Estrogen receptor β1 activation accelerates resistance to epidermal growth factor receptor-tyrosine kinase inhibitors in non-small cell lung cancer

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Abstract. Non-small cell lung cancer (NSCLC) is one of the leading causes of cancer-related deaths worldwide. Epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) have revolutionized the treatment of patients with advanced EGFR-mutant NSCLC. However, drug resistance eventually develops in the majority of patients despite an excellent initial response. The present study aimed to investigate the mechanism of acquired resistance to EGFR-TKIs and to explore strategies to overcome the resistance to EGFR-TKIs from a gender perspective. PC9 and Hcc827 cell lines, sensitized to EGFR-TKI, and secondary TKI-resistant PC9-ER (erlotinib resistant) and Hcc827-ER cell lines were evaluated for the expression of ERβ1. The proliferative ability of both cell lines was analyzed after transfection of siRNA-ERβ1 using Cell Counting Kit-8 and colony formation assays. Extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) and Akt activation were detected. The co-inhibition efficiency of erlotinib and fulvestrant was analyzed in PC9-ER xenografts. The expression of ERβ1 was investigated in tumor tissues of EGFR-TKI-treated patients, and its correlation with clinicopathological factors and progression-free survival (PFS) was assessed. The expression of ERβ1 was upregulated secondary to EGFR-TKIs in PC9 and Hcc827 cell lines, with β-estradiol dependence. Both PC9-ER and Hcc827-ER cell lines were re-sensitized to erlotinib after downregulation of the expression of ERβ1. ERK1/2 and Akt pathways were activated following the silencing of the expression of ERβ1 in PC9-ER and Hcc827 cell lines. The co-treatment of erlotinib and fulvestrant exhibited better growth inhibitory efficiency compared with the treatment of each agent alone in PC9-ER-derived xenografts. Primary NSCLC samples of 53 patients treated with EGFR-TKIs were analyzed. ERβ1 was highly expressed, and the strong expression of cytoplasmic ERβ1 was related to a shorter PFS. In conclusion, ERβ1 was activated in EGFR-TKI secondary resistance. The downregulation of ERβ1 sensitized the cells to EGFR-TKIs. ERβ1 may be a key molecule in EGFR-TKI therapy. In addition, anti-ERβ1 treatment may reverse TKI resistance.

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Introduction

The efficacy of epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs), such as gefitinib and erlotinib, in non-small cell lung cancer (NSCLC) therapy has been widely demonstrated (1). Patients with EGFR-sensitizing mutations treated with EGFR-TKIs have a significantly longer progression-free survival (PFS) compared with those treated with standard chemotherapy (1,2). However, despite an excellent initial response, drug resistance eventually develops in the majority of patients, limiting the mean drug-response duration to less than 1 year (3).

The estrogen receptor (ER) pathway is among the most well-studied pathways in breast cancer. Data on ER signaling in lung cancer have increased in recent decades. In the Women's Health Initiative, more than 16,000 postmenopausal women with an intact uterus and no breast cancer history were randomly allocated to supplemental estrogen and progesterone or no hormone replacement therapy (HRT). After 5.6 years of study and 2.4 years of follow-up, the hazard ratio for lung cancer incidence in the HRT group was 1.28 (P=0.12), and the hazard ratio for death in patients with NSCLC and death specifically from NSCLC was 1.61 (P=0.02) and 1.87 (P=0.004), respectively (4). Furthermore, a prospective cohort study confirmed

an increased, dose-dependent lung cancer risk among women who received HRT (5). Commonly expressed in patients with NSCLC, human NSCLC cell lines, and mouse models, ERβ is the major functional receptor of lung cancer (6,7).

Evidence supports an interaction between EGFR and ERβ pathways in the development of lung cancer. Aromatase is a candidate prognostic factor in patients with lung adenocarcinoma, particularly in those with EGFR mutations, and may also be a beneficial therapeutic target in these patients (8). The combination of anastrozole and gefitinib compared with either drug alone maximally inhibits cell proliferation, induces apoptosis, and affects downstream signaling pathways (9). Fulvestrant adds to the effects of EGFR inhibitors, including synergy in the EGFR-mutant, erlotinib-resistant H1975 cell line. Tumor stability is achieved in human tumor xenografts with either fulvestrant or EGFR inhibitors, but tumors regress significantly when both pathways are inhibited (10). However, the function of the full-length ERβ protein, ERβ1, in resistance to EGFR-TKIs in NSCLC is still unknown.

Therefore, the present study proposed that ERβ1 activation accelerates the resistance to EGFR-TKIs in NSCLC. Secondary TKI-resistant cell lines, PC9-ER and Hcc827-ER, and xenografts were established to investigate the function of ERβ1 in secondary resistance progression and efficiency by targeting ERβ1 to reverse resistance. Furthermore, the correlations between ERβ1 and follow-up outcome after EGFR-TKI therapy were analyzed in 53 patients with advanced NSCLC and in tumors biopsy confirmed with EGFR-sensitive mutants. The results established the activation and potential therapeutic effects of ERβ1 in NSCLC with resistance to EGFR-TKIs via the bypass signals of extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) and Akt pathways.

