	H6	Fmg020035	++	29.619	38.312
55	H7	Fmg020765	+	50.783	51.397
56	H10	Fmg030528	+++	43.665	37.322
57	I1	Fmg040182	+++	49.217	96.131
58	I5	Fmg120001	+++	80.681	130.405
59	I7	Fmg020038	+++	61.811	99.058
60	I9	Fmg020443	+++	47.161	53.287
61	J1	Fmg100067	+++	79.531	59.749
62	J5	Fmg010755	+	66.086	62.766
63	J7	Fmg030432	+++	76.274	63.891
64	J10	Fmg040344	++	45.781	99.132

CYP1B1, Cytochrome P450 1B1; ER, estrogen receptor; HIF-1α, hypoxia-inducible factor-1 alpha.

Table 3. Immunofluorescence staining intensities in ER-negative breast cancer tissue cores in the tissue microarray

No.	Position	Tissue ID.	Fluorescence intensity		
			ER	HIF-1α	CYP1B1
1	A1	Fmg020761	-	49.173	68.875
2	A4	Fmg130003	-	45.386	35.494
3	A8	Fmg020031	-	51.101	28.701
4	A10	Fmg020037	-	50.717	47.772
5	B1	Fmg080098	-	38.038	54.968
6	B7	Fmg080091	-	39.382	41.677
7	C10	Fmg010613	-	51.082	45.919
8	D7	Fmg010310	-	43.257	30.765
9	D8	Fmg090029	-	43.769	32.998
10	D9	Fmg050032	-	47.679	43.627
11	E5	Fmg060191	-	57.967	49.919
12	E6	Fmg010612	-	75.343	71.005
13	E7	Fmg030477	-	87.013	88.3
14	E10	Fmg010604	-	45.847	37.19
15	F4	Fmg060181	-	50.239	45.892
16	F6	Fmg020184	-	59.237	40.452
17	F7	Fmg020562	-	58.321	38.725
18	F10	Fmg020028	-	49.077	36.769
19	G3	Fmg080055	-	58.817	59.045
20	G8	Fmg010767	*	51.757	33.802
21	G9	Fmg140019	-	78.229	75.379
22	H3	Fmg010547	-	51.08	73.411

pathways [40]. To investigate whether ERα is activated by a hypoxia-induced HIF-1α protein signaling cascade, we analyzed the degree of ERα phosphorylation in lysates of cells cultured under hypoxia by immunoblotting. MCF-7 cells incubated under hypoxia led to the phosphorylation of ERα at S118, 167, and 305 in a time-dependent manner (**Figure 4B**). Furthermore, there was no change in ERα expression when MCF-7 cells were incubated for 0-60 min under hypoxia (**Figure 4C**). These kinases demonstrated increased activation in a time-dependent manner in MCF-7 cells under hypoxia for up to 60 min (**Figure 4D**). To further validate 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 the hypoxia-induced CYP1B1 expression (**Figure 4E**). Moreover, these inhibitors reduced hypoxia-mediated CYP1B1 mRNA expression and CYP1B1 promoter luciferase activity in breast cancer cells under hypoxia (**Figure 4F**). Under normoxic conditions, these inhibitors did not affect CYP1B1 transcriptional activity. Thus, hypoxia induces ligand-independent ERα activation as a non-genomic mechanism; it also induces the binding of ERα and HIF-1α (**Figure 4G**). PI3K and ERK are known hypoxia-induced modulators of HIF-1α signaling pathways.

HIF-1α positively correlates with CYP1B1 expression in breast cancer patients

To clinically evaluate the correlation between HIF-1α and CYP1B1, we performed immunofluorescence analysis using a breast cancer patient TMA. In the TMA specification sheet, ten normal breast tissues and 100 breast cancer tissues were classified according to the immunohistochemical (IHC) notation of the ER ("-" indicates ER-negative breast cancer tissue and "+" indicates ER-positive breast cancer tissue; **Figure 5A**, **5B**; **Table 1**). The fluorescence intensity results of CYP1B1 and HIF-1α in 100 breast cancer tissue samples from TMA (**Figure 5C**, **5D**; **Tables 2** and **3**) were scored using the ImageJ software (National Institutes of Health, Bethesda, MD, USA). The fluorescence intensity of CYP1B1 and HIF-1α in the ER-positive breast cancer tissue was higher

