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Original Paper

EGFR is a Therapeutic Target in Hormone Receptor-Positive Breast Cancer

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Key Words
Estrogen receptor • EGFR • Endocrine therapy • Tamoxifen resistance

Abstract
Background/Aims: Despite effective therapeutic strategies for treating hormone receptor-positive (HR+) breast cancer, resistance to endocrine therapy that is either de novo or acquired still occurs. We investigated epidermal growth factor receptor (EGFR) as a therapeutic target for overcoming endocrine resistance in HR+ breast cancer models. ***Methods:*** Using clinical data from 2,166 patients who had HR+ breast tumors and received tamoxifen, we analyzed survival rates. Levels of mRNA and protein expression were analyzed by real-time PCR and western blotting, respectively. Cell viability was analyzed by MTT assays and anchorage-independent growth by soft agar colony-formation assays. Efficacy of tamoxifen and/or gefitinib was analyzed using orthotopic xenograft mouse models. ***Results:*** EGFR expression was significantly associated with more advanced stage and higher grade. EGFR expression was different in luminal A-like (Lum A, 1.3%) versus luminal B-like (Lum B, 11.4%) subtypes. On multivariate analyses for survival Lum B subtype EGFR+ tumors showed a hazard ratio (HR) of 5.22 (95% CI, 1.29–21.15, P = 0.020) for overall survival (OS) and HR of 2.91 (95% CI, 1.35–6.28, P = 0.006) for disease-free survival (DFS). Levels of EGFR inversely correlated with ER-α expression. Basal ER-α level was completely blocked by TGFA or EGF treatment. With TGFA pretreatment, ER+ breast cancer cells were resistant to 4-hydroxytamoxifen (4-OHT). Conversely, downregulation of ER-α by TGFA was reversed by gefitinib with recovered sensitivity to 4-OHT. Tumorigenicity of EGFR and ER+ breast cancer cells were significantly decreased by combined tamoxifen and gefitinib. ***Conclusion:*** Aberrant EGFR expression was associated with poor prognosis in ER+ breast cancers, especially the Lum B subtype. Loss of

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ER by EGFR activation induced tamoxifen resistance. Therefore, EGFR could be a therapeutic target for overcoming recurrence of ER+ breast cancer with high EGFR expression.

Introduction

Approximately 70% of all breast cancers are hormone-receptor positive (HR+) and endocrine therapy has become one of the most important treatments for HR+ breast cancer. Tamoxifen, one of the most commonly used endocrine therapies, is a selective estrogen receptor (ER) modulator that acts as an ER antagonist in the breast but an ER agonist in other tissues [1]. Although most patients with HR+ cancer undergo adjuvant endocrine therapy, approximately 20-30% will eventually experience recurrence with distant metastasis [2]. Despite improvements in treatment, therapy resistance remains a major clinical problem [3, 4]. Primary endocrine resistance is defined as relapse in the first two years of adjuvant endocrine therapy. Secondary (acquired) resistance is defined as relapse after two years of adjuvant endocrine therapy or within 12 months of completing adjuvant endocrine therapy [5].

Resistance to endocrine therapy results from complex processes involving multiple signaling molecules and pathways [6, 7]. Expression and activity of receptor tyrosine kinases (RTKs) such as insulin-like growth factor receptor (IGFR), epidermal growth factor receptor (EGFR), and HER2 are significantly increased in resistant cells [8, 9]. EGFR expression occurs in approximately 14-91% of breast carcinomas and aberrant expression is associated with more aggressive breast tumor phenotypes and poorer prognoses [10-14]. Phase II clinical trials did not show any clinical benefit of gefitinib treatment [15-17]. Activation of mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinases (PI3K)/AKT pathways by EGFR and HER2 contributes to ER-α phosphorylation and ER-α phosphorylation at serine 118 might affect endocrine therapy [6, 18, 19]. Loss of ER-α expression is observed in ~15-20% of metastatic breast cancer patients [14, 20]. If these patients initially respond to tamoxifen, they eventually develop acquired resistance [3, 4]. Previous studies suggested that the disappointing results of phase II clinical trials might be associated with failure to identify appropriate populations to predict benefits from EGFR inhibitors [17, 21].

This study evaluated EGFR expression and its value as a prognostic factor to investigate the possibility of EGFR-targeted therapy for ER+ breast cancer patients. In our results, survival rates of patients with EGFR and ER+ breast cancer were poorer than patients with ER+ breast cancer. In addition, cotreatment with tamoxifen and gefitinib synergistically increased apoptotic cell death in EGFR and ER+ breast cancer *in vivo* and *in vitro*. Therefore, we demonstrated that combination therapy with tamoxifen and EGFR-targeted tyrosine kinase inhibitors (TKIs) against EGFR and ER was more effective than single treatment for patients with EGFR and ER (+) breast cancer.

Materials and Methods

Clinical samples

Patients were selected from the clinical database of the Breast Cancer Center at Samsung Medical Center, Korea, between January 2007 and July 2013. We included only patients treated with endocrine therapy with tamoxifen. Patients treated with aromatase inhibitors (AIs), including those who switched to AIs, were not included. A total of 2, 574 patients were selected, excluding patients diagnosed with bilateral tumors or distant metastases at preoperative work-ups or who underwent neoadjuvant chemotherapy. Study data were collected using a protocol approved by the Institutional Review Board of Samsung Medical Center, Korea (IRB number 2014-09-111).

We reviewed patient clinicopathological characteristics, including biological factors such as expression of ER, progesterone receptor (PR), HER2, EGFR, and Ki-67. Pathological tumor stage was assessed according to the American Joint Committee on Cancer Seventh Staging System. ER, PR, and HER2 expression were measured as previously described [22]. Immunostaining for EGFR was interpreted as positive when at least 10% of tumor cells showed moderate-to-strong membrane staining. The cut-off value for Ki-67 was 20.0%, per the recommendation of the St Gallen 2013 consensus [23], to classify luminal A-like (Lum A) and luminal B-like (Lum B) subtypes.

Differences in the frequencies of clinicopathological factors and subtypes were statistically analyzed using chi-square and Fisher's exact tests. Disease-free survival (DFS) was defined as time from surgery to date of documentation of relapse, including locoregional recurrence and/or distant metastasis. Overall survival (OS) was defined as the number of months from surgery to date of death. Survival curves were constructed using the Kaplan-Meier method. Hazard ratios were estimated using Cox regression for DFS/OS in a multivariate analysis.

Reagents

Dulbecco's modified Eagle's medium (DMEM), RPMI1640, and antibiotics were purchased from Life Technologies (Rockville, MD, USA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA). 4-Hydroxytamoxifen (4-OHT) was purchased from Sigma (St. Louis, MO, USA). Gefitinib was purchased from Selleck Chemicals (Houston, TX, USA). Anti-ER-α and anti-β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-EGFR and Anti-procaspase-3 were from Abcam (Cambridge, MA, USA). Anti-PARP-1 was from Cell Signaling Technology (Beverly, MA, USA). Human IL-8 ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA). ECL prime reagents were purchased from Amersham (Buckinghamshire, UK).

Cell culture and drug treatments

BT474 human breast cancer cells were grown in RPMI1640 media in a humidified atmosphere of 95% air and 5% CO_2 at 37°C supplemented with 10% FBS, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. MCF7 human breast cancer cells were grown in DMEM media under the same conditions. After seeding, cell lines were maintained in culture media supplemented with FBS for 24 h. Breast cancer cells were treated with TGFA, EGF, 4-OHT and/or gefitinib for 24 h to analyze viability and levels of protein and mRNA. Cell morphologies were photographed using a CK40 inverted microscope (Olympus, Tokyo, Japan).

Real-time PCR

Total RNA was extracted from cells with TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. Isolated RNA samples were used for RT-PCR. Samples (1 μg total RNA) were reverse-transcribed into cDNA in 20-μl reactions using first-strand cDNA synthesis kits for RT-PCR, according to the manufacturer's instructions (MBI Fermentas, Hanover, MD, USA).