Materials and methods

Cells and reagents. The human NSCLC cell lines Hcc827 and PC9 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). These cells were cultured under a humidified atmosphere of 5% CO_2 at 37˚C in RPMI-1640 medium (11835-030; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS). Erlotinib was kindly provided by Roche (Mannheim, Germany) and fulvestrant (129453-61-8) was purchased from Cayman Chemical (Ann Arbor, MI, USA). Erlotinib-resistant Hcc827-ER and PC9-ER cells were selected from a subculture that had acquired resistance to erlotinib using the following procedure. Cultured Hcc827 and PC9 cells were maintained in a medium containing 0.2 μM erlotinib for 7 days. After exposure to erlotinib, they were washed and cultured in drug-free medium for 14 days. Upon an increase in viable cells, they were seeded in a medium with an increasing concentration of erlotinib, from 0.4 to 4 μM, on 24-well culture plates for subcloning until a single clone was obtained. Then, EGFR mutations were detected by ARMS EGFR Mutation Detection kit (Amoy Dx, Shenzhen, China). Mutations of the PC9-ER Hcc827-ER were found to include E746-A750del. The cells were transfected with siRNA-ERβ1 using Lipofectamine 2000 according to the manufacturer's protocol. siRNA-ERβ1 oligos were ordered from Invitrogen (Shanghai, China): siRNA-ERβ1 sense sequence, CAGAUACUCUUUU AGACCATT; antisense sequence, UGGUCUAAAAGAGU AUCGTG), and a scrambled siRNA.

Ethical approval. All procedures performed in studies involving human participants were in accordance with the ethical standards of the Institutional and/or National Research Committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

Cell viability and proliferation analysis. For short-term cell viability assays, 3,000 cells were seeded in triplicate into 96-wells for 1 day, and then incubated for 5 days with various concentrations of erlotinib and fulvestrant. Nine duplicate wells were used for each group. At the end of the culture period, the viability of the cells was measured using the Cell Counting Kit-8 (CCK-8) assay according to the manufacturer's instructions. In brief, 90 μl of fresh serum-free medium and 10 μl of CCK-8 reagent were added to each well after decanting the old medium, and the culture was continued at 37˚C for 2 h. The optical density at 450 nm was measured using a microplate reader (Promega, Madison, WI, USA).

For long-term colony formation assays, 100,000-200,000 cells were plated/3-cm well the day before the start of treatment. The cells were retreated with fresh media with or without erlotinib and fulvestrant every 3 days until the appropriate confluence, as estimated by the control conditions, was reached. Each experiment was performed in triplicate. The plates were stained with 0.2% crystal violet/10% formalin, and the cell number was estimated under a microscope.

Quantitative real-time PCR. PC9, PC9-ER and Hcc827-ER cells were plated in 60-mm-diameter cell culture dishes. Total RNA was isolated from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from total RNA with Oligo(dT), Enzyme Mix and primers (Invitrogen). The primer sequences used were as follows: forward primer, 5'-GTCAGGCATGCGAGTAAC AA-3', reverse primer, 5'-GGGAGCCCTCTTTGCTTTTA-3'. For amplification, cDNA was initially denatured at 95˚C for 10 min, 40 cycles of 95˚C for 15 sec, 60˚C for 35 sec, and 72˚C for 40 sec (ABI PRISM 7300 Sequence Detection system). For each PCR reaction, a cDNA standard curve was used to generate relative expression changes in ER mRNA levels, which were normalized to the β-actin gene.

Western blotting. The cells were detached using trypsin, washed three times with phosphate-buffered saline, treated with lysis buffer [25 mM Tris-HCl pH 7.4, 1% Triton X-100, 150 mM NaCl, 5% ethylenediaminetetraacetic acid, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 mg of aprotinin and leupeptin], and incubated for 30 min on ice. Lung cancer tissues were lysed in PMSF, followed by homogenization and determination of the concentration of protein. The lysate was centrifuged for 10 min at 12,000 rpm, and the supernatant was collected. The concentrations of protein were measured using the Bradford method (Bio-Rad, Hercules, CA, USA). A 50 μg aliquot of protein/lane was electrophoresed on an 8-12%

sodium dodecyl sulfate-polyacrylamide gel and electroblotted on polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The transferred membranes were blocked with 5% non-fat dry skimmed milk in Tris-buffered saline (TBST) (25 mM Tris-HCl pH 7.4, 125 mM NaCl, 0.05% Tween-20) and incubated at 4˚C overnight with the appropriate primary antibodies, which were specific for the following proteins: ERβ1 (MCA1974ST; 1:500) (Serotec Biologicals, Raleigh, NC, USA). After being washed with TBST, the membranes were incubated with a horseradish peroxidase-labeled secondary antibody (1:2,000) for 1 h at 37˚C before detection using ECL Plus Western Blotting Detection Reagents (Pierce, Rockford, IL, USA). Glyceraldehyde 3-phosphate dehydrogenase was used as an internal control for protein loading and analysis.

Tumor xenograft model. PC9-ER ($5.0x10^6$ cells/mouse) cells were subcutaneously implanted into the posterior flank of 4-week old NOD SCID female mice. The tumor size was monitored as previously described (10). When the average tumor size reached 50 mm^3, erlotinib (10 mg/kg) and/or fulvestrant (10 mg/kg) were subcutaneously administered twice per week. After 22 days, the mice were sacrificed and the tumors were collected and divided into two groups. One group was fixed in 10% formalin and embedded in paraffin for pathological examinations and immunohistochemical analysis, and the other group was stored in liquid nitrogen, and then at -80˚C for further use (western blot analysis). The aforementioned measurements were performed by two qualified technicians in a double-blinded manner, and the mean of the two scores was obtained.

Patient selection and immunohistochemistry. The present study included 53 Chinese patients with advanced NSCLC who received EGFR-TKI therapy at the Tongji Hospital between July 2012 and December 2015. All diagnoses were histologically confirmed and evaluated as stage IV according to the current Tumor-Node-Metastasis (TNM) Staging System (IASLC 2009). Only patients with sufficient tissue for both EGFR mutation analysis and ERβ1 immunohistochemical staining were enrolled after obtaining appropriate approval from the Institutional Review Board (IRB) (IRB ID no. 20141101). Full consent was obtained from patients involved in the present study. Clinicopathological information from the patients was collected, and the database was tabulated in an anonymous manner. Responses were classified using standard Response Evaluation Criteria in Solid Tumors, version 1.1. The PFS was assessed from the first day of EGFR-TKI treatment until radiologic progression or death. Immunohistochemical staining was performed using an ERβ1 antibody, which was confirmed to be specific (11). Cells positive for ERβ1 appeared yellow or yellowish brown in the nucleus or cytoplasm, or they contained yellowish brown granules. A pathological examination and semi-quantitation based on the staining intensity and proportion of positive cells were performed as previously described (12,13).