23	H8	Fmg030512	*	48.59	50.035
24	H9	Fmg050004	-	49.738	40.934
25	I2	Fmg030520	-	38.076	89.016
26	I3	Fmg130067	-	43.532	77.659
27	I4	Fmg040162	-	40.397	51.021
28	I6	Fmg110075	-	44.696	56.791
29	I8	Fmg080106	-	61.073	84.497
30	I10	Fmg110115	-	59.771	85.638
31	J2	Fmg030644	-	67.28	59.883
32	J3	Fmg110037	-	59.521	60.27
33	J4	Fmg100037	-	65.868	55.786
34	J6	Fmg080053	-	80.665	68.146
35	J8	Fmg090030	-	80.725	73.647
36	J9	Fmg021845	-	68.593	55.872

*Measurement not possible. CYP1B1, Cytochrome P450 1B1; ER, estrogen receptor; HIF-1α, hypoxia-inducible factor-1 alpha.

than that in the ER-negative breast cancer tissue (**Figure 5E**). Pearson's correlation analysis was performed on ER-positive TMA samples (N=64), and a significant positive correlation was noted between CYP1B1 and HIF-1α levels ($P<0.0001$, $r^2=0.26$) (**Figure 5F**). When ER-positive breast cancer tissues were divided into low (IHC score: 1+, 2+) and high (IHC score: 3+) expression groups according to the ER expression status (TMA specification sheet), the expression of HIF-1α was higher in ER high patient tissues (**Figure 5G**). Additionally, after separating the groups using the 25^{th} or 75^{th} percentile of HIF-1α as a cutoff, the expression of CYP1B1 was established to be significantly increased in the ER-positive tissues with high HIF-1α expression (**Figure 5H**).

To validate the clinical significance of HIF-1α and CYP1B1 expression in the 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 HIF1α and CYP1B1 expression was confirmed (**Figure 6A-C**). Additional analysis was performed based on the brca_metabric dataset with the largest number of patient data. Analysis of the 1,459 ER-positive patients from the brca_metabric dataset revealed a significant positive correlation between the expression of CYP1B1 and HIF-1α (**Figure 6D**). In this dataset (metabric), patients with high CYP1B1 and HIF-1α expressions were classified as the ER high (N=26, top 25%) and ER low (N=33, bottom 25%) groups, based on the ER expression. Of the 18,042 genes expressed in this patient group (brca_metabric dataset), 304 genes (DEG; q-value <0.01, $0.58-|Log_2(FC)|$) that changed in the ER low group versus the ER high group were identified (red spots, increase =136 and blue spots, decrease =168) (**Figure 6E**). Gene ontology (GO) and enrichment analysis (metascape) were performed, based on the differentially expressed genes (DEG) of the ER/HIF1α/CYP1B1 triple high group. Genes associated with tumor recurrence were identified at the top of the analysis graph and presented as a percentage (% of number) and $-Log_{10}$ (q-value) of shared genes (**Figure 6F**, **6G**; **Table 4**); there were 43 such genes, including cell cycle-related genes such as aurora kinase, associated with tumor recurrence that were analyzed using hierarchical clustering and visualized as heat maps (**Figure 6H**; **Table 5**). Furthermore, we observed that the survival rate was significantly lower in patients with high expression of ER/HIF1α/CYP1B1 in breast cancer, compared to patients with low expression of these genes (**Figure 6I**). The pub2015 and TCGA patient datasets as well as the metabolic dataset showed a remarkable correlation between high ER/HIF1α/CYP1B1 expression and lower survival rates and cancer recurrence in breast cancer patients (**Figure 6J**, **6K**). Therefore, our results suggest that the overexpression of HIF-1α 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 can be treated with antihormonal therapy; therefore, the recurrence of breast cancer is high, which significantly and negatively impacts patient survival. Although many studies have been conducted to detect biomarkers for breast cancer recurrence [42, 43], recurrence and metastasis remain unresolved. Previous studies reported that HIF-1α 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 tar-