Gene expression was quantified by real-time PCR using SensiMix SYBR Kits (Bioline Ltd., London, UK) and 100 ng cDNA per reaction. Sequences of primer sets were: human ER-α (forward, 5′-CGC TAC TGT GCA GTG TGC AAT-3′ and reverse, 5′-CCT CAC AGG ACC AGA CTC CAT AA-3′), human EGFR (forward, 5′-CAT GTC GAT CTT CCA GA-3′ and reverse, 5′-GGG ACA GCT TGG ATC ACA CT-3′), human IL-8 (forward, 5′-AGG GTT GCC AGA TGC AAT AC-3′ and reverse, 5′-AAA CCA AGG CAC AGT GGA AC-3′) and GAPDH as an internal control (forward, 5′-ATT GTT GCC ATC AAT GAC CC-3′; reverse, 5′-AGT AGA GGC AGG GAT GAT GT-3′). An annealing temperature of 60°C was used for all primers. PCR was in standard 384-well plates with an ABI 7900HT real-time PCR detection system. For data analysis, raw threshold cycle (C_T) value was normalized to the housekeeping gene for each sample to obtain ΔC_T. Normalized ΔC_T was calibrated to control cell samples to calculate $\Delta\Delta C_T$.

Western blots

Cell culture media (supernatants) and cell lysates were used for immunoblots for EGFR, ER-α, PARP-1, procaspase, and β-actin. Proteins were boiled for 5 min in Laemmli sample buffer and electrophoresed in 8% or 10% SDS-PAGE gels. Separated proteins were transferred to PVDF membranes blocked with 10% skim milk in TBS with 0.01% Tween-20 for 15 min. Blots were incubated with anti-EGFR, ER-α, PARP-1,

procaspase, or β-actin in 1% TBS/T buffer (0.01% Tween 20 in TBS) at 4°C overnight. Blots were washed 3-4 times in TBS with 0.01% Tween 20 and incubated with anti-rabbit or mouse HRP-conjugated antibody (1/2, 000 dilution) in TBS/T buffer. After 1 h at room temperature, blots were washed three times, and ECL prime reagents used for development.

IL-8 ELISA

IL-8 protein was measured using ELISA kits for human IL-8 (KomaBiotech, Seoul, Korea) according to the manufacturer's instructions. A microtiter plate reader was used to measure absorbance at 450 nm.

Soft agar colony-formation assays

BT474 human breast cancer cells were seeded at 1×10^5 cells/well in six-well plates in growth medium with 0.7% agar (1.5 ml/well) on a layer of growth medium with 1.4% agar (2 ml/well). Growth medium (500 μl) with 10% FBS was added and 50 ng/ml TGFA, 10 μM 4-OHT, and/or 10 μM gefitinib was added on top of the agar of some plates. Cells were plated and cultured in a 37°C incubator for 2 weeks. After 2 weeks, viable colonies were stained with 0.01% crystal violet and observed using a CK40 inverted microscope (Olympus, Tokyo, Japan).

In vivo tumor growth inhibition by tamoxifen and/or gefitinib in orthotopic xenografts

We used 6- to 8-week-old female Balb/c nude mice (weight, 18-22 g; Orient Bio, Seoul, Korea) to establish a nude mouse xenograft model. Mice were kept in pathogen-free animal housing in accordance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and used according to protocols approved by the appropriate Institutional Review Board of Samsung Medical Center (Seoul, Korea).

Female Balb/c nude mice were implanted with 0.72 mg 17β-estradiol pellets (60-day release, Innovative Research). The next day, BT474 human breast cancer cells were cultured and resuspended in matrigel (BD Biosciences, Bedford, MA, USA) to 1.8×10^7 cells/120 μL and injected directly into right secondary mammary fat pads. Mice were randomly divided into three groups (n = 4/group) treated with saline only (Con, n=4), tamoxifen (5 mg/kg/dose, n=4, Tamoplex Tab, Teva Handok, Korea) and/or gefitinib (100 mg/kg/dose, n=4, Specssa Tab, Ildong, Korea). Treatment was 5 days/week until the end of experiments. Once tumors reached a volume of approximately 100 mm^3, mice were treated with drugs by oral gavage. The tumor size of mice in all three groups was measured using digital calipers at set time points, and volume was determined using the formula $V = 1/2 \times \text{length} \times (\text{width})^2$. Growth curves were calculated using average relative tumor volume per group (vehicle, tamoxifen, and/or gefitinib-treated) at the set time points. Tumors were removed and histological features were analyzed using hematoxylin and eosin (H&E), Ki67, EGFR, and ER-α, TUNEL staining.

Immunohistochemical staining

Xenograft tissues were formalin-fixed and paraffin-embedded. Tissue sections were cut and deparaffinized in xylene, dehydrated in graded alcohol and hydrated in water. Tissue sections (4 μm) were evaluated by H&E staining and then immunohistochemistry (IHC) was performed by Samsung Medical Center Animal Pathology Core Laboratory for Ki-67 (1:200, Dako, CA, USA), EGFR (1:200, Abcam, Cambridge, MA, USA), and ER-α (1:100, Santa Cruz, CA, USA) with appropriate positive and negative controls. After washing, tissue sections were incubated with appropriate biotinylated secondary antibody, goat anti-mouse or goat anti-rabbit (Dako, Campbellfield, Australia), followed by incubation with streptavidin-horseradish peroxidase complex. Slides were washed, and chromogen developed for 5 min with liquid 3, 3′-diaminobenzidine (Dako liquid DAB Plus, K3468). Sections were incubated with streptavidin (BD Pharmingen, CA, USA) before development with 3, 3′-diaminobenzidine tetrahydrochloride (BD Pharmingen). TUNEL staining was performed using ApopTag Peroxidase In Situ Apoptosis Detection kits (Millipore, CA, USA) according to the manufacturer's instructions. Quantitative data for Ki-67 and TUNEL were obtained by counting four fields per slide. Slides were analyzed using a Scanscope XT apparatus (Aperio Technologies, CA, USA).

Statistical analysis

Statistical significance was determined using Student's *t*-test. Results are presented as mean ± SEM. All quoted *P* values are two-tailed, and differences were considered significant for $P < 0.05$. All DFS and OS statistical analyses were performed using SPSS Statistics 23.0 (IBM Corp., NY, USA).

Results

Clinicopathological characteristics according to EGFR expression

Median follow-up duration was 69 months (range 1-143 months). Among the 2, 574 patients evaluated, 408 with unknown EGFR expression were excluded. Among the 2166 patients with HR+/HER2- tumors, 109 (5.0%) tumors showed EGFR expression and EGFR+ tumors showed higher nuclear grades (high grade; EGFR+ vs. EGFR-, 70.6% vs. 15.2%, *P*<0.001). In addition, EGFR expression correlated with HR (ER and PR) expression, and EGFR+ tumors showed higher ER negativity (16.5% vs. 0.1%, *P*<0.001), PR negativity (24.8% vs. 2.0%, *P*<0.001), p53 expression (49.5% vs. 17.5%, *P*<0.001), and Ki-67 levels (≥14.0%; 89.4% vs. 48.1%, *P*<0.001) than EGFR- tumors.

In 760 Lum B breast cancers, 87 (11.4%) tumors were EGFR+, but only 17(1.3%) tumors were EGFR+ in 1, 345 Lum A breast cancers (*P*<0.001). Clinicopathological characteristics according to subtypes are shown in Table 1.