Statistical analysis. Data are expressed as mean ± standard deviation from three independent experiments. Comparisons among groups were performed with the analysis of variance.

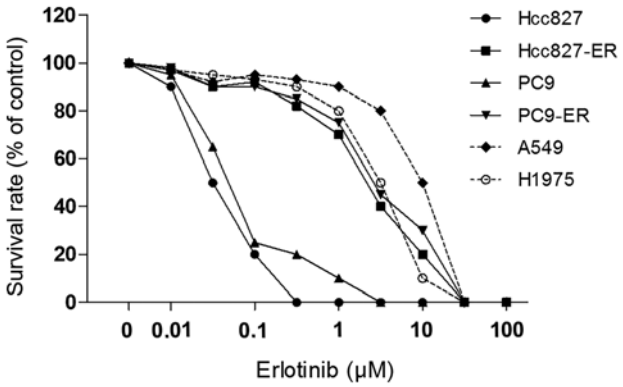

Figure 1. Dose-response curves of six NSCLC cell lines to erlotinib. Cell survival was determined by colony formation assay in the presence of various doses of erlotinib. The IC_{50} values for Hcc827 (19-Del), Hcc827-ER (19-Del), PC9 (19-Del), PC9-ER (19-Del), A549 (WT) and H1975 (L858R/T790M) cells are presented from dose-response curves.

Table I. Sensitivities to erlotinib and fulvestrant as assessed by CCK-8 assay in Hcc827 (19-Del), Hcc827-ER (19-Del), PC9 (19-Del), PC9-ER (19-Del), A549 (WT) and H1975 (L858R/T790M) cells.

	IC_{50} values (μM)	
Cell lines	Erlotinib	Fulvestrant
Hcc827	0.03±0.01	7.93±0.87
Hcc827-ER	7.57±0.67	15.76±2.33
PC9	0.05±0.002	8.05±1.22
PC9-ER	8.42±0.23	18.66±1.51
A549	10.06±1.03	34.02±0.52
H1975	6.23±1.50	22.04±3.61

Values are the mean ± SD of three independent experiments. CCK-8, Cell Counting Kit-8.

A P-value of <0.05 was considered to indicate a statistically significant result. Statistical tests were performed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA).

Results

Expression of ERβ1 is upregulated following resistance to erlotinib, with β-estradiol dependence. First, the effect of erlotinib on six NSCLC cell lines was compared using the CCK-8 assay. Four of the six NSCLC cell lines showed considerable resistance to erlotinib. Dose-response curves to erlotinib for all cell lines assessed by the colony formation assay are presented in Fig. 1. Among the six NSCLC lines, A549, H1975, PC9-ER and Hcc827-ER showed 100- to 200-fold greater resistance to erlotinib compared with the PC9 and Hcc827 cells. The drug sensitivity of all six human cancer cell lines to erlotinib and fulvestrant was examined using the CCK-8 assay, and the IC_{50} values are presented in Table I. The IC_{50} values of Hcc827 to erlotinib and fulvestrant were 0.03±0.01 and 7.93±0.87, and the IC_{50} values of PC-9 were 0.05±0.002 and 8.05±1.22,