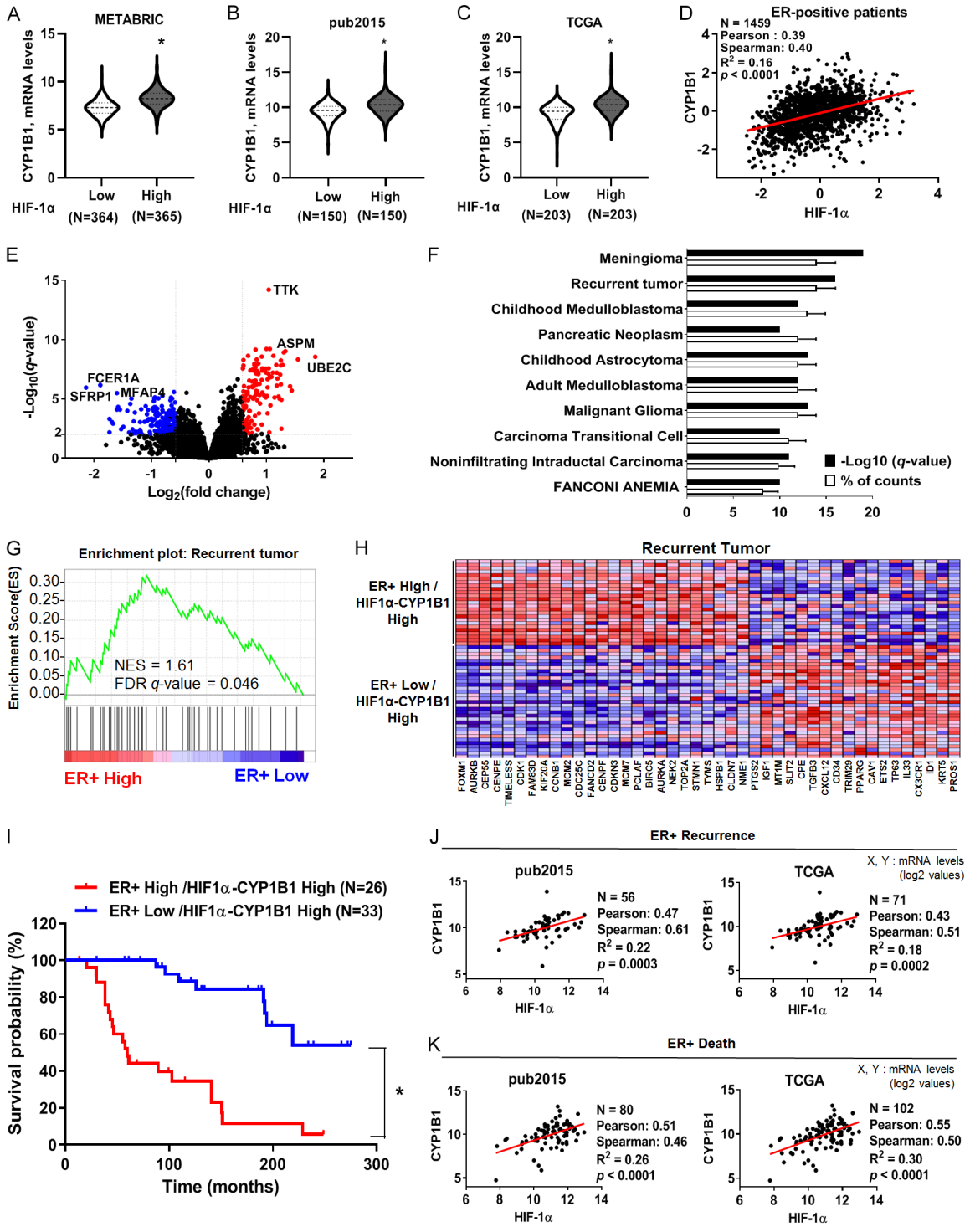

Figure 6. Clinical significance analysis of CYP1B1 and HIF-1α using the transcriptome dataset of breast cancer patients. Correlation analysis of mRNA levels of HIF1α and CYP1B1 in the open dataset of transcripts from ER-positive breast cancer patients in the cBioPortal database. In the brca_metabric (A), brca_tcga_pub2015 (B), brca_tcga (C) datasets and breast cancer patients were classified for the top/bottom 25% of *HIF-1α* expression among patients with ER-positive breast cancer. mRNA levels of *CYP1B1* were analyzed and presented as violin plots. In the brca_tcga_pub2015 and brca_tcga datasets, mRNA levels were Log_2-transformed. (D) Scatter plots showing HIF-1α and CYP1B1 mRNA expression in ER-positive breast cancer patients. Log_2-transformed values of mRNA levels were used as z-scores for all samples. (E) Volcano plot of differentially expressed genes (DEGs) of the ER high HIF-1α/CYP1B1