Survival analysis according to EGFR expression

On univariate analysis using the Kaplan–Meier method, EGFR+ tumors were associated with poorer survival than EGFR- tumors (5-year DFS, 84.3% vs. 97.9, $P < 0.001$; 5-year OS, 95.6 % vs. 99.5%, $P < 0.001$) According to subtypes, in Lum A breast cancers, EGFR showed significant association with OS (5-year OS, EGFR+ vs. EGFR-, 92.3% vs. 99.8%, $P < 0.001$), but no significant difference was seen in DFS (5-year DFS, EGFR+ vs. EGFR-, 92.3% vs. 98.9%, $P = 0.280$). However, in Lum B breast cancers, significant differences were seen in DFS (5-year

Table 1. Clinicopathological characteristics of luminal A and luminal B breast cancers according to EGFR expression. EGFR epidermal growth factor receptor, BCS breast-conserving surgery, ER estrogen receptor, PR progesterone receptor

Characteristics		Luminal B (n=760)			Luminal A (n=1345)		P
		EGFR+ (n=87)	EGFR- (n=673)	P	EGFR+ (n=17)	EGFR- (n=1328)	
Age (median, range)		44 (37-77)	45 (25-81)	0.646	42 (28-64)	43 (21-90)	0.390
Operation	Mastectomy	14 (16.1%)	200 (29.7%)	0.008	5 (29.4%)	328 (24.7%)	0.584
	Breast conserving surgery	73 (83.9%)	473 (70.3%)		12 (70.6%)	1000 (75.3%)	
T stage	T1	37 (42.5%)	364 (54.1%)	0.127	11 (64.7%)	948 (71.4%)	0.92
	T2	46 (52.9%)	267 (39.7%)		5 (29.4%)	329 (24.8%)	
	T3	4 (4.6%)	40 (5.9%)		1 (5.9%)	49 (3.7%)	
	T4	0	2 (0.3%)		0	2 (0.2%)	
N stage	N0	54 (62.1%)	344 (51.1%)	0.314	12 (70.6%)	878 (66.1%)	0.553
	Nmi	0	6 (0.9%)		1 (5.9%)	23 (1.7%)	
	N1	21 (24.1%)	216 (32.1%)		4 (23.5%)	321 (24.2%)	
	N2	7 (8.0%)	73 (10.8%)		0	70 (5.3%)	
	N3	5 (5.7%)	34 (5.1%)		0	36 (2.7%)	
Stage	IA	25 (28.7%)	243 (36.1%)	0.136	8 (47.1%)	721 (54.3%)	0.647
	IB	0	6 (0.9%)		1 (5.9%)	23 (1.7%)	
	IIA	37 (42.5%)	186 (27.6%)		5 (29.4%)	324 (24.4%)	
	IIB	10 (11.5%)	118 (17.5%)		3 (17.6%)	143 (10.7%)	
	IIIA	10 (11.5%)	85 (12.6%)		0	80 (6.0%)	
	IIIB	0	1 (0.1%)		0	2 (0.2%)	
	IIIC	5 (5.7%)	34 (5.1%)		0	36 (2.7%)	
Nuclear grades	low/intermediate	20 (23.0%)	458 (68.1%)	< 0.001	12 (70.6%)	1,239 (93.3%)	<0.001
	high	67 (77.0%)	215 (31.9%)		5 (29.4%)	89 (6.7%)	
ER	positive	71 (81.6%)	673 (100%)	< 0.001	16 (94.1%)	1,328 (100%)	<0.001
	negative	16 (18.4%)	0		1 (5.9%)	0	
PR	positive	63 (72.4%)	653 (97.0%)	< 0.001	14 (82.4%)	1,308 (98.5%)	<0.001
	negative	24 (27.6%)	20 (3.0%)		3 (17.6%)	20 (1.5%)	
Radiotherapy	yes	77 (88.5%)	539 (80.2%)	0.079	13 (76.5%)	1,075 (81.1%)	0.631
	no	10 (11.5%)	133 (19.8%)		4 (23.5%)	251 (18.9%)	
	unknown		1			2	
Chemotherapy	yes	82 (94.3%)	549 (81.8%)	0.003	10 (58.8%)	756 (57.1%)	0.884
	no	5 (5.7%)	122 (18.2%)		7 (41.2%)	569 (42.9%)	
	unknown		2			3	

DFS, EGFR+ vs. EGFR-, 81.6% vs. 96.1%, $P < 0.001$) and OS (5-year OS, 96.4% vs. 98.7%, $P = 0.005$) (Fig. 1A and 1B). As shown in Fig. 1C, EGFR expression is inversely correlated with ER-α expression.

Multivariate analysis showed that EGFR expression was also associated with increased risk of recurrence and death. In the Lum B subtype, compared with EGFR- tumors, EGFR+ tumors showed a hazard ratio of 5.22 (95% CI, 1.29–21.15, $P = 0.020$) (Table 2) for OS. EGFR+ tumors showed a hazard ratio of 2.91 (95% CI, 1.35–6.28, $P = 0.006$) (Table 3) for DFS. However, for Lum A subtype, EGFR expression was not a significant prognostic factor for either DFS or OS.

Response to tamoxifen in ER-α and EGFR-positive breast cancer cells

To investigate the relationship between ER-α and EGFR, we chose two breast cancer cell lines: BT474 and MCF7. BT474 breast cancer cells had features of high EGFR and low ER-α while MCF7 breast cancer cells had low EGFR and high ER-α expression (Fig. 2A and 2B). Using the two breast cancer cell lines, we investigated the effect of 4-OHT on ER-α-positive breast cancer cell viability. The viability of ER-α-positive breast cancer cells dose-dependently decreased with 4-OHT treatment for both BT474 and MCF7 breast cancer cells (Fig. 2C). Based on these results, we chose 10 μM 4-OHT for this study.

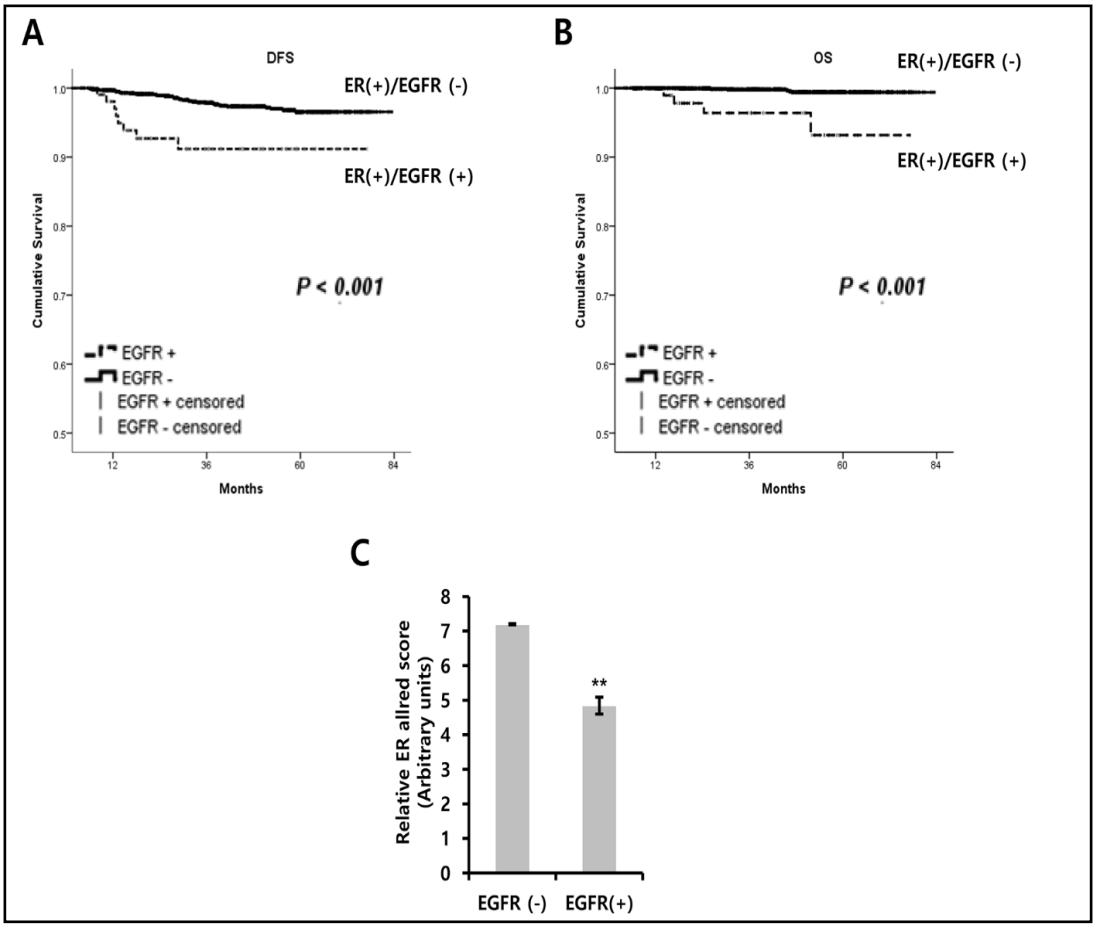

Fig. 1. EGFR expression is associated with poor prognosis of ER (+) breast cancer patients. (A) Disease-free survival. (B) Overall survival. (C) Correlation between ER-α and EGFR. Values are mean ± SEM. ** P<0.01 vs. EGFR (-).