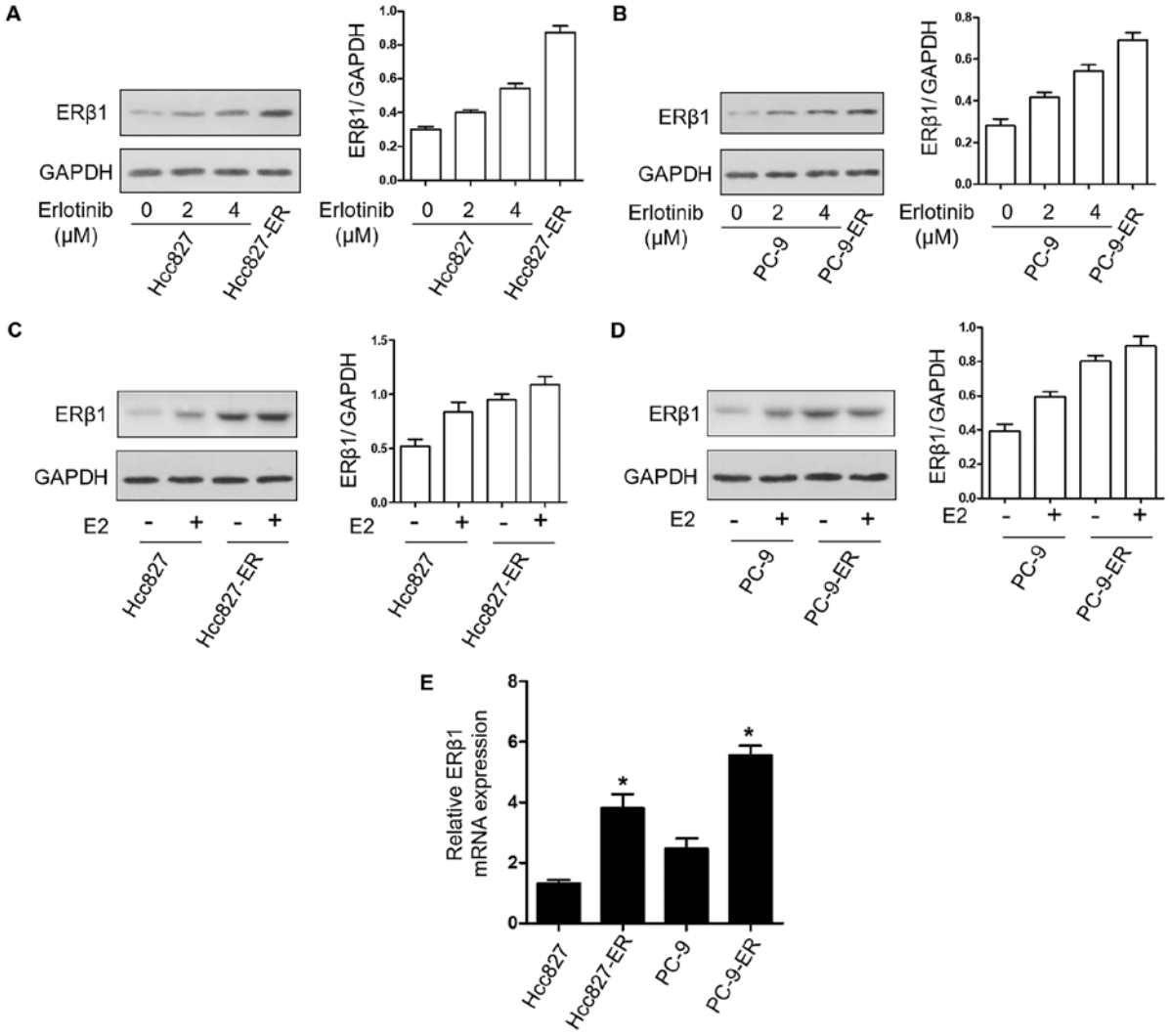

Figure 2. ERβ1 expression is upregulated after resistance to erlotinib, dependent on β-estradiol. (A and B) ERβ1 level was examined by western blotting in PC9 and Hcc827 cell lines as well as PC9-ER and Hcc827-ER cells treated with 0, 2 and 4 μM erlotinib after 48 h. (C and D) In the presence or absence of β-estradiol, ERβ1 was examined by western blotting in PC9 and Hcc827, PC9-ER and Hcc827-ER cells after 48 h. (E) ERβ1 mRNA levels were detected in PC9-ER and Hcc827-ER cells compared with PC9 and Hcc827 cells after 24 h by quantitative real-time PCR. *P<0.05.

respectively. A549, H1975, PC9-ER and Hcc827-ER cells had 100-fold or greater resistance to erlotinib (Table I).

To determine the effects of erlotinib resistance on the expression of ERβ1 protein in NSCLC cell lines, the expression of ERβ1 was examined by western blotting in PC9 and Hcc827 cell lines treated with the indicated concentrations of erlotinib and in PC9-ER and Hcc827-ER cells. ERβ1 had higher expression with increasing erlotinib concentrations; the expression was the highest in the PC9-ER and Hcc827-ER cells (Fig. 2A and B). In addition, in the PC9 and PC9-ER cells as well as in the Hcc827 and Hcc827-ER cells, treatment with 10 nM E2 upregulated the expression of ERβ1 (Fig. 2C and D). Additionally, ERβ1 mRNA levels were also higher in the resistant cells compared to levels noted in the parental cell lines (Fig. 2E). These results indicated that the expression of ERβ1 was upregulated after resistance to erlotinib, with β-estradiol dependence.

PC9-ER and Hcc827-ER cell lines are sensitive to erlotinib after downregulation of the expression of ERβ1. To determine the effects of ERβ1 knockdown on the development of resistance to erlotinib in long-term colony formation assays, PC9, PC9-ER, Hcc827 and Hcc827-ER cells were cultured in erlotinib in the presence or absence of siRNA-NC or siRNA-ERβ1 until colonies were formed. Strikingly, in this long-term assay, ERβ1 knockdown strengthened the effects to erlotinib in the PC9-ER and Hcc827-ER cells. Notably, the knockdown of ERβ1 was also effective when induced in PC9 and Hcc827 cells, that is, after initial erlotinib treatment for 14 days (Fig. 3A, B, D and F). To confirm the biological effects of ERβ1 knockdown, viability of the PC9, PC9-ER, Hcc827 and Hcc827-ER cells was assessed using CCK-8 assay after treatment as indicated. Downregulation of ERβ1 induced both PC9-ER and Hcc827-ER cells to grow slower when compared to the control group. In addition, ERβ1 knockdown induced the lowest proliferation in PC9 and Hcc827 groups (Fig. 3C and F), showing that the proliferation of both sensitive and resistant cell lines was suppressed by erlotinib after the downregulation of the expression of ERβ1.

ERK1/2 and Akt pathways are decreased following the silencing of the expression of ERβ1 in PC9-ER and Hcc827-ER