high breast cancer patient group and the ER low HIF-1α/CYP1B1 high group. By significance (q-value <0.01, $|Log_2(FC)| > 0.58$), red spot indicates upregulated genes and blue spot indicates downregulated genes. (F) Gene ontology analysis of DEGs in the ER/HIF1α-CYP1B1 high group. Bar charts visualizing the percentage of shared genes (% of number) and $-Log_{10}$ (q-value). (G) Recurrent tumor enrichment plot of ER high profile with high expression of HIF-1α/CYP1B1. (H) Heat map of the genes (22 genes in core enrichment) corresponding to the recurrent tumor analysis. Red and blue boxes indicate upregulated and downregulated gene levels, respectively (Z-scores). (I) Kaplan-Meier survival rate analysis of patients with ER high HIF-1α/CYP1B1 high breast cancer and the ER low HIF-1α/CYP1B1 high group. *, P<0.05 vs. ER-positive cancer patients with low ER expression. Correlation analysis of cancer recurrence and mortality in the public transcriptome analysis dataset (brca_tcga_pub2015 and brca_tcga) of ER-positive breast cancer patients. Scatterplots of the correlation between the mRNA expression of HIF-1α and CYP1B1 in ER-positive breast cancer patients who have relapsed (J) or died (K). The number of patients in each group, correlation coefficient, and *P*-values are displayed on each chart.

Table 4. Top 10 enriched gene set of ER/HIF1α-CYP1B1 triple high group

GO	Description	Number of gene			% of match	STDV of %	Log_{10} (*q*)
		input	GO list	matched			
C0025286	Meningioma	304	634	43	14	2	-19
C0521158	Recurrent tumor	304	735	43	14	2	-16
C0278510	Childhood Medulloblastoma	304	771	39	13	1.9	-12
C0555198	Malignant Glioma	304	724	38	12	1.9	-13
C0278876	Adult Medulloblastoma	304	762	38	12	1.9	-12
C4086152	Childhood Astrocytoma	304	615	36	12	1.9	-13
C0030297	Pancreatic Neoplasm	304	764	36	12	1.9	-10
C0007138	Carcinoma, Transitional Cell	304	623	32	11	1.8	-10
C0007124	Noninfiltrating Intraductal Carcinoma	304	486	30	9.9	1.7	-11
C3469521	Fanconi anemia, complementation group A (disorder)	304	359	25	8.2	1.6	-10

CYP1B1, Cytochrome P450 1B1; ER, estrogen receptor; HIF-1α, hypoxia-inducible factor-1 alpha; STDV, standard deviation.

Table 5. Gene lists of gene set enrichment analysis (GSEA) (recurrent tumor)

No.	Symbol	Rank metric score	Running ES	Core enrichment
1	FOXM1	1.385	0.0276	Yes
2	AURKB	1.336	0.0615	Yes
3	CEP55	1.296	0.0904	Yes
4	CENPE	1.233	0.0984	Yes
5	TIMELESS	1.14	0.0655	Yes
6	CDK1	1.133	0.0936	Yes
7	FAM83D	1.079	0.0896	Yes
8	KIF20A	1.061	0.1119	Yes
9	CCNB1	1.055	0.1378	Yes
10	MCM2	1.014	0.1473	Yes
11	CDC25C	1.005	0.1718	Yes
12	FANCD2	0.984	0.1843	Yes
13	CENPF	0.972	0.2041	Yes
14	CDKN3	0.959	0.2273	Yes
15	MCM7	0.953	0.2427	Yes
16	PCLAF	0.932	0.246	Yes
17	BIRC5	0.906	0.2639	Yes
18	AURKA	0.884	0.2698	Yes
19	NEK2	0.851	0.2784	Yes
20	TOP2A	0.823	0.2902	Yes

get the HIF-1α signaling pathway. Inhibition of HIF-1α by YC-1 is known to decrease cell growth and metastasis in breast cancer [46]. Additionally, 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 those of the breast, colon, lung, brain, skin, prostate, ovarian, and liver cancers [50-54]. As CYP1B1 expression is increased in tumor tissues

21	STMN1	0.822	0.3134	Yes
22	TYMS	0.761	0.3196	Yes
23	HSPB1	0.632	0.2914	No
24	CLDN7	0.579	0.2809	No
25	NME1	0.555	0.2851	No
26	PTGS2	-0.517	0.2231	No
27	IGF1	-0.527	0.215	No
28	MT1M	-0.535	0.2262	No
29	SLIT2	-0.539	0.2338	No
30	CPE	-0.542	0.2414	No
31	TGFB3	-0.546	0.2377	No
32	CXCL12	-0.56	0.2152	No
33	CD34	-0.564	0.2234	No
34	TRIM29	-0.576	0.209	No
35	PPARG	-0.585	0.2064	No
36	CAV1	-0.623	0.1665	No
37	ETS2	-0.653	0.1352	No
38	TP63	-0.658	0.1422	No
39	IL33	-0.662	0.1494	No
40	CX3CR1	-0.673	0.1378	No
41	ID1	-0.711	0.1042	No
42	KRT5	-0.768	0.0569	No
43	PROS1	-0.834	0.0307	No