Table 2. Multivariate analysis of disease-free survival in HR+ and HER2- tumors according to subtype. Adjusted for age, stage, nuclear grade, progesterone receptor, cytokeratin 5/6 and chemotherapy

DFS	B	Standard error	Wald	P	Hazard ratio	95% confidence interval	
						lower	upper
Luminal A							
Age	0.012	0.033	0.124	0.725	1.012	0.949	1.079
Stage	0.774	0.345	5.032	0.025	2.169	1.103	4.265
High grade vs. Low/intermediate grades	1.097	0.510	4.626	0.031	2.995	1.102	8.138
EGFR+ vs. EGFR-	0.711	1.140	0.389	0.533	2.037	0.218	19.019
Luminal B							
Age	-0.025	0.022	1.322	0.250	0.975	0.935	1.018
Stage	0.887	0.212	17.488	0.000	2.427	1.602	3.678
High grade vs. Low/intermediate grades	0.655	0.293	5.006	0.025	1.926	1.085	3.419
EGFR+ vs. EGFR-	1.069	0.392	7.450	0.006	2.914	1.352	6.280

Table 3. Multivariate analysis of overall survival in HR+ and HER2- tumors according to subtype. Adjusted for age, stage, nuclear grade, progesterone receptor, cytokeratin 5/6 and chemotherapy

OS	B	Standard error	Wald	P	Hazard ratio	95% confidence interval	
						lower	upper
Luminal A							
Age	0.010	0.091	0.012	0.913	1.010	0.845	1.207
Stage	1.048	0.853	1.507	0.220	2.851	0.535	15.178
High grade vs. Low/intermediate grades	2.828	1.225	5.329	0.021	16.915	1.533	186.703
EGFR+ vs. EGFR-	2.414	1.840	1.721	0.190	11.178	0.303	411.778
Luminal B							
Age	0.010	0.049	0.038	0.845	1.010	0.916	1.113
Stage	1.814	0.579	9.812	0.002	6.133	1.972	19.076
High grade vs. Low/intermediate grades	1.146	0.698	2.698	0.100	3.147	0.801	12.356
EGFR+ vs. EGFR-	1.653	0.713	5.371	0.020	5.225	1.291	21.150

Basal ER-α expression is downregulated in BT474 cells by EGFR ligands TGFA and EGF

We examined the alteration of ER-α mRNA and protein expression by EGFR ligands TGFA and EGF. As shown in Fig. 3A and 3B, the levels of ER-α mRNA and protein expression were dramatically decreased by TGFA and EGF, respectively. Relative to controls, basal levels of ER-α mRNA decreased to 0.45 ± 0.01-fold with 50 ng/ml TGFA (Fig. 3A) and 0.49 ± 0.05-fold with EGF (Fig. 3B). As a positive control, we measured expression of the pro-inflammatory chemokine IL-8 with TGFA or EGF treatment. Our results showed that the levels of IL-8 mRNA and protein expression were significantly increased by TGFA or EGF treatment (Fig. 3C and 3D). These results demonstrated that EGFR ligands such as TGFA and EGF are important for ER-α transcriptional suppression.

TGFA-induced ER-α downregulation is acquired tamoxifen resistance in BT474 breast cancer cells

We investigated whether TGFA induced ER-α downregulation to result in acquired tamoxifen resistance. After 50 ng/ml TGFA pretreatment for 24 h, we treated cells with or without 10 μM 4-OHT for 24 h. Cell viability was significantly decreased by tamoxifen treatment (Fig. 4A). However, after TGFA treatment, cell viability was slightly decreased by 4-OHT treatment (Fig. 4A). These results suggested that ER-α downregulation by EGFR ligands suppressed cell response to a 4-OHT.

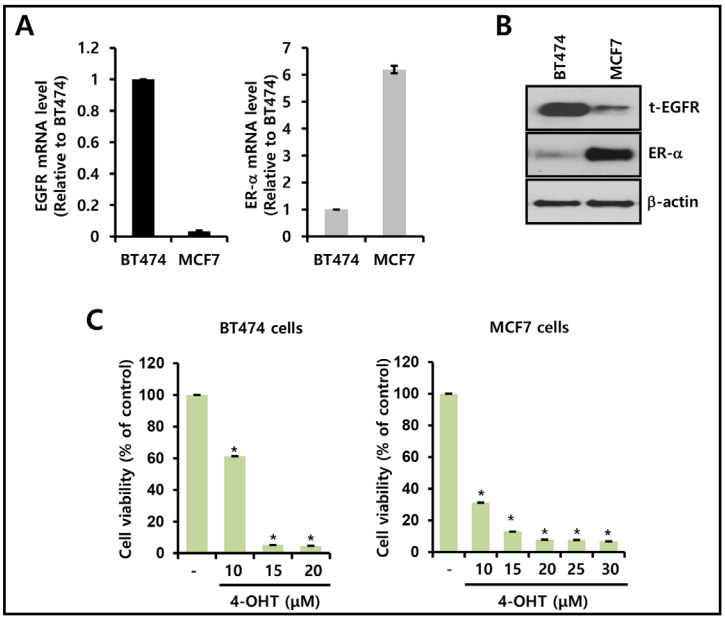

Fig. 2. Response to tamoxifen in ER-α and EGFR-positive breast cancer cells. (A, B) EGFR and ER-α mRNA and protein were detected by real-time PCR (A) and western blots (B). (C) Cells were treated with 4-OHT at the indicated concentration for 24 h. Cell viability was analyzed by MTT assays. Results are representative of three independent experiments. Values are mean ± SEM. * P<0.05 vs. (-) control.

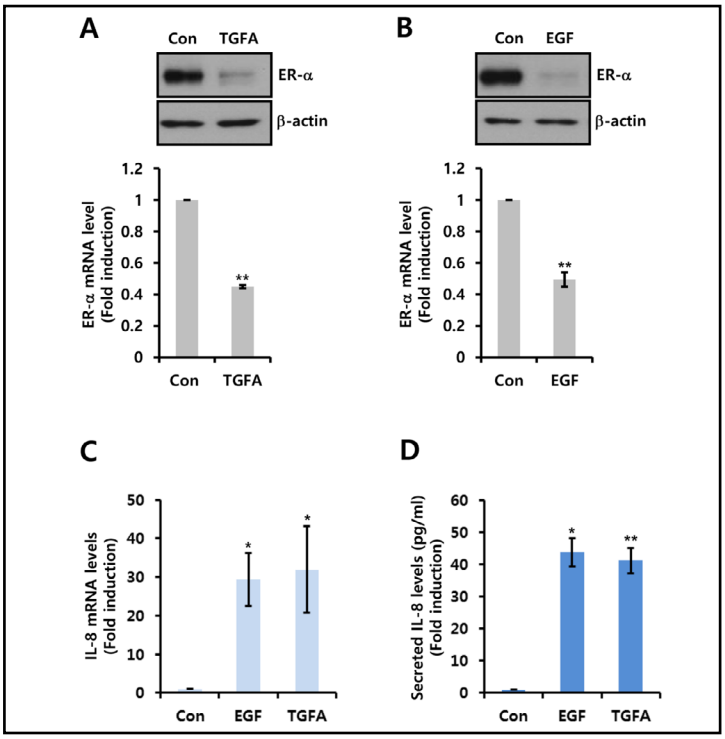

Fig. 3. Basal levels of ER-α expression are downregulated by EGFR ligands TGFA and EGF in BT474 breast cancer cells. (A, B) After 24-h serum starvation, we treated cells with 50 ng/ml TGFA and EGF. Levels of ER-α mRNA and protein were detected by real-time PCR and western blots. (C, D) Under the same conditions, IL-8 mRNA and protein were detected by real-time PCR and ELISA. Results are representative of three independent experiments. Values are mean ± SEM. * P<0.05, ** P<0.01 vs. Con. Con: control.