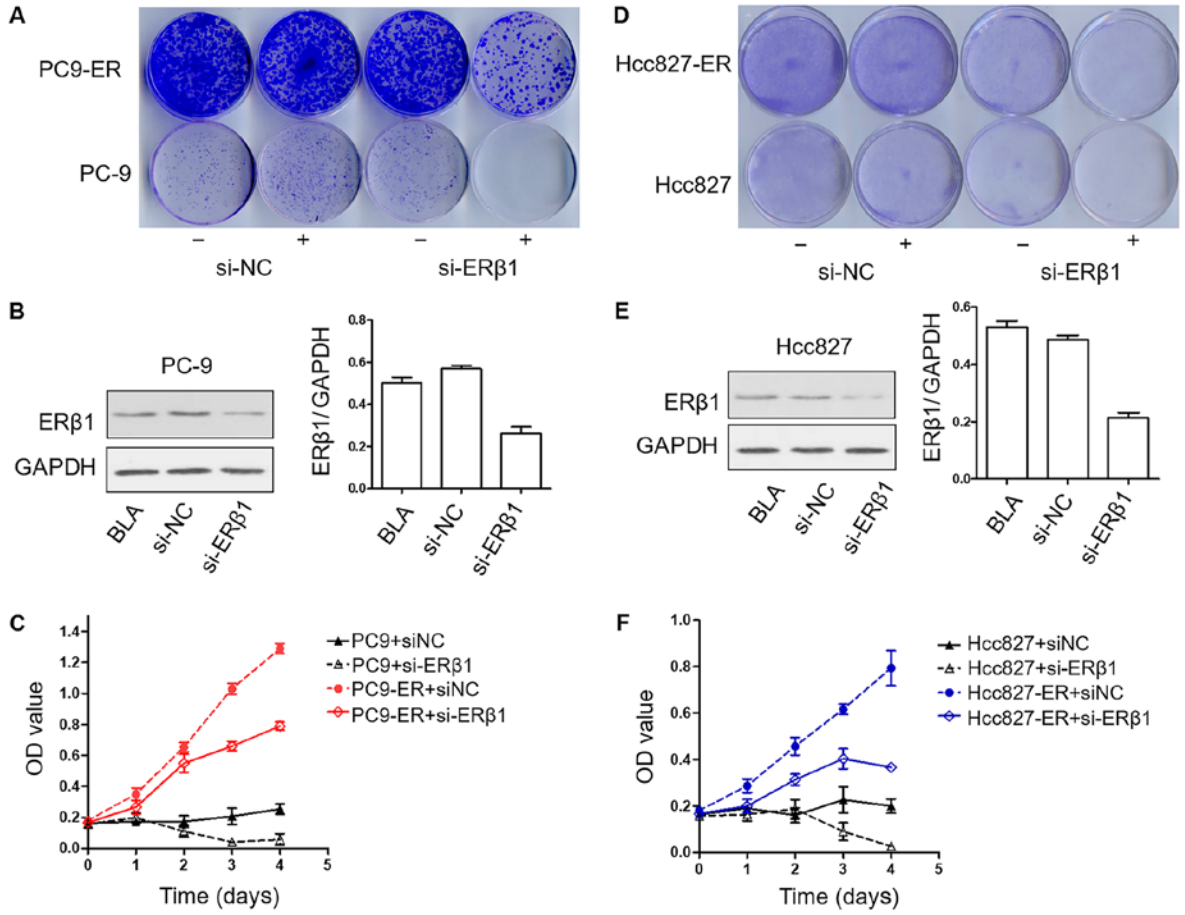

Figure 3. PC9-ER and Hcc827-ER cell lines are re-sensitized to erlotinib after downregulation of ERβ1 expression. (A and D) Proliferation of PC9, Hcc827, PC9-ER and Hcc827-ER cells transfected with siRNA-ERβ1 by colony formation assay. (B and E) Knockdown efficiency of siRNA-ERβ1 in the PC-9 and Hcc827 cell lines. (C and F) In PC9, PC9-ER, Hcc827 and Hcc827-ER cells transfected with siRNA-NC or siRNA-ERβ1, cell viabilty was assessed for the indicated days by CCK-8 assay.

cell lines. In an effort to investigate the signaling involved in the re-sensitization to erlotinib treatment after ERβ1 silencing in PC9-ER cells, the expression levels of tERK1/2 (4695), pERK1/2 (4376) (both from Cell Signaling Technology, Inc., Danvers, MA, USA), tAkt (BS1810) and pAkt (BS4007) (both from Bioworld Technology, Inc., Nanjing, China) were evaluated in PC9, PC9-ER, Hcc827 and Hcc827-ER cells. The levels of pERK1/2 and pAkt were decreased by siRNA-ERβ1 treatment in the PC9-ER and Hcc827-ER cells. Levels were the lowest in the PC9 (Fig. 4A) and Hcc827 (Fig. 4B) cells. In addition, the lack of E2 strengthened the downregulation of ERK1/2 and Akt pathways by ERβ1-knockdown.

Co-treatment of erlotinib and fulvestrant results in better tumor inhibition efficiency in PC9-ER cell-derived xenografts compared with treatment of each agent alone. NOD SCID mice harboring tumors derived from human NSCLC PC9-ER cells were randomly assigned to a vehicle control, erlotinib 10 mg/kg, fulvestrant 10 mg/kg (subcutaneously) groups for a total of 22 days. Erlotinib and fulvestrant alone delayed tumor growth. However, treatment with both erlotinib and fulvestrant elicited significantly reduced tumor volume compared with all other treatments (P<0.001), with minimal tumors after several weeks of therapy (Fig. 5A and B). The expression of pERK and pAkt in tumor tissues was lower in the single-drug group, and it was the lowest in the co-inhibition group (Fig. 5C). Consistent with the observations concerning tumor size, the combination (erlotinib plus fulvestrant) led to the inhibition of the expression of ERβ1 in tumor tissues (Fig. 5D).

Strong expression of cERβ1 is related to a shorter PFS in EGFR-TKI-treated patients. A total of 53 patients with NSCLC treated with EGFR-TKIs were analyzed. Most patients were never/light smokers (40, 75.47%) and had adenocarcinoma (46, 86.79%). A total of 48 patients (90.57%) carried EGFR-sensitizing mutations (19-Del or L858R). The expression of ERβ1 was positive in 79.24% (42/53) of the patients with different intracellular distribution patterns, including nuclear (nERβ1) and cytoplasmic (cERβ1) ERβ1 (Fig. 6A and B). No significant correlations were observed between the expression of ERβ1 and EGFR mutations (P=0.093) or sex (P=0.370). Moreover, neither nuclear nor cytoplasmic expression of ERβ was associated with sex (P=0.586, and P=0.105, respectively) or any other clinicopathological characteristic (data not shown).