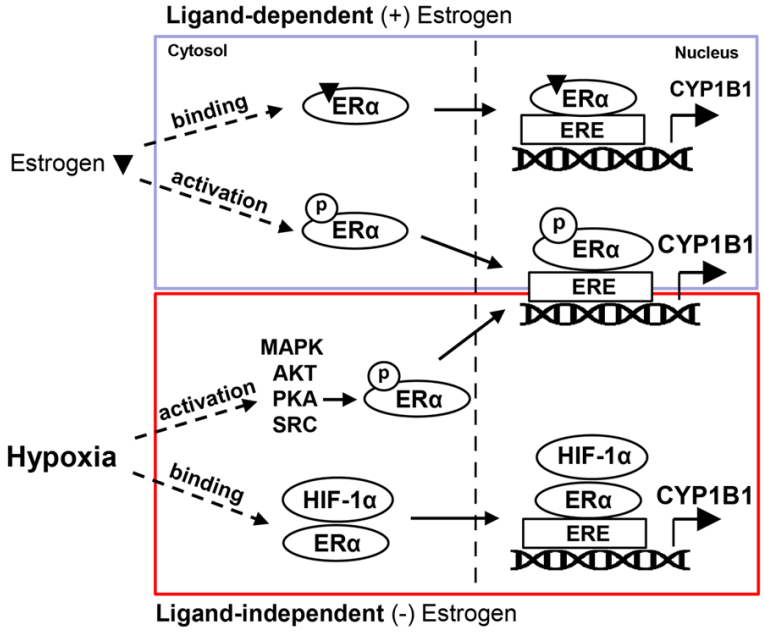

Figure 7. Mechanism of hypoxia-mediated CYP1B1 upregulation through ER activation. In the presence of estrogen as an ER ligand, estrogen directly binds ER or induces CYP1B1 expression through ER activation through a ligand-dependent pathway. Under hypoxia, even in the absence of estrogen, a ligand-independent pathway can be induced in ER-positive breast cancer cells by the activation of kinases that phosphorylate ER or by binding of increased HIF-1α to ER to induce CYP1B1 expression.

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-1α 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-1α in human ERα-positive breast cancer. We observed that hypoxia-induced CYP1B1 expression occurred only in the ERα-positive breast cancer MCF-7 cells. HIF-1α can bind ERα directly, and this complex translocates from the cytoplasm to the nucleus and binds to the ERE site of the CYP1B1 promoter in MCF-7 cells. We also found that hypoxia activates ERα in a ligand-independent manner in the absence of E2 through the activation of signaling kinases (**Figure 7**). In **Figure 1D**, **1F**, siHIF-1α reduced CYP1B1 protein levels and transcriptional activity by approximately 50%, possibly due to another signaling pathway that could have activated Erα, besides hypoxia. Since ERα activation can be induced by the MAP kinase signaling pathway activated by various physiological stimuli in breast cancer cells, CYP1B1 inhibition by HIF-1α knockdown may be incomplete. Further studies are needed to elucidate the mechanism of unambiguous CYP1B1 expression in breast cancer. As shown in **Figure 3A**, HIF-1α expression was induced by hypoxia. However, ERα expression was not altered by hypoxia in MCF-7 cells. Cho et al. [58] reported

decreases in ERα protein and gene expression under hypoxia, which are inconsistent with our results. However, Yang et al. [59] reported no change in ERa protein expression under hypoxic conditions compared with under normoxic ones.

Moreover, immunofluorescence analysis of tissue microarrays from the ER-positive breast cancer patients confirmed a positive correlation between HIF-1α and CYP1B1. A positive correlation of cancer recurrence with mortality and high ERα, HIF-1α, 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-1α and CYP1B1 prevented cancer recurrence in recurrent ERα-positive breast cancer patients. Our findings provide the foundation for further studies and promote the use of HIF-1α and CYP1B1 as therapeutic targets in the treatment of recurrent tumors.

We report the mechanism by which hypoxia-induced HIF-1α regulates CYP1B1 through the activation of ERα in breast cancer cells and the role of hypoxia and ERα positivity in tumor relapse. Our findings confirm the role of hypoxia in the pathogenesis and progression of ERα-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.

Acknowledgements

We would like to thank Editage (www.editage.co.kr) for English language editing. 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-2020R1C1C1012-542), the National Research Council of Science & Technology (NST) (No. CAP-18-02-KRIBB), Korea Food Research Institute (No. E0210-203), and the Korea Basic Science Institute (No. C210700, C270100, and C270300).

Disclosure of conflict of interest

None.

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