We measured levels of the apoptosis-related proteins expression such as PARP-1 and procaspase-3. The cleavage form of PARP-1 significantly increased with 4-OHT and levels of procaspase-3 decreased with 4-OHT (Fig. 4B). With TGFA pretreatment, 4-OHT-induced increases in the cleavage form of PARP-1 decreased (Fig. 4B). We also investigated the anchorage-independent growth of BT474 breast cancer cells with TGFA and/or 4-OHT treatment. Anchorage-independent growth of BT474 breast cancer cells significantly decreased with 4-OHT treatment and recovered after TGFA pretreatment (Fig. 4C). Based on these results, we demonstrated that ER-α downregulation by EGFR ligands contributed to acquired tamoxifen resistance.

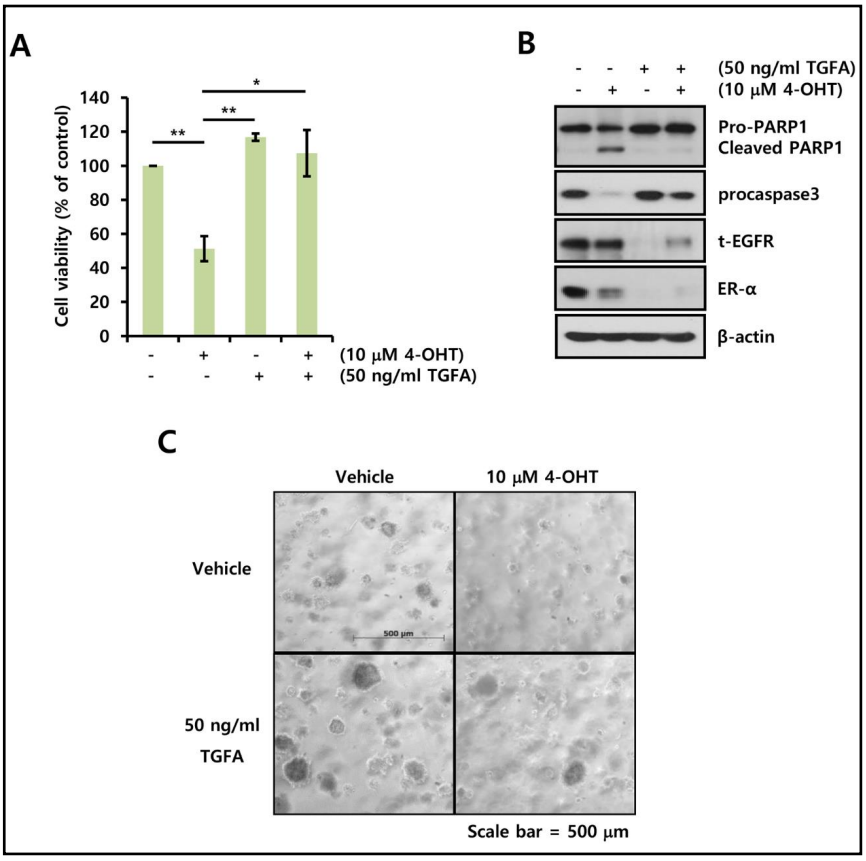

Fig. 4. TGFA-induced ER-α downregulation resulted in acquired tamoxifen resistance in BT474 breast cancer cells. (A, B) After 24 h serum starvation, we pretreated cells with or without 50 ng/ml TGFA for 24 h and cultured with or without 10 μM 4-OHT for 24 h. (A) Cell viability was analyzed by MTT assays. (B) Levels of PARP-1 and procaspase-3 protein were detected by western blots. (C) Cells were pretreated with or without 50 ng/ml TGFA for 24 h, trypsinized, and reseeded with or without 10 μM 4-OHT in agarose plates for colony-forming assays. Results are representative of three independent experiments. Values are mean ± SEM. * P<0.05, ** P<0.01 vs. (-) control.

EGFR ligand-induced ER-α downregulation is prevented by specific EGFR inhibitor gefitinib in BT474 cells

We investigated whether EGFR ligand-induced ER-α downregulation was prevented by gefitinib. After 24 h serum starvation, we pretreated cells with 10 μM gefitinib for 30 min, then cultured for 24 h with or without 50 ng/ml TGFA or EGF. Basal levels of ER-α protein decreased with TGFA or EGF treatment (Fig. 5A and 5B). In contrast, downregulation of ER-α by TGFA (Fig. 5A) or EGF (Fig. 5B) was prevented by gefitinib treatment. Under the same conditions, levels of ER-α mRNA patterns were similar to protein expression (Fig. 5C and 5D). These results demonstrated that ER-α expression was downregulated by an EGFR-dependent pathway in ER-α-positive breast cancer cells.

Combination of tamoxifen and gefitinib synergistically induces apoptosis and suppresses tumor growth

We examined the effect of 4-OHT and/or gefitinib on BT474 breast cancer cells. After 24 h serum starvation, we cultured cells with or without 10 μM 4-OHT and/or gefitinib for 4 h. Cell viability decreased with 4-OHT or gefitinib (Fig. 6A). Under tamoxifen and gefitinib cotreatment conditions, cell viability was completely decreased (Fig. 6A). In addition, we examined levels of the apoptosis-related proteins PARP-1 and procaspase-3. The cleavage form of PARP-1 was significantly increased by a combination of 4-OHT and gefitinib while the levels of procaspase-3 were decreased (Fig. 6B). We also investigated the anchorage-independent growth of BT474 breast cancer cells with 4-OHT and/or gefitinib treatment. Anchorage-independent growth synergistically decreased with a combination of 4-OHT and gefitinib

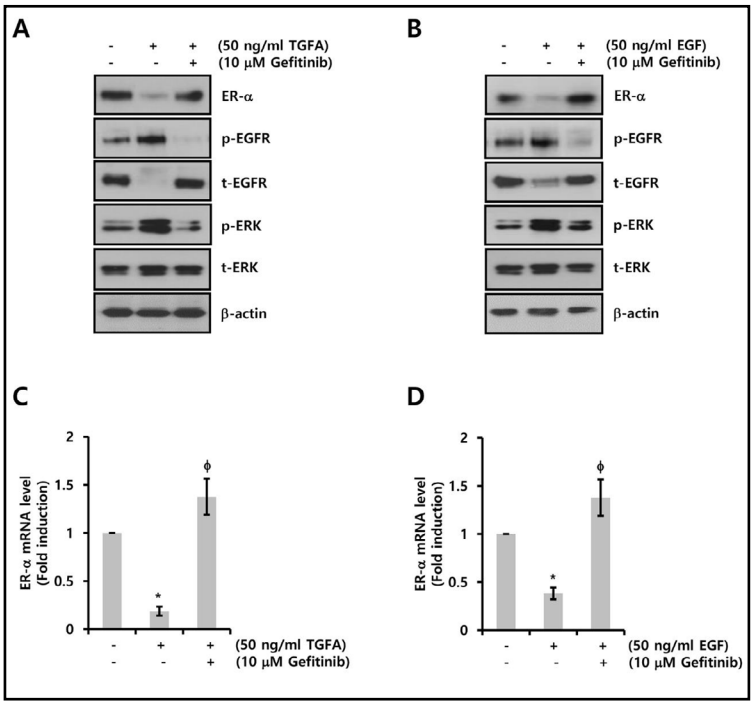

Fig. 5. EGFR ligand-induced ER-downregulation is prevented by the specific EGFR inhibitor gefitinib in BT474 breast cancer cells. After 24-h serum starvation, we pretreated cells with or without 10 μM gefitinib for 30 min and then treated cells with 50 ng/ml TGFA (A, C) and EGF (B, D). Levels of p-, t-EGFR, p-, t-ERK and ER-α protein were detected by western blots (A, B). Levels of ER-α mRNA were detected by real-time PCR (C, D). Results are representative of three independent experiments. Values are mean ± SEM. * P<0.05 vs. (-) control, ϕ P<0.05 vs. TGFA- or EGF-treated cells.

compared with single treatment with tamoxifen or gefitinib (Fig. 6C).