At the time of data collection (Jan 1, 2016), 31 patients (58.59%) presented with progressive disease. Notably, patients with strong expression of cERβ1 had a poorer PFS after EGFR-TKI treatment (P=0.009) compared with those with weak or without expression (P=0.009) (Fig. 6C and D). Strong nERβ1 immunoreactivity was not significantly associated

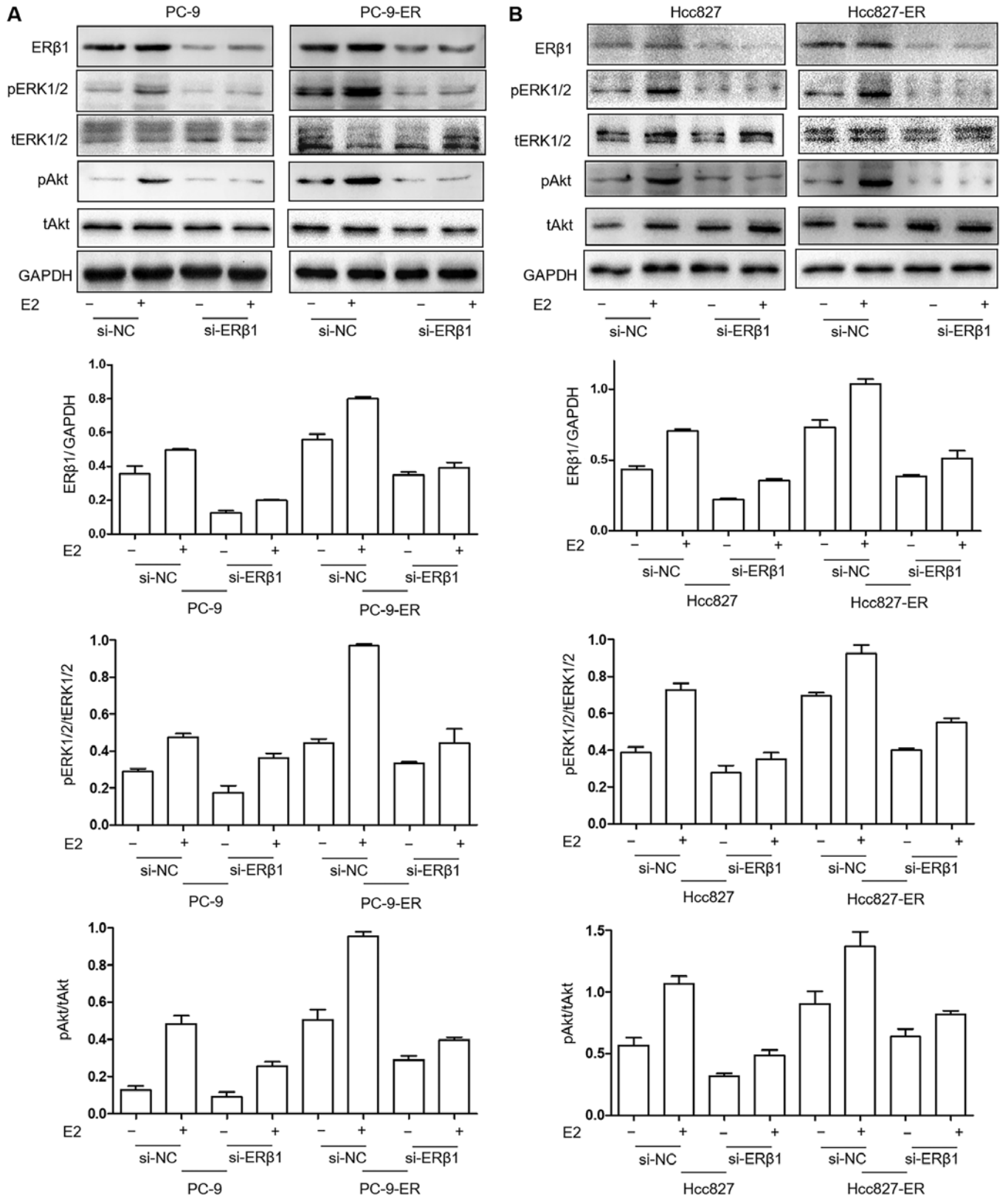

Figure 4. ERK1/2 and Akt pathways are regulated following the downregulation of ERβ1 expression in the PC9, PC9-ER, Hcc827 and Hcc827-ER cell lines. In the presence of siRNA-ERβ1, the expression levels of tERK1/2, pERK1/2, tAkt and pAkt were evaluated in (A) PC9, PC9-ER, (B) Hcc827 and Hcc827-ER cells after 48 h.

with worse PFS (P=0.340). When categorized by positive or negative expression of ERβ1, no statistical significance was achieved in the PFS in the nuclear or cytoplasmic expression subgroup. By Cox multivariate analysis, strong expression of cERβ1 was found to be independent factors predicting worse prognosis [P=0.019, (hazard ratio) HR=2.547, 95% confidence interval (CI) 1.165-5.564], which were in agreement with our Kaplan-Meier plots (Table II).

Table II. PFS by Cox univariate and multivariate analyses in the NSCLC patients.

	Univariate		Multivariate	
Variable	P-value	HR (95% CI)	P-value	HR (95% CI)
cERβ1 (strong vs. weak/neg)	0.014	6.052 (1.212-5.489)	0.019	2.547 (1.165-5.564)
nERβ1 (strong vs. weak/neg)	0.356	0.853 (0.659-3.190)	0.532	1.295 (0.575-2.917)
Differentiation (trend)	0.210	1.574 (0.301-1.301)	0.858	0.866 (0.179-4.198)
TNM stage (trend)	0.287	1.136 (0.870-1.605)	0.685	1.145 (0.594-2.210)
Age (linear, years)	0.486	0.486 (0.940-1.030)	0.769	1.008 (0.953-1.067)
Sex (male vs. female)	0.994	0.000 (0.455-2.185)	0.726	0.844 (0.328-2.175)

PFS, progression-free survival; HR, hazard ratio; CI, confidence interval; TNM, tumor-node-metastasis.

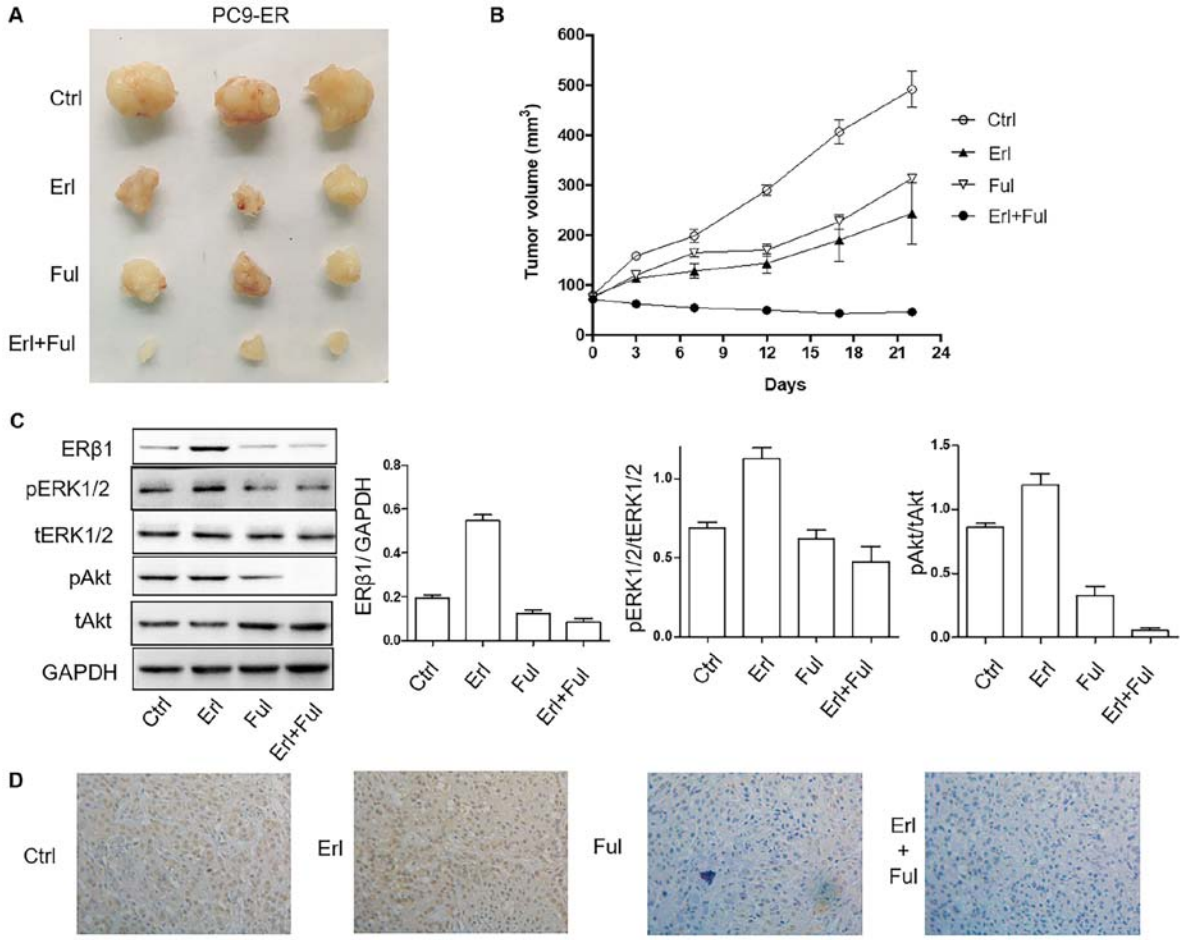

Figure 5. Co-treatment with erlotinib (Erl) and fulvestrant (Ful) exhibit better tumor growth inhibition efficiency in PC9-ER cell-derived xenografts than each single treatment alone. (A) NOD SCID mice harboring tumors derived from human NSCLC PC9-ER cells were assigned to groups: vehicle control, erlotinib 10 mg/kg, fulvestrant 10 mg/kg, and erlotinib plus fulvestrant. (B) Treatment with both erlotinib and fulvestrant elicited significantly reduced tumor volume compared with all other treatments after 17 days of therapy ($P<0.05$). (C) Levels of pERK and pAkt in tumor tissues were decreased in the single-drug groups, but were lowest in the co-treatment group. (D) Combination of erlotinib and fulvestrant led to the inhibition of the expression of ERβ1 in tumor tissues.

Discussion

In the present study, secondary resistant cell lines were constructed, and it was demonstrated that ERβ1 upregulation occurred following the development of resistance to EGFR-TKIs, accompanied by the activation of ERK1/2 and Akt signals. Fulvetrant plus erlotinib reversed TKI resistance *in vitro* and *in vivo*, revealing a new approach to overcome EGFR-TKI resistance. Consistently, patients given EGFR-TKI therapy exhibited a longer PFS in subpopulations with lower expression of cERβ1. The present study discovered a new mechanism of EGFR-TKI resistance from a gender perspective.

The aim of the present study was to investigate the mechanism of acquired resistance to EGFR-TKIs and explore