We examined the effect of tamoxifen and/or gefitinib on orthotopic xenografts models. An experimental schematic model is in Fig. 7A. Tumor growth was maximally delayed in tamoxifen-and-gefitinib combination groups (Fig. 7B). In addition, analysis of tumor tissues from each condition revealed reduced connective tissue areas in tamoxifen-and-gefitinib combination groups (Fig. 7C, H&E). We also observed that expression of the Ki67 cell-proliferation marker protein decreased in tamoxifen-and-gefitinib combination groups (Fig. 7C). Under the tamoxifen and gefitinib treatment, Ki67 positive cells decreased to 15.1 ± 0.2% of control level (36.0 ±

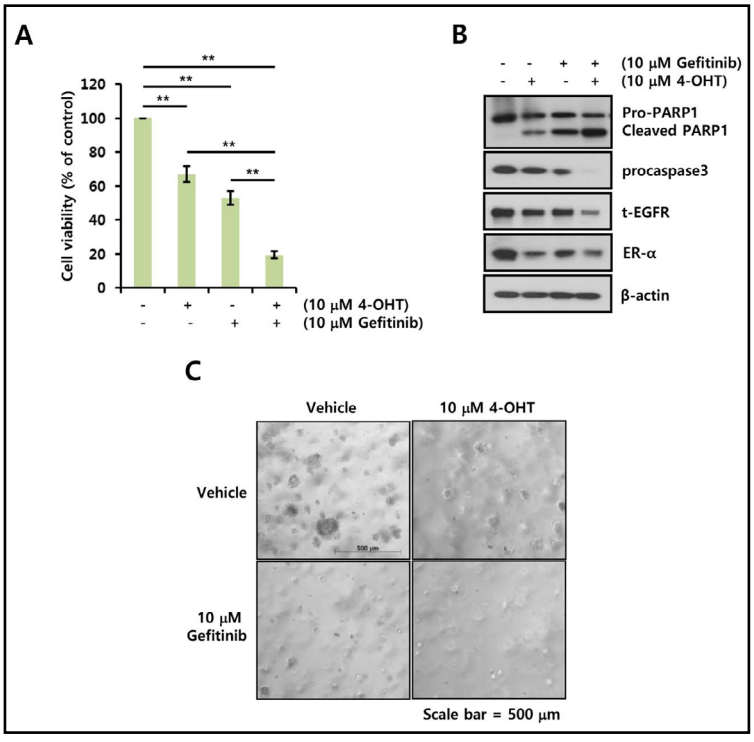

Fig. 6. Combination of tamoxifen and gefitinib synergistically induces apoptosis in in vitro. (A, B) After 24 h serum starvation, we treated cells with or without 10 M 4-OHT and/or gefitinib for 4 h. (A) Cell viability was analyzed by MTT assays. (B) Levels of PARP-1 and procaspase-3 protein were detected by western blots. (C) Cells were treated with or without 10 μM 4-OHT and/or gefitinib in agarose plates for colony-forming assays. Values are mean ± SEM. * P<0.05, ** P<0.01 vs. (-) control.

1.0%). To verify apoptotic cell death, we analyzed TUNEL assay. Our results showed that TUNEL positive cells is increased by tamoxifen and gefitinib cotreatment compared with vehicle only (Fig. 7D). These results demonstrated that EGFR activity was associated with regulation of ER-α expression. Inhibition of EGFR increased the therapeutic effect in ER-α (+) breast cancer.

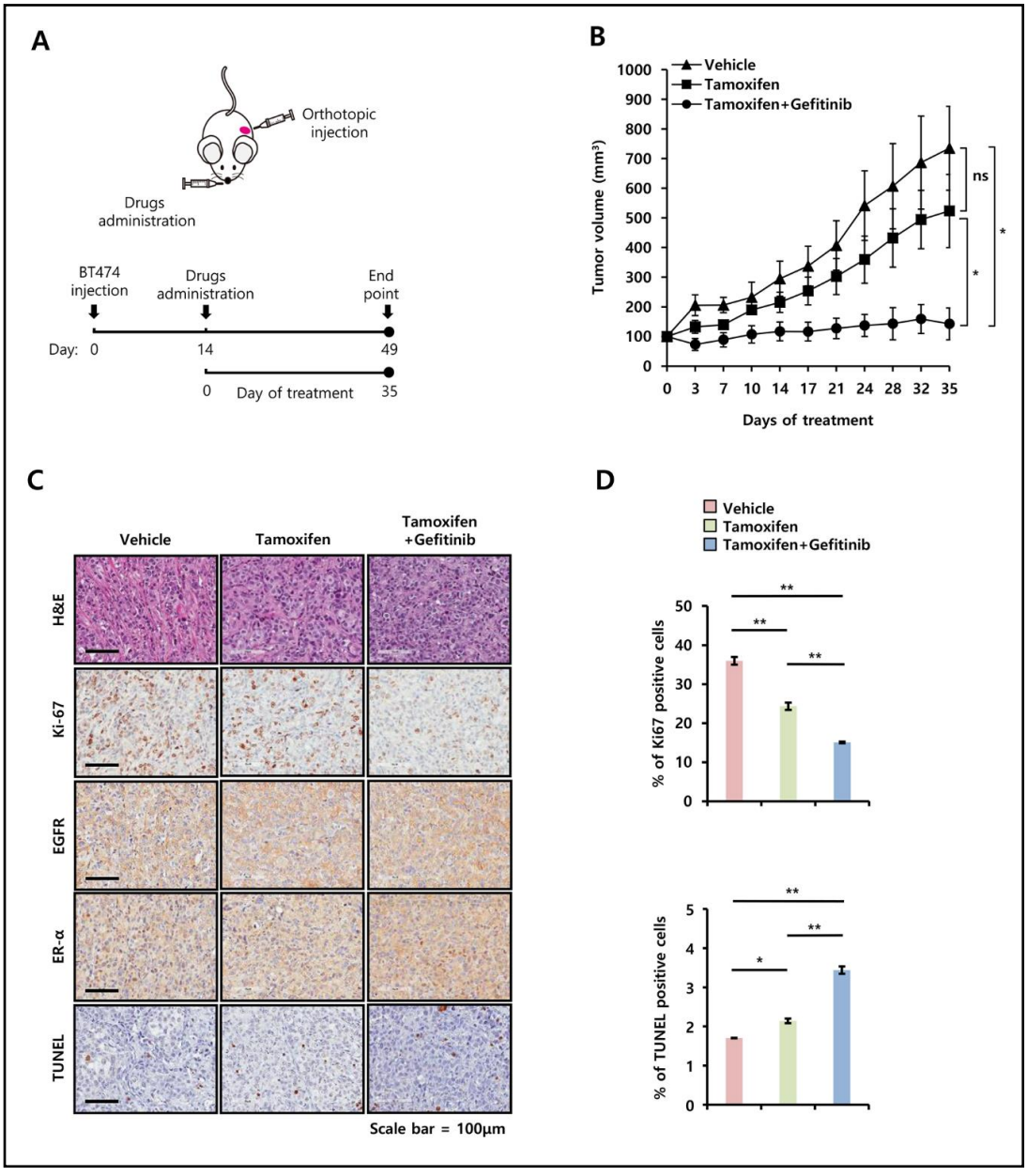

Fig. 7. Pharmacological effect of tamoxifen and/or gefitinib in orthotopic xenograft models. (A) Experimental schematic of drug injection into the xenograft model. (B) Tumor sizes for each group (n = 4) were analyzed for 35 days. Values are mean ± standard errors. * P<0.05 vs. vehicle. (C) After 35 days, tumor tissues were collected from Veh-, tamoxifen- and/or gefitinib-treated groups and stained with H&E or for Ki67, EGFR, ER-α and TUNEL. (D) Immunohistochemical scores. Quantitative data for Ki-67 and TUNEL positive cells were obtained by counting four fields. Values are mean ± SEM. * P<0.05, ** P<0.01 vs. (-) control. Scale bar = 100 μm.

Discussion

A clinical aspect of tamoxifen-resistant breast cancer is often increased expression of RTKs such as EGFR and HER2 [24, 25]. Interaction between RTK and ER-α is one of the most important mechanisms of endocrine resistance [7]. Preclinical studies support the hypothesis that endocrine resistance could be overcome using TKIs through bidirectional RTK-ER-α crosstalk [16, 17, 26]. Smith *et al.* reported that treatment with the EGFR TKI gefitinib does not have any clinical benefit for patients with early breast cancer [17]. However, this study did not prospectively identify an appropriate subgroup of tumors, such as those overexpressing EGFR [17]. Therefore, we investigated the efficacy of combination therapy against EGFR and ER-α using EGFR and ER (+) breast cancer cells and orthotopic xenograft mouse models.

EGFR is the only member of the HER family that can be activated following heterodimerization with other HER receptors in addition to homodimerization [27]. EGFR expression is reported in 14-91% of breast cancers [12, 13, 28, 29] and is negatively correlated with ER status. EGFR expression is particularly high in ER- breast cancer [29, 30]. Tutsi *et al.* [29] reported that EGFR was positive in 26.9% (277/1029), which was 57% (199/349) of ER- breast cancers; EGFR was positive in only 11% (78/680) of ER+ breast cancers. In addition, EGFR is an independently significant prognostic factor for DFS (P = 0.017) and OS (P = 0.010). Our study found expression of EGFR in 5%, which was lower than reported in previous studies. This result might be due to the study population, which included only HR+/HER2- breast cancers. In addition, EGFR expression was different in Lum A and B subtypes: EGFR was positive in 11.1% of Lum B cancers, but only 1.3% of Lum A cancers. Although EGFR expression was low, EGFR was an independent prognostic factor for HR+/HER2- breast cancer patients. These results were consistent with reports linking EGFR overexpression with a more aggressive breast tumor phenotype and poorer patient prognosis [10-12]. However, in this study, EGFR was significantly associated with prognosis in the Lum B subtype. EGFR was not a prognostic factor in the Lum A subtype.

Further analysis determined that EGFR+ tumors had more primary resistance than EGFR- tumors (EGFR+, 42.9 % [6/8] vs. EGFR-, 8.8% [6/68], P = 0.001). Although the number of patients was small, these results suggest that EGFR could be associated with primary endocrine resistance. Two studies reported that EGFR inhibitors had a positive effect for therapy-naïve patients. Osborne *et al.* [31] reported a trend towards prolonged PFS in endocrine therapy-naïve patients compared to patients who received prior endocrine therapy. Christianly *et al*. reported a marked advantage in PFS and clinical benefit rate in patients with HR+ metastatic breast cancer who received anastrozole in combination with gefitinib [32]. In subset analysis of endocrine therapy-naïve patients, the PFS for patients receiving anastrozole and gefitinib indicated a benefit over patients receiving anastrozole and placebo (20.2 months vs. 8.4 months [HR 0.39, 95% CI, 0.16–0.97]), but no significant difference from prior endocrine therapy (11.2 months vs. 7.1 months [HR 0.65, 95% CI, 0.32–1.33]) [32]. These findings also suggest that EGFR expression could be associated with primary endocrine resistance and EGFR inhibitors could be effective for endocrine therapy-naïve patients or patients receiving adjuvant endocrine therapy. We need well-designed clinical studies to determine the efficacy of EGFR inhibitors.

A complete loss of ER-α expression is uncommon and ~10-20% of patients with metastatic breast cancer show conversion to ER-α-negative type cancer [14, 33]. During establishment of tamoxifen-resistant cells, long-term culture of MCF7 breast cancer cells with 4-OHT decreases ER-α expression in tamoxifen-resistant breast cancer cells [34, 35]. Consistent with these reports, we observed that ER-α expression was regulated by EGFR expression and activity. Furthermore, levels of ER-α expression were significantly decreased by the EGFR ligands EGF and TGFA. On contrast, EGF-induced ER-α downregulation was prevented by gefitinib or lapatinib. We demonstrated that ER-α expression was regulated through an EGFR-dependent pathway. Breast cancers with high EGFR expression may be associated with loss of ER-α and induced endocrine resistance.

Although the mechanisms of endocrine resistance are complex, tamoxifen-resistant breast cancer cells have increased expression or constitutive activation of EGFR and/or downstream MAPK or Akt activation [34, 36]. We reported that the TKI neratinib induces more effective apoptotic cell death than gefitinib in tamoxifen-resistant breast cancer cells [34]. Consistent with these results, we showed that the combination of gefitinib and 4-OHT more effectively induced apoptotic cell death than 4-OHT alone in BT474 breast cancer cells. In addition, tumor growth was markedly blocked by tamoxifen and gefitinib cotreatment in orthotopic xenograft mouse models. Therefore, TKIs should preferentially be combined with endocrine therapy for EGFR and ER-α (+) breast cancer patients.

Conclusion

In this study, we investigated the possibility of EGFR-targeted therapy for patients with ER (+) breast cancers with high EGFR expression. Clinically, EGFR expression inversely correlated with ER-α expression in breast cancers. In addition, the DFS and OS of EGFR and ER (+) breast cancer patients was poorer than patients with ER (+) breast cancer, especially luminal B subtype. We found that cotreatment with tamoxifen and gefitinib synergistically increased apoptosis in EGFR and ER (+) breast cancer in *in vitro* and *in vivo* models. We demonstrated that a combination therapy of tamoxifen and EGFR-targeted TKIs against ER-α and EGFR were more effective than single treatment with gefitinib or tamoxifen for patients with EGFR and ER (+) breast cancer. Future clinical trials with appropriate patient selection parameters are needed to provide evidence regarding the optimization of individualized therapeutic regimens to treat endocrine-resistant breast cancer.

Abbreviations

EGFR (Epidermal Growth Factor Receptor); TGFA (Transforming Growth Factor Alpha); HR+ (Hormone Receptor-Positive); ER (Estrogen Receptor); TKI (Tyrosine Kinase Inhibitor); 4-OHT (4-Hydroxytamoxifen); Lum A (Luminal A-like); Lum B (Luminal B-like); DFS (Disease Free Survival); OS (Overall Survival).

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Disclosure Statement

The authors have no conflicts of interest to declare.

References

1 Osborne CK: Tamoxifen in the treatment of breast cancer. N Engl J Med 1998;339:1609-1618.
2 Early Breast Cancer Trialists' Collaborative Group: Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: An overview of the randomised trials. Lancet 2005;365:1687-1717.