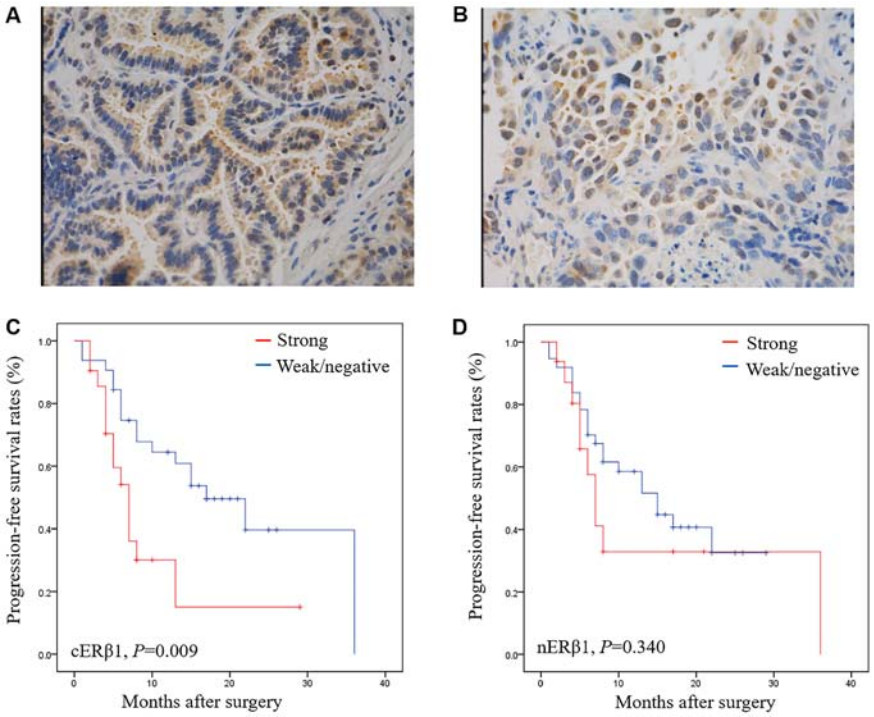

Figure 6. Representative immunohistochemical staining (IHC) of cERβ1 and nERβ1 in tissue specimens obtained from 53 patients with advanced NSCLC treated with EGFR-TKIs and Kaplan-Meier curves of PFS in patients with strong or weak/negative expression of cERβ1 and nERβ1 after EGFR-TKI treatment. Representative IHC images of ERβ in tissue specimens from NSCLC patients treated with EGFR-TKIs. IHC staining of ERβ protein reflecting the different patterns of intracellular localization of ERβ: (A) 'ERβ positive in the nucleus' (nERβ1) and (B) 'ERβ positive in the cytoplasm'(cERβ1). Magnification, x400. (C) Kaplan-Meier curves showing that patients with strong expression of cERβ showed a poorer PFS after EGFR-TKI treatment than those without such expression pattern. (D) Strong nERβ1 immunoreactivity was not significantly associated with worse PFS.

strategies to overcome the resistance to EGFR-TKIs from a gender perspective. Following stimulation with increasing concentrations of erlotinib, the expression of ERβ1 in PC9 and Hcc827 cells was found to be gradually upregulated, consistent with the progressive resistance. Established with the stepwise escalation of EGFR-TKI concentration, PC9-ER, and Hcc827-ER cells had 100-fold or greater resistance compared to sensitive sublines respectively, as previously described (14). In addition, following knockdown of ERβ1, pERK1/2 and pAkt were decreased in the PC9-ER and Hcc827-ER cells. These findings suggest that ERK1/2 and Akt pathways are activated following resistance-induced ERβ1 upregulation. EGFR-TKIs may alter ERβ1 expression through the ERK1/2 and Akt pathways, the bidirectional signaling between ERβ1 and EGFR. Several mechanisms are believed to be responsible for acquired resistance to EGFR-TKIs, including secondary EGFR T790M and minor mutations, MET amplification, activation of MET/HGF axis, acquisition of an epithelial-to-mesenchymal transition signature, and transformation from nNSCLC to small cell lung cancer (SCLC) (14). In addition, the present study demonstrated that ERβ1 upregulation following resistance with activation of the ERK1/2 and Akt pathways, may be another mechanism in EGFR-TKI resistance.

The present study revealed that a combination of erlotinib and a novel ERβ inhibitor fulvestrant significantly inhibited the growth of PC9-ER xenografts in nude mice. Additional treatment of fulvestrant could overcome secondary resistance to EGFR-TKIs *in vivo*. Strategies for overcoming EGFR-TKI resistance include targeting T790M EGFR or other receptor TKs, alternatively activated bypass pathway molecules, and various downstream signaling molecules. The present study demonstrated a better efficiency by co-treatment of erlotinib and fulvestrant compared with alternatives, and complementary application of fulvestrant reversed resistance to erlotinib *in vitro* and *in vivo*. A combination with fulvestrant may provide a prolonged effectiveness of EGFR-TKIs within the range of tolerated toxicity. Previous studies also revealed that a combination of anastrozole and gefitinib compared with either drug alone can maximally inhibit cell proliferation, induce apoptosis and affect downstream signaling pathways (14). Fulvestrant adds to the effects of EGFR inhibitors, including synergy in the EGFR-mutant, erlotinib-resistant H1975 cell line. Tumor stability is achieved in human tumor xenografts with either fulvestrant or EGFR inhibitors, but tumors regress significantly when both pathways are inhibited (10). However, more targeted applications with different genetic abnormalities in a heterogeneous tumor population warrant further exploation. The targeted inhibition strategy from a gender perspective provides a new idea for the clinical treatment of EGFR-TKI resistance.

Patients with strong expression of cERβ1 had a poorer PFS after EGFR-TKI treatment. No statistical significance was achieved in the median PFS between strong nERβ expression and weak/negative expression. Numerous published reports have examined the ER status in relation to the survival of patients with NSCLC (11). High cERβ1 staining was identified as a negative prognostic factor for lung cancer, independent of other prognostic factors (15). nERβ1 positivity was observed in the majority of lung cancer cases (15,16) and found to be a favorable prognostic indicator in various studies. Moreover,

lack of nuclear ERβ or the loss of EGFR expression were reported to be independent prognosis markers associated with shorter overall survival (17). However, most studies used antibodies to total ERβ that could not distinguish different ERβ isoforms. In addition, previous studies involving ERβ and EGFR signals rarely identified the difference in ER subtypes.

In conclusion, ERβ1 activation may accelerate EGFR-TKI resistance. Co-targeting ERβ1 can re-sensitize resistant cell lines. Anti-estrogen treatment may be a potential strategy with which to reverse TKI resistance.

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