3 Cleator SJ, Ahamed E, Coombes RC, Palmieri C: A 2009 update on the treatment of patients with hormone receptor-positive breast cancer. Clin Breast Cancer 2009;9:S6-S17.
4 Musgrove EA, Sutherland RL: Biological determinants of endocrine resistance in breast cancer. Nat Rev Cancer 2009;9:631-643.
5 Cardoso F, Costa A, Senkus E, Aapro M, André F, Barrios CH, Bergh J, Bhattacharyya G, Biganzoli L, Cardoso MJ, Carey L, Corneliussen-James D, Curigliano G, Dieras V, El Saghir N, Eniu A, Fallowfield L, Fenech D, Francis P, Gelmon K, et al.: 3rd eso–esmo international consensus guidelines for advanced breast cancer (abc 3). The Breast 2017;31:244-259.
6 Arpino G, Wiechmann L, Osborne CK, Schiff R: Crosstalk between the estrogen receptor and the her tyrosine kinase receptor family: Molecular mechanism and clinical implications for endocrine therapy resistance. Endocr Rev 2008;29:217-233.
7 Giuliano M, Schifp R, Osborne CK, Trivedi MV: Biological mechanisms and clinical implications of endocrine resistance in breast cancer. Breast 2011;20:S42-49.
8 Jin K, Kong X, Shah T, Penet MF, Wildes F, Sgroi DC, Ma XJ, Huang Y, Kallioniemi A, Landberg G, Bieche I, Wu X, Lobie PE, Davidson NE, Bhujwalla ZM, Zhu T, Sukumar S: The hoxb7 protein renders breast cancer cells resistant to tamoxifen through activation of the egfr pathway. Proc Natl Acad Sci U S A 2012;109:2736-2741.
9 Knowlden JM, Hutcheson IR, Barrow D, Gee JM, Nicholson RI: Insulin-like growth factor-i receptor signaling in tamoxifen-resistant breast cancer: A supporting role to the epidermal growth factor receptor. Endocrinology 2005;146:4609-4618.
10 Normanno N, Bianco C, Strizzi L, Mancino M, Maiello MR, De Luca A, Caponigro F, Salomon DS: The erbb receptors and their ligands in cancer: An overview. Curr Drug Targets 2005;6:243-257.
11 Normanno N, De Luca A, Maiello MR, Mancino M, D'Antonio A, Macaluso M, Caponigro F, Giordano A: Epidermal growth factor receptor (egfr) tyrosine kinase inhibitors in breast cancer: Current status and future development. Front Biosci 2005;10:2611-2617.
12 Normanno N, Bianco C, De Luca A, Maiello MR, Salomon DS: Target-based agents against erbb receptors and their ligands: A novel approach to cancer treatment. Endocr Relat Cancer 2003;10:1-21.
13 Salomon DS, Brandt R, Ciardiello F, Normanno N: Epidermal growth factor-related peptides and their receptors in human malignancies. Crit Rev Oncol Hematol 1995;19:183-232.
14 Drury SC, Detre S, Leary A, Salter J, Reis-Filho J, Barbashina V, Marchio C, Lopez-Knowles E, Ghazoui Z, Habben K, Arbogast S, Johnston S, Dowsett M: Changes in breast cancer biomarkers in the igf1r/pi3k pathway in recurrent breast cancer after tamoxifen treatment. Endocr Relat Cancer 2011;18:565-577.
15 Green MD, Francis PA, Gebski V, Harvey V, Karapetis C, Chan A, Snyder R, Fong A, Basser R, Forbes JF, Australian New Zealand Breast Cancer Trials Group: Gefitinib treatment in hormone-resistant and hormone receptor-negative advanced breast cancer. Ann Oncol 2009;20:1813-1817.
16 von Minckwitz G, Jonat W, Fasching P, du Bois A, Kleeberg U, Luck HJ, Kettner E, Hilfrich J, Eiermann W, Torode J, Schneeweiss A: A multicentre phase ii study on gefitinib in taxane- and anthracycline-pretreated metastatic breast cancer. Breast Cancer Res Treat 2005;89:165-172.
17 Smith IE, Walsh G, Skene A, Llombart A, Mayordomo JI, Detre S, Salter J, Clark E, Magill P, Dowsett M: A phase ii placebo-controlled trial of neoadjuvant anastrozole alone or with gefitinib in early breast cancer. J Clin Oncol 2007;25:3816-3822.
18 Sarwar N, Kim JS, Jiang J, Peston D, Sinnett HD, Madden P, Gee JM, Nicholson RI, Lykkesfeldt AE, Shousha S, Coombes RC, Ali S: Phosphorylation of eralpha at serine 118 in primary breast cancer and in tamoxifen-resistant tumours is indicative of a complex role for eralpha phosphorylation in breast cancer progression. Endocr Relat Cancer 2006;13:851-861.
19 Yamashita H, Nishio M, Kobayashi S, Ando Y, Sugiura H, Zhang Z, Hamaguchi M, Mita K, Fujii Y, Iwase H: Phosphorylation of estrogen receptor alpha serine 167 is predictive of response to endocrine therapy and increases postrelapse survival in metastatic breast cancer. Breast Cancer Res 2005;7:R753-764.
20 Gutierrez MC, Detre S, Johnston S, Mohsin SK, Shou J, Allred DC, Schiff R, Osborne CK, Dowsett M: Molecular changes in tamoxifen-resistant breast cancer: Relationship between estrogen receptor, her-2, and p38 mitogen-activated protein kinase. J Clin Oncol 2005;23:2469-2476.

21 Polychronis A, Sinnett HD, Hadjiminas D, Singhal H, Mansi JL, Shivapatham D, Shousha S, Jiang J, Peston D, Barrett N, Vigushin D, Morrison K, Beresford E, Ali S, Slade MJ, Coombes RC: Preoperative gefitinib versus gefitinib and anastrozole in postmenopausal patients with oestrogen-receptor positive and epidermal-growth-factor-receptor-positive primary breast cancer: A double-blind placebo-controlled phase ii randomised trial. Lancet Oncol 2005;6:383-391.
22 Bae SY, Jung SP, Jung ES, Park SM, Lee SK, Yu JH, Lee JE, Kim SW, Nam SJ: Clinical characteristics and prognosis of pregnancy-associated breast cancer: Poor survival of luminal b subtype. Oncology 2018;95:163-169.
23 Goldhirsch A, Winer EP, Coates AS, Gelber RD, Piccart-Gebhart M, Thurlimann B, Senn HJ: Personalizing the treatment of women with early breast cancer: Highlights of the st gallen international expert consensus on the primary therapy of early breast cancer 2013. Ann Oncol 2013;24:2206-2223.
24 Gee JM, Robertson JF, Gutteridge E, Ellis IO, Pinder SE, Rubini M, Nicholson RI: Epidermal growth factor receptor/her2/insulin-like growth factor receptor signalling and oestrogen receptor activity in clinical breast cancer. Endocr Relat Cancer 2005;12:S99-S111.
25 Mass R: The role of her-2 expression in predicting response to therapy in breast cancer. Semin Oncol 2000;27:46-52; discussion 92-100.
26 Goldhirsch A, Ingle JN, Gelber RD, Coates AS, Thurlimann B, Senn HJ, Panel m: Thresholds for therapies: Highlights of the st. Gallen international expert consensus on the primary therapy of early breast cancer 2009. Ann Oncol 2009;20:1319-1329.
27 Deb TB, Su L, Wong L, Bonvini E, Wells A, David M, Johnson GR: Epidermal growth factor (egf) receptor kinase-independent signaling by egf. J Biol Chem 2001;276:15554-15560.
28 Changavi AA, Shashikala A, Ramji AS: Epidermal growth factor receptor expression in triple negative and nontriple negative breast carcinomas. J Lab Physicians 2015;7:79-83.
29 Tsutsui S, Ohno S, Murakami S, Hachitanda Y, Oda S: Prognostic value of epidermal growth factor receptor (egfr) and its relationship to the estrogen receptor status in 1029 patients with breast cancer. Breast Cancer Res Treat 2002;71:67-75.
30 Flageng MH, Knappskog S, Haynes BP, Lonning PE, Mellgren G: Inverse regulation of egfr/her1 and her2-4 in normal and malignant human breast tissue. PLoS One 2013;8:e74618.
31 Osborne CK, Neven P, Dirix LY, Mackey JR, Robert J, Underhill C, Schiff R, Gutierrez C, Migliaccio I, Anagnostou VK, Rimm DL, Magill P, Sellers M: Gefitinib or placebo in combination with tamoxifen in patients with hormone receptor-positive metastatic breast cancer: A randomized phase ii study. Clin Cancer Res 2011;17:1147-1159.
32 Cristofanilli M, Valero V, Mangalik A, Royce M, Rabinowitz I, Arena FP, Kroener JF, Curcio E, Watkins C, Bacus S, Cora EM, Anderson E, Magill PJ: Phase ii, randomized trial to compare anastrozole combined with gefitinib or placebo in postmenopausal women with hormone receptor–positive metastatic breast cancer. Clinical Cancer Research 2010;16:1904-1914.
33 Hoefnagel LD, Moelans CB, Meijer SL, van Slooten HJ, Wesseling P, Wesseling J, Westenend PJ, Bart J, Seldenrijk CA, Nagtegaal ID, Oudejans J, van der Valk P, van Gils CH, van der Wall E, van Diest PJ: Prognostic value of estrogen receptor alpha and progesterone receptor conversion in distant breast cancer metastases. Cancer 2012;118:4929-4935.
34 Kim S, Lee J, Oh SJ, Nam SJ, Lee JE: Differential effect of egfr inhibitors on tamoxifen-resistant breast cancer cells. Oncol Rep 2015;34:1613-1619.
35 Knowlden JM, Hutcheson IR, Jones HE, Madden T, Gee JM, Harper ME, Barrow D, Wakeling AE, Nicholson RI: Elevated levels of epidermal growth factor receptor/c-erbb2 heterodimers mediate an autocrine growth regulatory pathway in tamoxifen-resistant mcf-7 cells. Endocrinology 2003;144:1032-1044.
36 Hutcheson IR, Knowlden JM, Madden TA, Barrow D, Gee JM, Wakeling AE, Nicholson RI: Oestrogen receptor-mediated modulation of the egfr/mapk pathway in tamoxifen-resistant mcf-7 cells. Breast Cancer Res Treat 2003;81:81-93.